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Parturient Hormones: Cytokine, and Oxytocin Effects on Prostaglandin Synthesis

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT

Birth in our Canadian society is a daily phenomenon that occurs approximately 1,100 times per day, but unfortunately not always within the normal time frame, after 39-40 weeks of pregnancy. The present thesis addresses the question: What factors initiate premature labour?

Although the process is incompletely understood, it is known that immune and endocrine factors as well as the fetus itself contribute to the initiatory signals that bring about parturition. Indeed, cytokines, oxytocin, and steroids have been shown to affect the tone, amplitude and frequency of myometrial contractions. Given that their effects can be abolished by the addition of cyclooxygenase(Cox) inhibitors, the changes, in vivo, of Cox-1 and Cox-2 gene expression during pregnancy and the effects of cytokines and oxytocin on prostaglandin synthesis were examined.

Analysis by Polymerase Chain Reaction and Northern blotting indicated that uterine Cox-2 transcript levels increased approaching delivery and that the Cox-1 mRNA levels remained relatively unchanged throughout pregnancy. Immunohistochemical staining for the Cox-2 and OTR proteins revealed both to be co-expressed in the myometrium and in endometrial epithelial cells. Within the stroma, no OTR staining was found but intense staining for Cox-2 was noticed during labour.

Following the in vivo work, the effects of cytokines and oxytocin on various cell lines were tested. When IL-1β and TNF-α were added to CUS-V2, a stromal cell line I developed, an increase in Cox-2 mRNA levels and an approximately 2 fold increase in $PgF_{2\alpha}$ released per 24 hours was observed. Under the same conditions, no changes in the levels of Cox-1 or GAPDH mRNA were observed. The effect of oxytocin was more pronounced. Its addition to CHO cells expressing constitutively the rat oxytocin receptor led to a 107 fold increase in the amount of PgE_2 released in a 24 hour period and a increase in Cox-2 protein levels as determined by Western blot analysis. Therefore, since uterine oxytocin and oxytocin receptor, in the rat, are regulated by estrogen and progesterone, and these steroids are in turn regulated by placental born factors, a clearer

understanding is emerging of their interaction. These three systems might synergistically or additively interact to produce an amplified signal that initiates strong synchronous contractions and a delayed, but a parallel, increase in cervical compliance by inducing prostaglandins.

ABRÉGÉ

Dans notre société Canadienne, il y a approximativement 1,100 naissances par jour. Malheureusement, toutes les naissances non pas lieu dans la période normale, c.à.d. apres 39-40 semaines de grossesse. Les expériences dans la presente thèse adressent la question suivante: quels sont les facteurs qui jouent un rôle dans le travail prématuré?

Même si notre compréhension des mécanismes est incomplet, nous savons que des facteurs immunologiques et endocriniens ainsi que le fétus lui-même contribuent aux signaux qui ainsi la parturition. En effet, les cytokines, l'ocytocine(OT) et certains stéroïdes sont connus pour affecter le tonus, l'amplitude et la fréquence des contractions myométriales. Vu que leur effet peut être aboli par l'addition d'inhibiteur de cyclooxygenase(Cox), les changements *in vivo* de l'expression du gene Cox-1 et Cox-2 ont été examinés durant la grossesse ainsi que les effets des cytokines et de l'ocytocine sur la synthèse des prostaglandines.

Des analyses par PCR(polymerase chain reaction) ainsi que de Northern blot ont indiqués les niveaux d'expression des ARNm de Cox-2 dans l'utérus sont augmentes en approchant l'accouchement et que les niveaux de ARNm Cox-1 restent relativement constant durant toute la gestation. Par immunohistochimie, la présence des protéines Cox-2 et du récepteur de l'ocytocine(ROT) a été demontré dans le myomètre et dans les cellules épithéliales de l'endomètre. Dans le stroma, un marquage intense pour Cox-2 mais pas pour OTR a été trouvés durant la parturition.

Basé sur les résultats obtenus *in vivo*, les effets des cytokines et de l'OT ont été etudiés dans différentes lignées cellulaires. L'ajout d'interleukin-1β et de TNF-α une lignée cellulaires stromale uterin(CUS-V2) que j'ai développé entraînait une augmentation des nivaux d'expression des ARNm Cox-2 et une augmentation de deux fois de la quantité de prostaglandin $F_{2\alpha}$ relâché pendant une période de 24 heures. Dans les mêmes conditions, aucun changement dans les taux d'ARNm de Cox-1 ou GAPDH n'a été observé. L'effet de l'OT était plus prononcé.

L'ajout de l'ocytocine à des cellules CHO transfécté stablement avec le ROT du rat entraînait une augmentation de 107 fois dans la quantité de prostaglandin E_2 libérée pendant une période de 24 heures et une augmentatiion des niveaux de Cox-2 déterminés par l'analyse de Western blot. Lorsque dans l'utérus du rat, OT et ROT sont contrôlés par l'oestrogène et la progestérone et lorsque ces deux stéroïdes sont eux-mêmes contrôlés par des facteurs placentaire, il en résultera une meilleure compréhension de leur interaction. Ces trois systèmes interagisent probablement d'une manière synergistique ou additive en vu de produire un signal amplifié qui mene à initiation de contractions fortes et synchrones du myomètre.

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List of Abbreviations

ACTH	adrenocorticotropin hormone
ATP	adenosine triphosphate
Ca^{2+}	calcium
CDK's	cyclin dependent kinases
cDNA	complementary deoxyribonucleic acid
CKI's	CDK inhibitors
COX	cyclooxgenase
CRB	creb binding protein
CRF	corticotropin
CSF-1	colony stimulating factor-1
CUE-P	cemal uterus epithelial cells-Plasmid transfected
CUE-V	cemal uterus epithelial cells-retroViral transfected
CUS-V2,4	cemal uterus stromal cells-retroViral transfected
DAG	diacylglycerol
DMEM	dulbecco's modified eagle's medium
dPRP	decidual prolactin related protein
E	estrogen
E_1	estrone
E_2	estradiol
E_3	estriol
EGF	epidermal growth factor
ER	endoplasmic reticulum
ET	endothelin
FGF	fibroblast growth factor
FSH	follicle stimulating hormone
GAP	GTP'ase activating protein
GAPDH	glyceraldehyde phosphodehydrogenase
GH	growth hormone
Grb2	growth factor receptor bound protein 2
HBSS	hank's balanced salt solution
hCG	human chorionic gonadotropin
hCS	human chorionic somatomammotropin

HPV	human papillomavirus
IGF-I	insulin-like growth factor-I
IL-1	interleukin 1
ISGF	interferon stimulated gene factor
JAK1,2	janus kinase1,2
kb	kilobases
LH	luteinizing hormone
LHRH	luteinizing hormone-releasing hormone
LIF	leukemia inhibitory factor
LTH	decidual luteotrophin
LTR	long terminal repeat
MAP	mitogen activated proteins
Mg^{2+}	magnesium
MHC	major histocompatibility complex
MLCK	myosin light chain kinase
mRNA	messenger ribonucleic acid
NGF	nerve growth factor
NpI	neurophysin I
NSAID	non-steroidal antiinflammatory drugs
OT	oxytocin
OTR	oxytocin receptor
P	progesterone
PAF	platelet-activating factor
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PGDH	15-hydroxy-prostaglandin dehydrogenase
PgE_2	prostaglandin E_2
$PgF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PgI_2	prostacyclin
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PRL	prolactin
PTP1C	phosphatase 1C
PVN	paraventricular nucleus

Rb	retinoblastoma
rPL	rat placental lactogen
rPLP	rat prolactin-like hormones
RT-PCR	reverse transcriptase-PCR
SDS	sodium dodecyl sulphate
SH2	src-homology domain 2
SH3	src-homology domain 3
SON	supraoptic nucleus
SOS	son of sevenless
SV40	simian virus 40
TGF	transforming growth factor
TIMP	tissue inhibitors of mettaloproteinases
TNF	tumour necrosis factor
VEGF	vascular endothelial cell growth factor
VP	vasopressin
WT	wilm's tumour

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Preface

The Guidelines Concerning Thesis Preparation of the Faculty of Graduate Studies and Research at McGill University states the following:

"The candidate has the option, subject to the approval of the Department, of including as part of the thesis the text, or duplicated published text(see below), of an original paper, or papers. In this case the thesis must still conform to all other requirements explained Guidelines Concerning Thesis Preparation. Additional material (procedural and design data as well as descriptions of equipment) must be provided in sufficient detail(e.g. in appendices) to allow a clear and precise judgement to be made of the importance and originality of the research reported published or to be published. It must include a general abstract, a full introduction and literature review and a final overall conclusion. Connecting texts which provide logical bridges between different manuscripts are usually desirable in the interests of cohesion.

It is acceptable for theses to include as chapters authentic copies of papers already published, provided these are duplicated clearly on regulation thesis stationery and bound as integral part of the thesis. Photographs or other materials which do not duplicate well must be included in their original form. In such instances, connecting texts are mandatory and supplementary explanatory material is almost always necessary.

The inclusion of manuscripts co-authored by the candidate and others is acceptable but the candidate is required to make an explicit statement on who contributed to such work and to what extent, and supervisors must attest to the accuracy of the claims, e.g. before the Oral Committee. Since the task of the Examiners is made more difficult in these cases, it is in the candidate's interest to make the responsibilities of authors perfectly clear. Candidates following this option must inform the Department before it submits the thesis for review."

I have decided to submit my thesis with 4 published and 1 publishable paper.

This thesis is organized in five Parts. Part I is the "Introduction and Literature Review" written as three large chapters. Part II is the "Experimental Results" section containing the scientific results and their interpretation. Three separate chapters are contained within Part II, each containing the standard Summary, Introduction, Materials and Methods, Results, Discussion and References. A small preface at the beginning of each chapter serves as connecting text.

Chapter 1 contains a published paper entitled Characterization and Co-Culture of Novel Nontransformed Cell Lines Derived From Rat Endometrial Epithelium and Stroma in *In Vitro Cell Developmental Biology 31:140-148(1995).* The authors are A Arslan, G Almazan and HH Zingg. I am the first author and the primary investigator who established the cell lines. Dr. Almazan provided prepackaged defective retrovirus encoding the temperature sensitive viral oncogene SV40 Large T antigen. I have included a second manuscript, Development of Immortalized Endometrial Epithelial and Stromal Cell Lines from the Mink (Mustela vison) Uterus and Their Effects on the Survival In Vitro of Mink Blastocysts in Obligate Diapause, placed in appendix B, where I was involved in immortalizing mink uterus endometrial cells. The authors are GM Moreau, A Arslan, DA Douglas, J Song, LC Smith and BD Murphy and it was published in *Biology of Reproduction 53:511-518(1995).*

Chapter 2 consists of a published paper entitled Regulation of Cox-2 Gene Expression in Rat Uterus in Vivo and In Vitro. The authors are A Arslan and HH Zingg and it was published in *Prostaglandins* 52:463-481.1996. I was also the first author for this manuscript and the primary investigator who designed and undertook the various experiments.

Chapter 3 consists of a publishable paper entitled Cox-2 Induction by Oxytocin. Its authors are A Arslan and HH Zingg. As part of this chapter I have included a published paper entitled Oxytocin Receptor Gene Expression

in the Rat Uterus During Pregnancy and the Estrous Cycle and in Response to Gonadal Steroid Treatment which is placed in Appendix C. Its authors are A Larcher, J Neculcea, C Breton, A Arslan, F Rozen, C Russo and HH Zingg and it was published in *Endocrinology 136:5350-5356(1995).* For this study, I had developed and sequenced specific PCR primers to assess the expression of the oxytocin receptor and with Agnes showed the gene is regulated by 17-β estradiol which served as the initial results of this manuscript.

Part III is the "Discussion" for the thesis. Part IV is the "Bibliography" which lists all the references for the thesis. Finally, Part V contains the "Claims for Original Research".

Acknowledgments

I feel indebted to Dr. H. Zingg, and the Laboratory of Molecular Endocrinology, Dr. B. Turcotte, and Dr. B. Rehfuss for their kind advice and Jeana Neculcea and Cathy Russo for their technical assistance. Furthermore, I feel obliged to the department of Physiology and its staff members and fellow graduate students for their interaction and encouragement.

I would like to extend my appreciation and gratitude to my parents, Zeynep and Cemal Arslan whose support for my endeavours kept my drive and curiosity awake and willfull.

And last but not least in importance are the funding agencies which made this research possible. I would like to thank the Medical Research Council of Canada, the Fonds pour la Formation de Chercheurs et l'Aide a la Reserche and the Royal Victoria Hospital for their financial support.

PART I

INTRODUCTION AND LITERATURE REVIEW

CHAPTER 1

My thesis work consisted of investigating how various endocrine factors initiate labour. I have chosen to subdivide the introduction into three chapters. The first chapter presents the basic histology, molecular biology and endocrinology of the uterus and fetal membranes and the respective changes which occur from the estrous cycle to pregnancy. The second chapter details current scientific understanding of parturition both in cellular and molecular terms. And finally, the last chapter details the technique of immortalizing uterine cell lines and their value for new results. For our studies, since our animal model for human labour was the rat, most of the introduction emphasizes pregnancy in rodents and its relation to other species.

Histology and Endocrinology of Pregnancy

1.1 Histology of Uterus

The uterus can best be defined as the reproductive organ located in the pelvic cavity where the differentiated fertilized ovum, the blastocyst, implants, and develops for its gestational length. If one were to dissect out the uterus, fix and section it, a cross-sectional profile would show two compartments(Fig.1). An initial layer next to the lumen consisting of epithelial and stromal cells defined as the endometrium, and a smooth muscle layer known as the myometrium(Wheaten et al, 1987). The myometrium itself is composed of a inner circular and outer longitudinal myometrial cells and cell bundles. Surrounding the myometrium is a serosal layer of connective tissue. In addition to the cell types that define the different compartments, in rodents, the uterus also has mesometrial and antimesometrial divisions. The stromal area between the circular and longitudinal muscle layers is known as the mesometrial triangle or mesometrium and it is where the metrial gland forms in the course of

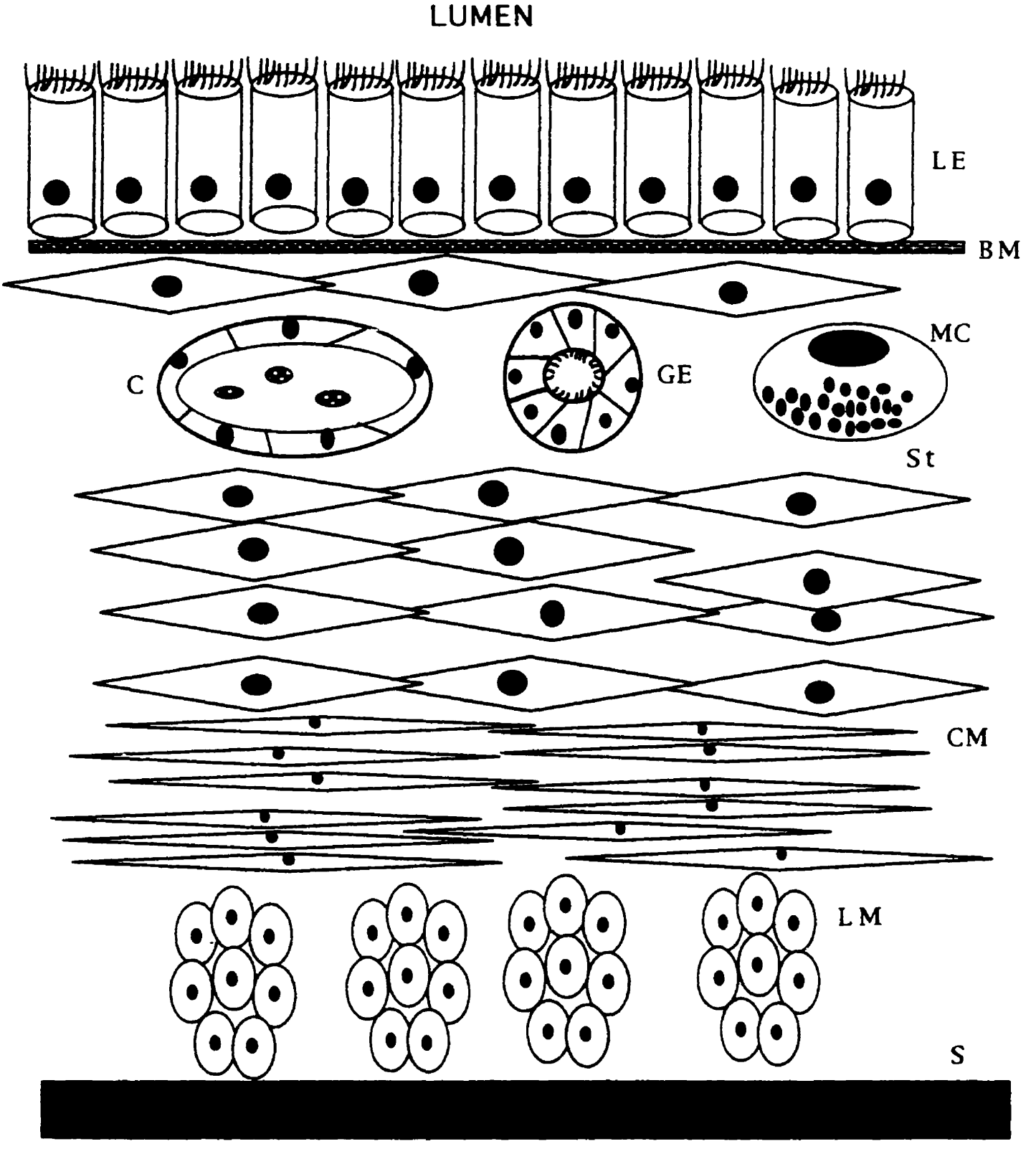


Fig.1 Schematic diagram of the Uterus. LE: luminal epithelium; GE: glandular epithelium; C: capillary; St: stroma; CM: circular myometrium; LM: longitudinal myometrium; BM: basement membrane; S: serosa; MC:mast cells.

pregnancy and where arteries enter the uterus and bifurcate towards the endometrium leading to capillary branching just beneath the epithelial cells(Hunt et al, 1988; DeFeo et al, 1967). The divisions are important.

1.1.1 Endometrium

In most species, the endometrium usually consists of a single layer of columnar luminal epithelial cells which are continous with glandular epithelial cells and a multi-layered stroma which itself consists of stromal, vascular and bone marrow derived cells such as monocytes, macrophages, dendritic cells, Langerhan cells, Natural Killer(NK) cells, and cytotoxic and T_H cells(Lysiak et al, 1992; Wiley et al, 1990; Julian et al, 1992). The epithelial cells being similar to most mucosal epithelial cells are adjoined by tight junctions, desmosomes and gap junctions(Rodriguez-Boulan et al, 1989). On the apical face of the cells, microvilli extend into the lumen. On the basolateral side, the cells are anchored above the basal laminae consisting of collagen typeIV, entactin, laminin, and heparan sulfate proteoglycan(Mulholland et al, 1992). The sub-epithelial stromal cells form a distinct stromal layer. They are connected by gap junctions formed by connexin 26 and 43(Orlando-Mathur et al, 1996). Below this layer is the remainder of the stroma which is embedded in a rich layer of extracellular matrix consisting of collagen typeI, III, V and VI, laminin, and proteoglycans(Mulholland et al, 1992; Farrar et al, 1992).

The endometrium could also be considered as an endocrine gland. The following hormones have been shown to be produced:oxytocin, prostaglandins, cytokines, Insulinlike growth factor(IGF), IGF-binding proteins, prolactin, endothelin, β-endorphin, prolactin like proteins, relaxin, growth factors such as epidermal growth factor, uteroglobin and Transforming Growth Factor-β which opposes cell division in many cell types(Lefebvre et al, 1992; Behrman et al, 1983; Tabibzadeh et al, 1991; De et al, 1992; Yanagisawa et al, 1988; Inoue et al, 1989; Arai et al, 1990; Yallampali et al, 1994; Maggi et al, 1993; Cameron et al, 1991; Kapur et al, 1992; Girvigian et al, 1994; Handwerger et al, 1992; Soares et al, 1991; Li et al, 1989; Downing et al, 1986; Nelson et al, 1992; Wiehle et al, 1990; Tamada et al, 1990; Das et al, 1992).

1.1.2 Myometrium

The myometrium provides the contractile force that is needed to deliver the fetus. Individual myometrial cells and their shortening form the basic unit of pressure. As mentioned above there are two layers: an inner circular layer and outer longitudinal layer which are interspersed with connective tissue, myometrial stromal cells and, interestingly, macrophages. Both cell types have a resting membrane potential of -56mV(Kuriyama et al, 1976) and function as a single unit(Puri et al, 1982; Lye et al, 1993; Chan et al, 1977). In the course of pregnancy both hypertrophy and hyperplasia increase their size and numbers respectively. For example in the rat, the surface area of the cells increases from 3.7 X $10^{-5}cm^2$ in the non pregnant state to 1.04 X $10^{-4}cm^2$ at day 21 of pregnancy which lends itself to increased membrane capacitance and Ca^{2+} current(Mirronean et al, 1994). Given that the neuronal input to the myometrium diminishes substantially during the course of parturition(Stjernquist et al, 1994), endocrine factors have been shown to be prominent both in terms of depolarizing the sarcolemmal membranes, and, inducing action potentials and, as well, generating secondary signals such as IP_3 which increase cytosolic Ca^{2+} levels. They are thought not only to interact with myometrial cells but as well with pacemaker cells which determine the frequency of action potentials that are propagated to adjoining cells(Lodge et al, 1994; Crane et al, 1991). An interesting observation made by some investigators is that subplacental myometrium functions differently then periplacental sites(Moonen et al, 1986). It is thought that the local stretching and hormonal milieu keeps the local pacemaker cells in a more active state, therefore, determining the nature and the amounts of contractions. The contractions themselves increase in tone, frequency and amplitude approaching term(Fuchs et al, 1976). Not all endocrine factors effect each of these variables in the same manner.

Myometrial cells function basically in the same manner as do most smooth muscle cells. An advantage of smooth muscle over skeletal muscle is due to the arrangement of their actin-myosin filaments they can be stretched or contracted to a greater extent. For example, they can contract to a fifth of their initial size. This versatility is essential in the uterus where fetal development stretches the uterus to larger volumes and at the

end of this development they need to contract in a stretched state(reviewed by Huszar et al, 1986 and Daifotis et al, 1992).

The manner in which smooth muscle cells generate force is by activation of myosin ATPase and actin availability which then permits both thick and thin filaments to interact and slide past each other. This mechanism is under the control of the secondary signal Ca^{2+}. If cytosolic Ca^{2+} levels rise, contractions progress, and if they are lowered, contractions cease(Izumi et al, 1994). The cytosolic Ca^{2+} level itself, usually 0.1μM in resting state, is determined by three sources: 1- extracellular pool availability, 2-intracellular pool availability, and 3-Ca^{2+} pumps(Fig.2). Whereas the extracellular Ca^{2+} pool, 1.5mM in plasma, is under the control of voltage-gated calcium channels and ligand activated calcium channels(Izumi et al, 1994), the intracellular pool which is stored in sarcoplasmic reticulum and mitochondria is regulated and controlled by the secondary signal IP_3 and Ca^{2+} itself(Somlyo et al, 1994; Majerus et al, 1990; Nishizuka et al, 1992). Increased IP_3 and Ca^{2+} bind the $InsP_3$ receptor and ryanodine receptor, respectively, on the sarcoplasmic reticulum(SR) and permit endogenous Ca^{2+} which is weakly bound to calsequesterin to be released into the cytosol(Berridge et al, 1993; Clapham et al, 1995). As soon as Ca^{2+} is released it's removed either by the uterine SERCA2b and SERCA3 SR calcium pumps or PMCA1a,b and PMCA2 sarcolemmal calcium pumps(O'Reilly et al, 1994)(Fig.3). Therefore any endocrine factor which depolarizes the membrane, opens calcium channels or inhibits the pumps will increase cytosolic Ca^{2+} levels. A classic example of such an agent is oxytocin which increases the rate of spontaneous contractions and whose effects can be abolished with the co-addition of the Ca^{2+} chelating agent EGTA or EDTA(Izumi et al, 1994). In contrast, any factor which inhibits these steps will maintain the uterus in a quiescent state.

In smooth muscle cells, unlike in skeletal myofibers, the Ca^{2+} doesn't directly activate the myosin ATPase. Cytosolic calcium is bound by calmodulin which itself binds to the myosin light chain kinase(MLCK). Upon binding calmodulin, MLCK becomes activated and phosphorylates the 20kD regulatory light chain of myosin on serine 19(Somlyo et al, 1994; Sanbor et al, 1994). This phosphorylation step permits actin to bind to

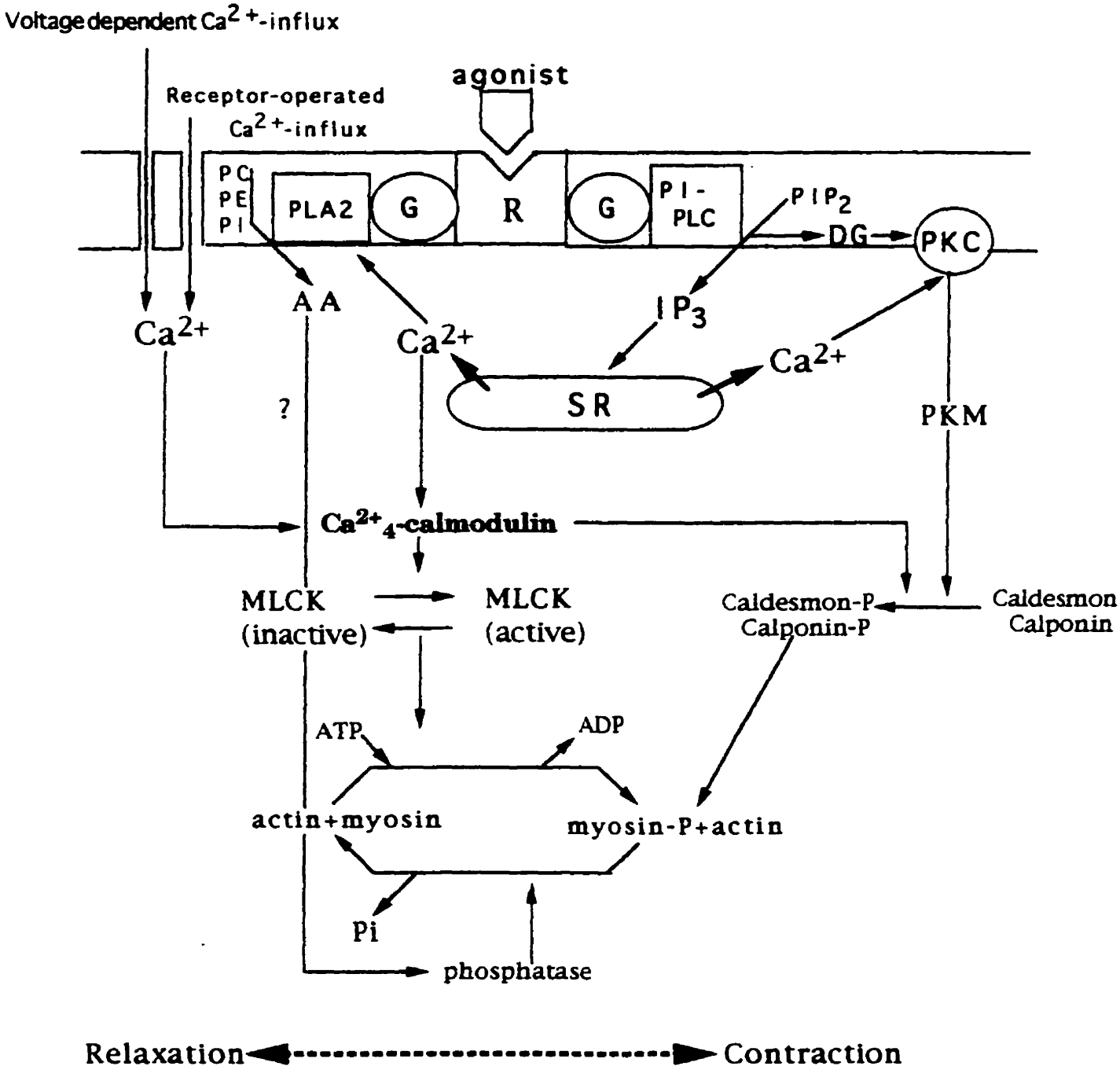


Fig.2 Schematic diagram depicting the different variables in Ca^{2+} Influx. AA:arachidonic acid; PKM:protein kinase M; MLCK:myosin light chain kinase; SR:sarcoplasmic reticulum; DG:Diacylglycerol; PKC: protein kinase C; PLC:phospholipase C. Figure obtained from Izumi, 1994.

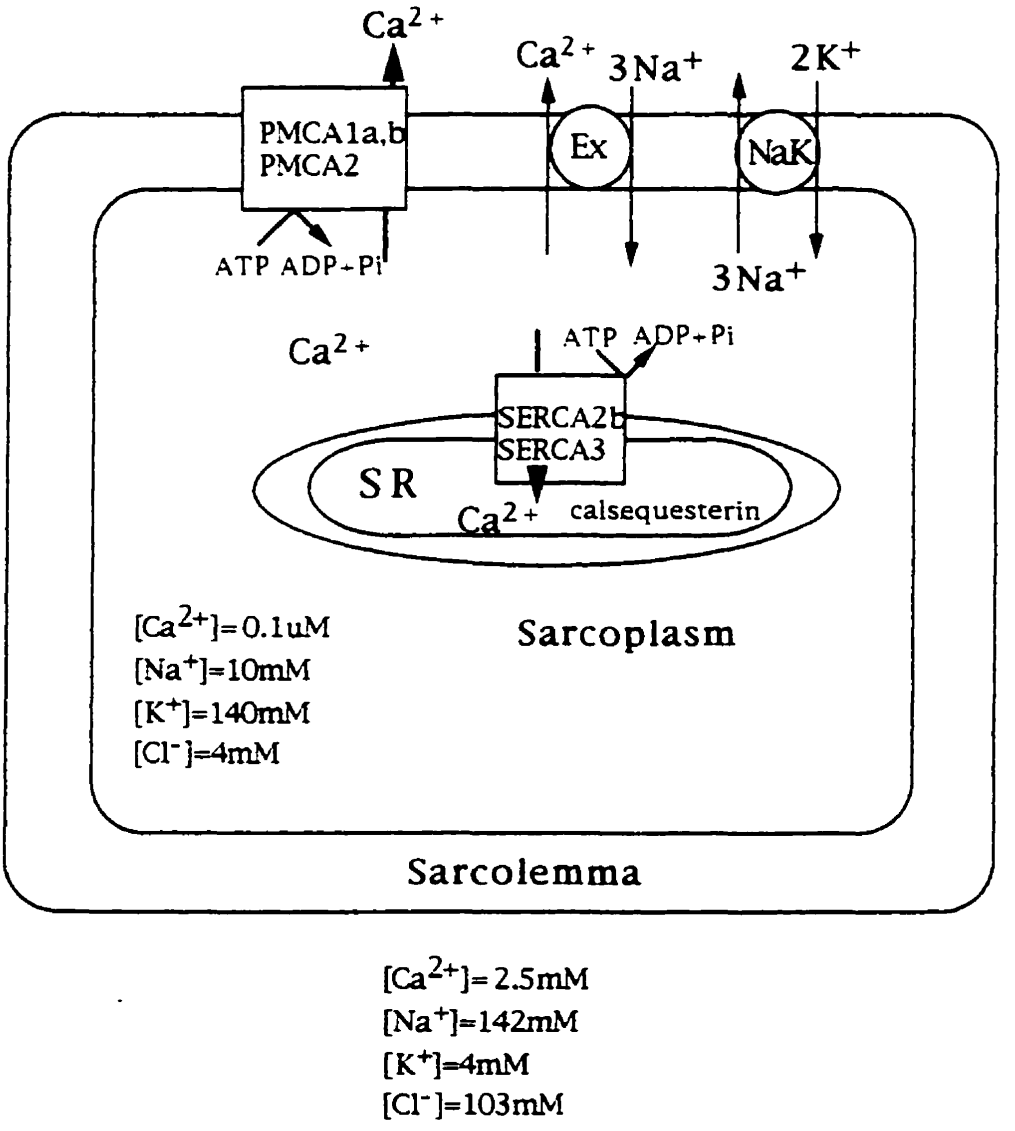


Fig.3 Schematic figure depicting the different variables in cytosolic calcium extrusion. SR:sarcoplasmic reticulum. Figure obtained from Huszar and Walsh, 1986.

myosin and activation of the myosin ATPase, hydrolysis of ATP and sliding of myosin heads along the actin filament(Huszar et al, 1986).

Opposing the effects of calcium in these cells is the cytosolic secondary signal cAMP. The cytosolic levels of cAMP is determined by two enzymes:1-adenylate cyclase, and 2-phosphodiesterases(Kafinas et al, 1987; Tang et al, 1992). Activation of or inhibition of these enzymes will determine cAMP content. Increased cAMP levels in myometrial cells amounts to decreased phospholipaseC activity and decreased levels of IP_3(Anwer et al, 1990), impermeable gap junctions and therefore asynchronous contractions(Sakai et al, 1992), and finally, inhibition of MLCK activity whose consequence is evident(Conti et al, 1981). For many years, it has been known that β2-adrenergic receptors are coupled to cAMP formation in the uterus, and clinicians have used agonists, such as isoproteronol, successfully to inhibit contractions, but due to desensitization researchers have looked for other candidates(Anwer et al, 1989). These include relaxin which is also coupled to cAMP and nucleotide phosphodiesterase inhibitors such as theophyline and papaverine(Anwer et al, 1989; Berg et al, 1983).

ESTROUS CYCLE

1.2 Estrous cycle

Rats are nonseasonal, spontaneously ovulating, polyestrous mammals whose average cycle length is approximately 5.4 days. The cycle was reduced to defined stages by Heape who used the term estrous in describing the female rats permissiveness to lordosis(Heape et al, 1900). He used the prefixes pro, di, and met, and suffix estrous to describe the intervening stages between estrous. Simply stated, proestrous was defined to be the period in the cycle where the animal is approaching sexual receptivity, and diestrous was defined to be the period after ovulation and fertilization where ovarian secretions prepare the reproductive tract for the ensuing pregnancy. Metestrous was defined as the period following estrous in the absence of conception where the estrous changes subside. If

conception failed, the animal returns to proestrous and the cycle begins anew. In general, proestrous is 12 to 14 hrs in length; estrous lasts 25-27 hrs, and diestrous spans 55 to 57hrs (reviewed by Freeman, 1988).

The different stages can be identified according to vaginal smears. During diestrous most of the cells in the smear are leukocytes and the vaginal mucosa is thin and bluish in texture. During proestrous, the smear contains nucleated epithelial cells which have been shed and the vagina is gaping, reddish-pink, moist and with numerous striations. During estrous the shed epithelial cells are squamous in appearance with degenerate nuclei and the vagina is similar in appearance to proestrous but the striations are more prominent and there is less moisture(Turner, 1961).

1.2.1 Endocrine Hormones

At the cellular and anatomic levels, scientists have known for decades that changes must and do occur in the reproductive tract prior to implantation and during pregnancy. Exactly what signals directed or controlled not only the sequence but the spatial, temporal, and time dependent events remained an enigma. Although the central nervous system was most likely involved, the endocrine glands and their endocrine/exocrine secretions or hormones captivated their attention. Their dedication and results proved them correct. The development of sophisticated techniques such as gene ablation and transgenetics(Kendall et al, 1995), and monoclonal anti-sera(Kohler et al, 1975) permitted them to not to only define the hormone functions but also their additive and synergistic interactions. The following hormones became central to the understanding of the female reproductive cycle.

1.2.1.1 FSH/LH

Follicle Stimulating Hormone(FSH) and Luteinizing Hormone(LH) are two glycoproteins that are produced and stored in vesicles and released by the gonadotrophs in the anterior pituitary. Both are dimers of approximately 32kDa which share the same alpha subunit, comprised of 89 amino acids,

but bear different beta subunits of 115 amino acids. Their plasma content is determined by positive and negative factors on the gonadotrophs. Whereas Luteinizing Hormone-Releasing Hormone(LHRH) which is a hypothalamic derived decapeptide that is released into the hypophyseal portal vasculature, stimulates their synthesis and release, the ovarian steroids and hormone inhibin suppresses their release and synthesis. If one were to measure their plasma content in short intervals, one observes that both hormones are released in a pulsatile fashion whose frequency, tone and amplitude change according to the estrous cycle and reproductive stage. Furthermore, both of their receptors are seven transmembrane domain receptors which are G-protein coupled(reviewed by McCann et al, 1988; Ojeda et al, 1988).

1.2.1.2 Estrogen/Progesterone

Steroids in general are produced in large amounts in three tissues. They include the adrenal gland where cortisol, aldosterone and small amount of androgens are produced, the ovary where the estrogens, estradiol(E2), estrone(E1), and estriol(E3) and progestins are produced and the placenta where progesterone synthesis occurs and, depending on the species, estrogen synthesis as well(Yen et al, 1991; Adashi et al, 1992; Kaplan et al, 1988). Within the rat reproductive cycle the amount of steroids produced by the ovary differs between the stages and it is this tissue which is involved in influencing not only uterine motility, leukocyte infiltration and ovulation but the cycle length collectively.

The site and synthesis of steriods in the ovary has been shown to be the growing follicles. Initially, the primary oocytes which number approximately 11,000 per ovary in the rat start developing upon sexual maturation. Once the animal starts cycling, FSH induces the differentiation of primary follicles into Antral or Graffian follicles which move towards the periphery of the ovary for ovulation(Zeleznik et al, 1974; Zeleznik et al, 1977). The steroids themselves are produced in granulosa cells which are the inner cells surrounding the zona pellucida and the theca interna and externa cells which are the outermost cells. FSH promotes estrogen synthesis in granulosa cells which in turn increases the level of LH

receptors(Fig.4; Gore-Langton et al, 1994) preparing the follicle for ovulation by LH. The remaining ruptured follicle differentiates and is defined as the corpus luteum which, under LH stimulation, synthesizes and releases progesterone and estrogen. The produced steroids, in turn, prepare the uterus for implantation by making the required changes (reviewed by Hogan et al, 1994).

These changes include, at proestrous, water imbibition, epithelial cell grow from the inducion of proto-oncogenes and increased chemotaxis and immune cell influx(Lubahn et al, 1993; McDonnell et al, 1995). For example, it has been shown that 17-β estradiol induces transforming growth factor(TGF)-α(Nelson et al, 1992) in epithelial cells and the oncogene c-fos and c-jun(Kirkland et al, 1992; Chiappetta et al, 1992), both are leucine-zipper transcription factors involved in the G1-phase of the cell cycle. Furthermore, in addition to TGF-α, 17-β estradiol also induces oxytocin(Lefebvre et al, 1994), which is highly expressed during proestrous and estrous but not in diestrous, the progesterone receptor(Kastner et al, 1990; Inaba et al, 1988), and colony stimulating factor(CSF)-1 in the endometrium(De et al, 1993). Therefore, in the uterus, the effects of estrogens are pleiotropic. Progesterone, similarly, has many effects. These include myometrial quiescence(Alexandrova et al, 1980), inhibition of epithelial cell growth(Kirkland et al, 1992), and preparation of the stroma for decidualization(Sananes et al, 1978).

What are steroids and how are they synthesized? The answer to these questions have been addressed by many researchers. The precursor to steroids is cholesterol which is obtained by the follicle cells from two sources: diet and liver synthesis. The cholesterol is metabolized to various steroids by the many carbon specific hydroxylases and hydroxyl specific dehydrogenases which are mostly found in the mitochondria(reviewed by Zubay, 1988). Fig.5 shows the synthetic pathway of the most common steroids and the enzymes involved(Gore-Langton et al, 1994). Not all steroid metabolites are active. For example E4, estetrol, an estrogen, is an inactive metabolite(Yen, 1991), and similar examples are found for progesterone. Both in the ruminant placenta and rat and human corpus luteum, progesterone is converted to 17α,20α-dihydroxypregn-4-en-3-one(Yen, 1991; Tsang et al, 1987; Taya et al, 1981). This product is

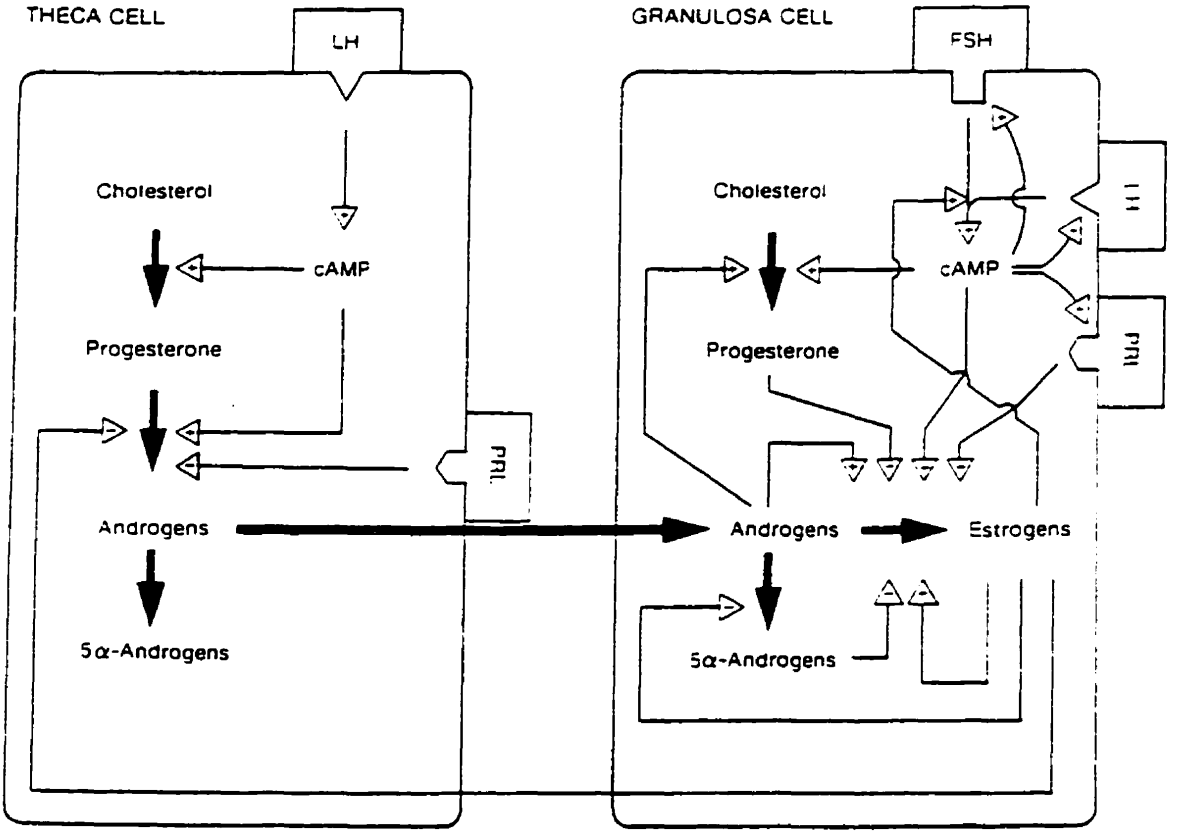


Fig.4 Gonadotropin stimulation of steroid synthesis in the ovary. Notice that androgen synthesis occurs in thecal cells, and subsequently, they are aromatized to estrogens within the granulosa cells. Diagram taken from Gore-Langton and Armstrong,1994.

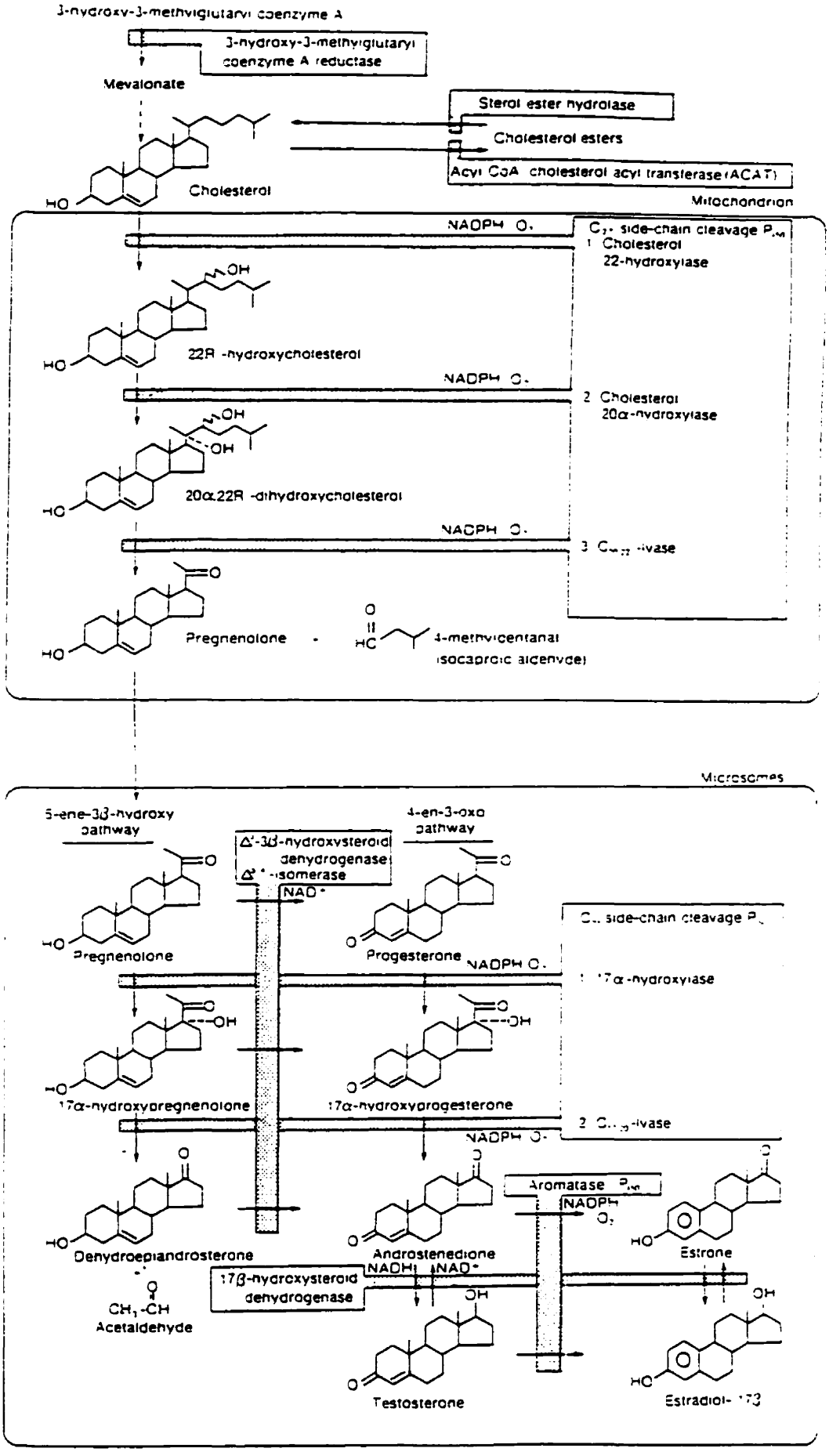


Fig.5 Synthesis of ovarian steroids. Diagram taken from Gore-Langton and Armstrong, 1994.

biologically inactive and when its serum levels rise, progesterone levels decline. Its levels are regulated and increased by fetal cortisol which increases 17-hydroxylase activity (Anderson et al, 1975).

1.2.1.3 Prolactin

In rats, unlike in many other species, prolactin is required for the continued viability of the corpus luteum up to day 6 of pregnancy. It is produced and stored in vesicles and released in a pulsatile fashion within the lactotroph cells, also located in the anterior pituitary. Being similar to other pituitary hormones, the expression and release of prolactin has elements of both positive and negative regulation. It has been demonstrated that adrenergic hormones, epinephrine and norepinephrine, and oxytocin stimulate prolactin release and, in contrast, dopamine inhibits it(Freeman, 1988).

Past day 6, the expression of decidual luteotrophin(LTH), decidual prolactin related protein(dPRP), and prolactin like protein(PLP)-B in the decidua and placental lactogen I which is expressed in the giant trophoblasts keep the corpus luteum viable up to day11 of pregnancy. Past day11, placental lactogen II, PLP-A,C, and PLP-Iv which are expressed in the giant trophoblasts and spongiotrophoblasts, and PLP-B which is expressed in spongiotrophoblasts takes over this function. It has been shown PL-I and PL-II not only bind the prolactin receptor but are functional analogs(reviewed by Soares et al, 1991). The prolactin receptor in the ovary is found in two forms which differ in the cytoplasmic domains(Shirota et al, 1990). Therefore, in addition to having multiple ligands, the prolactin receptor signalling adds another level of complexity to prolactin actions. These changes are summarized in Fig.6 which depicts the expression and serum concentrations of these proteins during pregnancy.

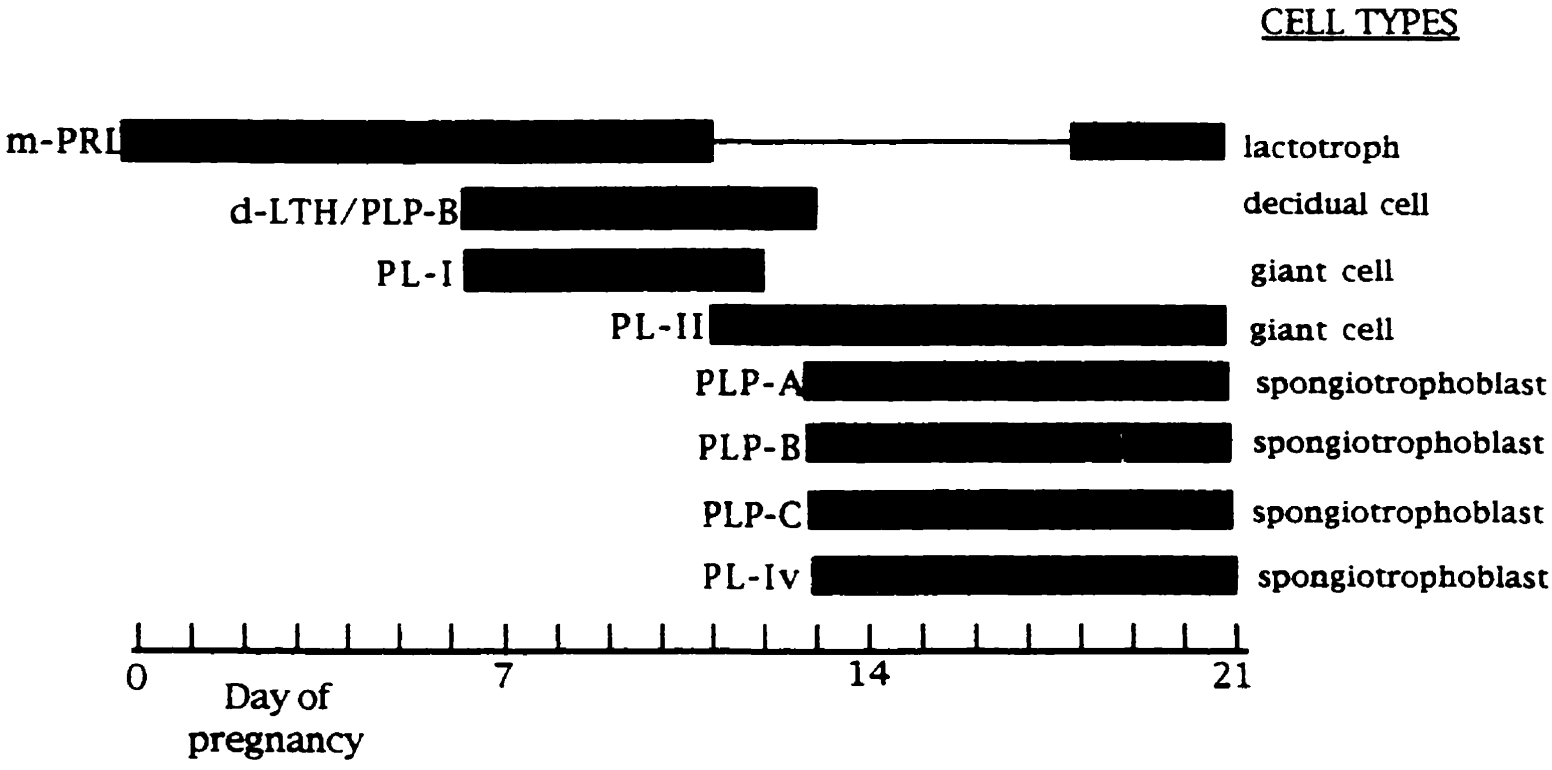


Fig.6 Schematic diagram of the temporal expression of the PRL gene family during pregnancy in the rat. m-PRL:maternal anterior pituitary PRL; d-LTH:decidual LTH. Figure obtained from Soares et al.,1991.

1.2.2 Corpus Luteum

In most species, the corpus luteum synthesizes estrogens and progestins that are required for implantation. Its life span is determined by luteotrophic and luteolytic factors. Prolactin, as previously mentioned, which is secreted upon activation of the cervical neuroendocrine reflex, allows the corpus luteum to be viable up to day 11 of gestation. This extended period is defined as pseudopregnancy as mechanical stimulation of the cervix at estrous will also reproduce these effects. If fertilization doesn't occur, the corpus luteum is actively regressed or luteolized and the cycle continues uninterrupted(Auletta et al, 1988).

Luteolysis in sub-primates is uterine dependent. If hysterectomy is performed post-estrous the corpus luteum remains viable spanning a normal gestational period. For example, in the rat this would be 23 days and in the rabbit this would be 30 days(Shaikh et al, 1977). The active luteolytic component has been reduced to Prostaglandin $F_{2\alpha}$ which not only rises in the ovarian vein during diestrous and at the end of gestation but when administered leads to regression of the corpus luteum(Horton et al, 1976; Smith et al, 1993). Postulated mechanisms include an inhibition of LH receptor function by increasing cytoplasmic calcium levels. Since the LH receptor is coupled to cAMP, it has been shown that elevated calcium levels interfere with the cAMP effects(Auletta et al, 1988).

1.2.3 Steroid Receptor Expression

Like most ligands, steroid function is dependent on binding and activation of its receptors. Within the cells, steroid receptors are expressed in the cytosol bound to heat shock proteins 90kDA, 70kDA, 56 kDA, 45kDA(Pratt et al, 1992; Mantel-Guiochon et al, 1993). Upon binding the lipophilic steroids, they usually homodimerize, become detached from hsp90 and are transported into the nucleus(Halachmi et al, 1994) where they bind to deoxyribonucleic acid(DNA) near the promoter to initiate transcription(Funder et al, 1993; Luisi et al, 1991). The DNA sequence is usually a palindrome spaced by 3 nucleotides(Truss et al, 1993; Richard et al, 1990). Once bound, at the promoter it interacts with the basal

transcriptional machinery(RNA polymerase II, transcriptional complexes of A,B,D,E,F, and H) and enhancer elements such as acidic, glutamine rich or proline rich proteins which increase the rate of initiation and therefore, eventually increasing the number of mRNA transcripts(Webb et al, 1995; Struhl et al, 1996; Verrijzer et al, 1995; Ptashne et al, 1990; Rastinejad et al, 1995; Hernandez et al, 1993; Herbomel et al, 1990; Choy et al, 1993; Levine et al, 1989; Johnson et al, 1995; Buratowski et al, 1995; Greenblatt et al, 1991; Johnson et al, 1989; Blackwood et al, 1991; Harrison et al, 1991; Beg et al, 1993; van Holde et al, 1992; Felsenfeld et al, 1992; Krisna et al, 1994; Berger et al, 1996; Mitchell et al, 1989; Bradshaw et al, 1991; Keller et al, 1993; Vivat et al, 1992; Ing et al, 1992; Wahli et al, 1991).

As previously mentioned, the uterus expresses the estrogen and progesterone receptors but their expression levels and activity differ between myometrium and endometrium. Whereas the myometrium expresses estrogen and progesterone receptors constitutively, this is not the case for the endometrium(Brenner et al, 1991). In most species, occupancy of the progesterone receptor leads to downregulation of estrogen receptors in epithelial and stromal cells. Eventually progesterone receptors are only expressed in the stroma(Brenner et al, 1991; Okulicz et al, 1993). A slight variation occurs in glandular epithelial cells. These cell types, even under high progesterone concentrations, still express estrogen and progesterone receptors. As progesterone levels continue to rise during pregnancy, the endometrium becomes quite unresponsive to estrogen. A slight variation occurs in capillary endothelial cells; they express the estrogen receptor more or less in a constitutive fashion(Brenner et al, 1991).

The progesterone receptor has a Kd of 5nM for progesterone (Gronemeyer et al, 1987). The estrogen receptor has a Kd of 0.5nM for17-β estradiol(Metzger et al, 1988).

Within the past decade, the cDNA for most steroid, retinoid and thyroid receptors and the related orphan receptors have been cloned and expressed. Analysis of the coding regions and proteins has shown conserved functional domains that have been evolutionarily preserved(Schwabe et al, 1993; Luisi et al, 1991; Bourguet et al, 1995;

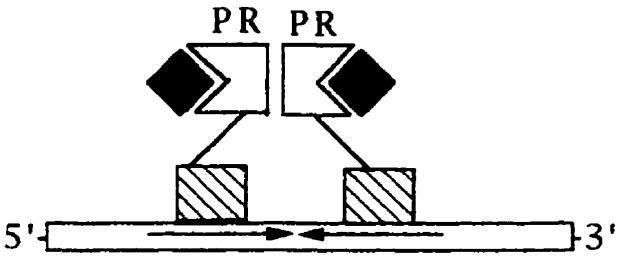
Danielian et al, 1992; Issemann et al, 1990; Murray et al, 1988; Liu et al, 1993; Wang et al, 1989; Wilson et al, 1992; Kliewer et al, 1992; Leid et al, 1992; Naar et al,1991). Fig.7A shows a typical steroid receptor(Schwabe et al, 1993). The A/B domain differs between receptors and its involved in transcriptional activation. The C domain is involved in DNA binding and it recognizes specific nucleotide sequences present in the genome. The D domain is a variable hinge domain. The E domain is the ligand binding domain which recognizes the specific ligand(Mangelsdorf et al, 1995; Giguere et al, 1994). These domains function independent of each other allowing the formation of chimaeric receptors. For example, exchanging the estrogen ligand binding domain with the cortisol binding domain results in estrogen inducing cortisol related genes and the opposite applies for cortisol(Truss et al, 1993). Fig.7B,C highlights the above mentioned points.

1.2.4 Endocrine Changes During the Cycle

The following endocrine changes occur during the estrous cycle. In the afternoon of proestrous, the rising plasma estrogen levels accompanied with low progesterone levels stimulate the secretion of pituitary LH, FSH and prolactin. For the LH hormone, receptor activation leads to increased steroid synthesis in the Antral follicles, ovulation which occurs in the morning of estrous and the extrusion of the first polar-body from the ovum(Evans et al, 1988; Ayalon et al, 1972; Mandl et al, 1963). As the rising hormone levels are transient, they return to basal levels in late estrous. Approaching and within diestrous the corpus luteum production of both estradiol and progesterone increases which is evident in their plasma levels(Thorncroft et al, 1971). In the second half of diestrous, the corpus luteum begins to regress and the progesterone levels drop but the estradiol levels keep rising due to sustained and increasing production from the developing follicles and the cycle renews. Fig.8 presents the serum values of these hormones(Smith et al, 1975).

A)

B)



C)

PRE	5'AGAACAXXXTGTTCT3'
ERE	5'AGGTCAXXXTGACCT3'
GRE	5'AGAACAXXXTGTTCT3'

Fig.7 A: Generalized structure of a steroid receptor, A/B-transactivation domain, C-zinc finger DNA binding domain, D- variable hinge region, E-ligand binding domain, F-variable C-terminal domain; B: Progesterone receptor homodimerization and binding to DNA. C: Response elements for progesterone(PRE), estradiol(ERE) and glucocorticoid hormone(GRE).

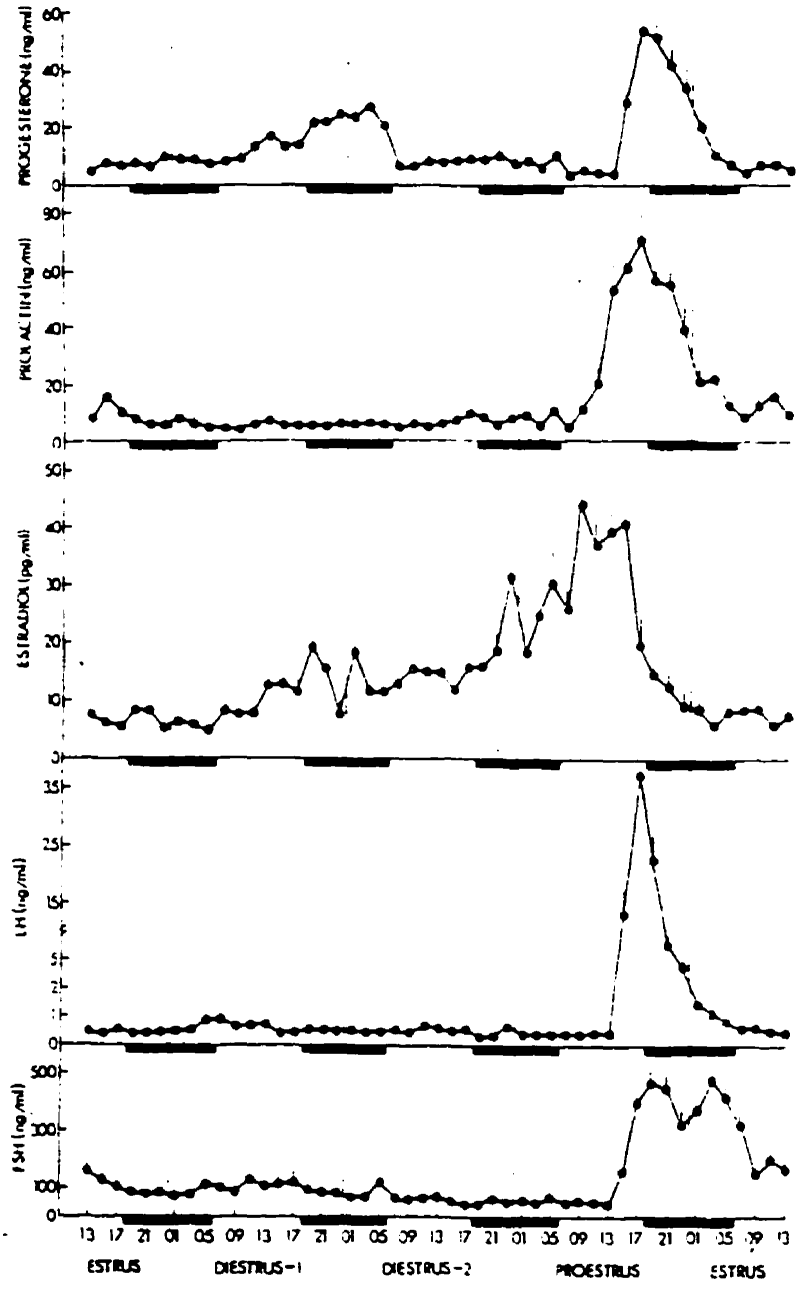


Fig.8 Measurements of rat serum progesterone, prolactin, estradiol, LH and FSH during the 4-day estrous cycle. The time of day during each stage is indicated along the horizontal coordinate. Diagram taken from Smith et al.,1975.

1.3.1 Implantation

The surging plasma LH levels in proestrous, as previously stated, induces ovulation and the release of the first polar body. The released follicles, now containing the zona pellucida and attached granulosa cells, remain in the oviduct until feritilization which normally occurs in the morning of estrous. Sperm entry induces the release of the second polar body and, finally, making the follicle into a haploid cell. Fusion of the maternal and paternal nuclei form the initial zygote which begins cleavage approximately within 25-27 hrs. The zygote continues to divide until the eight cell stage, now referred to as the morula, whereby it begins to compact. Up to this stage the cells are equipotent, meaning each cell has the capacity to give rise to an entire organism. From the morula stage, the embryo begins to differentiate into the blastocyst. The differentiated blastocyst has a blastocoel, a mural and polar trophoectoderm and an inner cell mass. Whereas the mural trophoectoderm interacts with the luminal antimesometrial epithelial cells to implant, the polar trophoectoderm will form the ectoplacental cone and finally the chorioallantois placenta. The inner cell mass gives rise to the embryo and extraembryonic membranes(Fig.9 summarizes the lineage). At this stage the embryo has entered the uterus and is ready for implantation. At the time of implantation, which occurs around day4-5 of pregnancy, the luminal epithelial cells which by now lose their microvilli become tightly juxtaposed around the hatched blastocyst and attaches to the trophoblast cells. Once the trophoblasts binds to the epithelium, it extravasates into the sub-epithelial decidua and begins to proliferate and expand(reviewed by Hogan et al, 1994; Cross et al, 1994; Parr et al, 1989).

Implantation requires changes within the epithelium. Ovariectomy at day4 delays implantation. If the steroids estrogen and progesterone are injected upto 30 days later, implantation resumes(Parr et al, 1989). Therefore steroids modify the epithelium receptivity. From these initial experiments reproductive biologists have begun to identify some of these induced genes. For example genetic ablation of the leukemia inhibitory factor(LIF) prevents implantation and supplementation of the recombinant

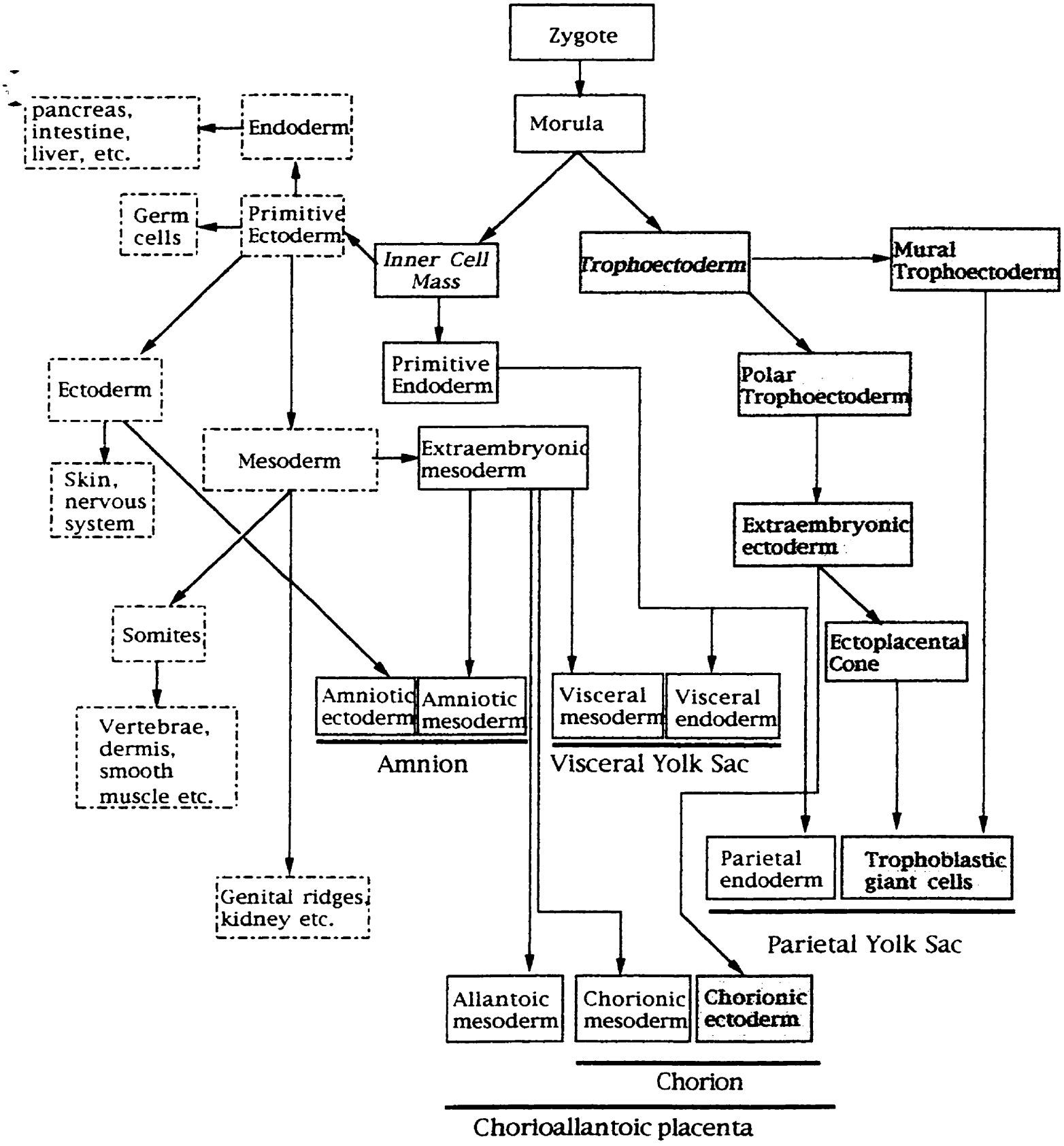


Fig.9 Schematic diagram of fetal membrane development.

protein, by injection, rescues the embryo(Stewart et al, 1992). Another factor identified is Interleukin-1β(Strickland et al, 1992). Neutralization of this cytokine by antibodies also prevents implantation. In summary, it is believed steroids alters the differentiation state of the epithelium which allows the embryo to attach.

1.3.2 Decidualization

Just prior to implantation a few changes occur only in the endometrial sites apposed to the implanting blastocyst. One of these changes is increased blood flow accompanied with vasodilation and edema. The uterus gains weight mostly due to water retention. The other changes consist of an increase in histamine and glycogen content and the process of decidualization. Stromal cells, at these sites, proliferate and differentiate into epitheloid-like cells which are polyploid at certain stages. This phenomenon of decidualization has been compared to an acute inflammatory reaction as the process involves not only increased prostaglandin formation but an influx and involvement of immune cells such as macrophages and mast cells. The exact function of these decidual cells remains to be determined but certain hypotheses put forward include restriction of the trophoblastic invasion, a source of nourishment, the formation of a cleavage zone for placental separation, and protection of the embryo from the maternal immune system (reviewed by DeFeo et al, 1967).

Decidualization begins from the anti-mesometrial side and gradually reaches the mesometrial face around day 9. At this stage the mesometrial decidual cells contain abundant glycogen which will provide energy for the implanted embryo. Collectively, the number of decidual cells and the size of the decidual region reach a maximum at around day11 of pregnancy, thereafter, it begins to recede reaching a minimum towards the end of pregnancy(Strickland et al, 1992).

Artificially, scientists are able to induce the decidualization reaction. After an infertile copulation, the corpus luteum persists extending the full length of pseudopregnancy. During pseudopregnancy, various agents

added to the uterus around day4 or day5 will transform the stroma into a decidual mass or collectively referred to as a deciduoma. These agents include scratching the surface, injection of substances such as oily fluids and air to mention a few. As stated above, the uterus is only receptive to these changes on day4 and day5 of pregnancy which has been shown to be under hormonal control(Table I)(DeFeo et al, 1967; O'Shea et al, 1983).

Whereas ovariectomy plus progesterone supplementation permits the uterus to become decidualized, estrogen supplementation has no effect on decidualization. On the other hand, both steroids given together, improves the response(DeFeo et al, 1967; Sananes et al, 1978; Bell et al, 1981; Vladimirsky et al, 1977). Therefore, the given changes are progesterone dependent. This conclusion is further supported from the PR-knockout mice which are infertile and lack the decidual response(Lydon et al, 1995). In addition to steroids, prostaglandins, as well, seem to play an essential role. Data such as the dramatic rise in the content of prostaglandin E_2(PgE_2) at day7 of pregnancy and the effects of non-steroidal antiinflammatory drugs(NSAID) which have been shown to inhibit prostaglandin synthesis and decidualization(Vladimirsky et al, 1977; Castracane et al, 1974) support their involvement. The effects of PgE_2 have been shown to be vasodilation of capillaries(Kennedy, 1979), immuno-suppression(Kelley, 1994), transformation of stromal cells to decidual cells(Kennedy et al, 1982) and finally, inhibition of myometrial contractility(Honda et al, 1993). The link between progesterone and prostaglandins is as follows: progesterone is able to increase the number of endometrial PgE_2 receptors with approximately three fold greater affinity for PgE_2(Kennedy et al, 1983), as assessed from binding studies. Therefore, the corpus luteum by producing progesterone increases the endometrial PgE_2 receptors gradually approaching implantation. If the pregnancy is fertile, the embryo elicits a decidual reaction, compared and similar to an acute inflammatory reaction, with an increase in PgE_2 content and an autocrine mediated activation of its receptor. Whether PgE_2 is the sole determinant in the decidual reaction remains questionable. Although studies have indicated for their involvement(Kennedy et al, 1982), the recent cyclooxygenase1 and 2 knockout mice indicate implantation and pregnancy still occur in homozygous inbred mice(Welsh et al, 1993; Roberts et al, 1996). These experiments raise interesting questions such

Table I. INFLUENCE OF DAY OF SCRATCH ON CAPACITY FOR DECIDUOMA FORMATION

Strain	Day2[a]	Day3[a]	Day4[a]	Day5[a]
Sprague-Dawley	218 +/- 9 (n=5)	643 +/- 113 (n=11)	2080 +/- 69 (n=12)	262 +/- 32 (n=10)
Long-Evans	209 +/- 12 (n=6)	948 +/- 132 (n=14)	1318 +/- 64 (n=18)	381 +/- 37 (n=24)
Wistar	268 +/- 18 (n=10)	1210 +/- 56 (n=9)	1521 +/- 92 (n=10)	276 +/- 24 (n=10)

[a]Uterine weight(mg) +/- SE that was obtained 5 days after trauma.

as:1) Is the liberated arachidonic acid rerouted to the lipooxygenase pathway, and 2) Are leukotrienes involved in the decidualization process?

1.3.3 Fetal Membrane Development

Postimplantation, the embryo needs to form various membranes to absorb nutrients. Of these membranes, the first to be formed is the choriovitteline placenta. The cell types for this placenta are derived from the primary endoderm which gives rise to visceral and parietal extraembryonic endoderm cells. The parietal cells form the Reichert's membrane and atop this membrane sit the giant mural trophoectoderm cells. Surrounding the trophoectoderm is the decidua capsularis. In between the parietal and visceral membranes, blood vessels form the mesenchymal cells allowing for a primitive circulatory system. As the embryo grows, the amnion which is derived from the primitive ectoderm, eventually surrounds it with its amniotic fluid. And finally, the allantois forms near the amnion and ectoplacental cone, and eventually fuses with the chorion and becomes the chorioallantois placenta at around day10 of gestation(Cross et al, 1994; Soares et al, 1987; Perry et al, 1981; Paavola et al, 1995). The aforementioned fetal membranes are important for parturition which will be discussed later. Fig.10 depicts the given changes.

1.3.4 Placentation

Although certain mammals are oviparous(egg-laying), the monotremes- duckbill platypus and spiny anteaters- being such examples, the majority which belong to the subclass eutheria are viviparous and require a placenta. Within this subclass, the placenta, which is a general term to describe a fetal-maternal interface, can't be readily defined by its shape, size or the manner which it becomes attached to the uterus. In regard to evolutionary development, it has become divergent. Broadly, they are categorized as follows: 1) hemochorial placentation- the trophoblasts traverse past the epithelium and invade the maternal vasculature, primates and rodents are examples, 2) endotheliochorial placentation- the trophoblasts again traverse past the epithelium but do not invade the

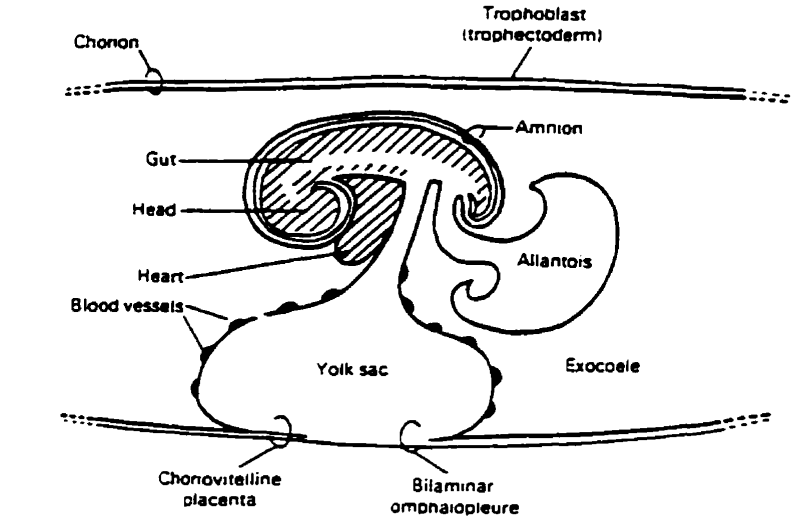


Fig. 3.

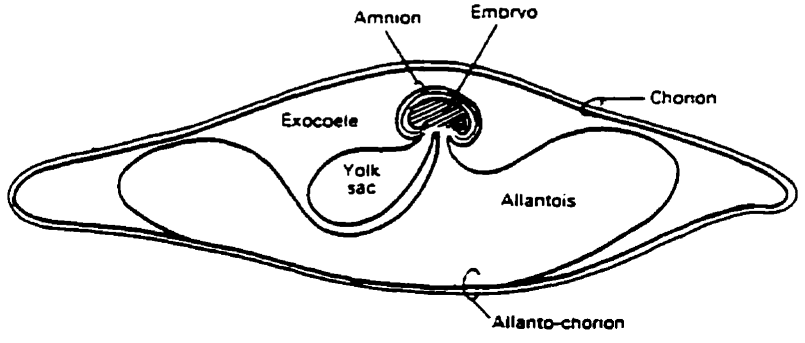


Fig.10 Fetal membranes of the pig at days18(top) and days21(bottom). Notice the change from a choriovitelline to a allonto-chorion placenta. Diagram taken from Perry, 1981.

maternal vasculature, cats and dogs are examples, 3) synepitheliochorial placentation- the trophoblasts fuses with the epithelium, sheep and cows are examples, 4) epitheliochorial placentation- the trophoblasts only attaches to the epithelium, camels and pigs are examples(Roberts et al, 1996; Ramsey, 1982). Therefore a heterogenous pool of shapes and attachments bear the same effects. To make the subsequent discussion more applicable to the forthcoming chapter, only rodents will be considered.

In rodents, the chorioallantois, which is another term for hemochorial, and choriovitteline(which is absent in humans) placentas are quite important for fetal viability approximately from day7 onwards. Not only are they are involved in nutrient absorption, gas exchange, and immunosuppression but their endocrine hormones sustain the corpus luteum past day12 unto day22 in the mouse and day23 in the rat. They produce many hormones such that it has been described as a condensed hypothalamic-pituitary axis. Of these many hormones, an emphasis will be placed on those that have been implicated in either initiating labour or involved in the process.

The choriovitelline placenta regresses and becomes non-functional on day14 of pregnancy. The chorioallantois placenta overtakes this function at around mid-gestation. The latter placenta can be compartmentalized into two parts: a junctional zone and a labyrinth zone(Fig.11). Differences between the two includes the absence of fetal vasculature cells in the junctional zone and the identification of different trophoblast cells in both layers(Soares et al, 1991; Perry et al, 1981; Ramsey, 1982).

At least four types of trophoblasts have been identified in the placenta: cytotrophoblasts, syncytiotrophoblasts, spongiotrophoblasts and giant trophoblast cells. Cytotrophoblasts are the undifferentiated precursor cells derived from the polar trophoectoderm. They differentiate into synctiotrophoblasts and help form the labyrinth zone. These cell are polyploid and bear extended and extensive microvilli for absorbing maternal nutrients and excreting fetal waste. They express inert MHCI and lack Major Histocompatibility Antigens(MHC)II expression making them ideal barriers to maternal blood and minimizing an alloreactive immune

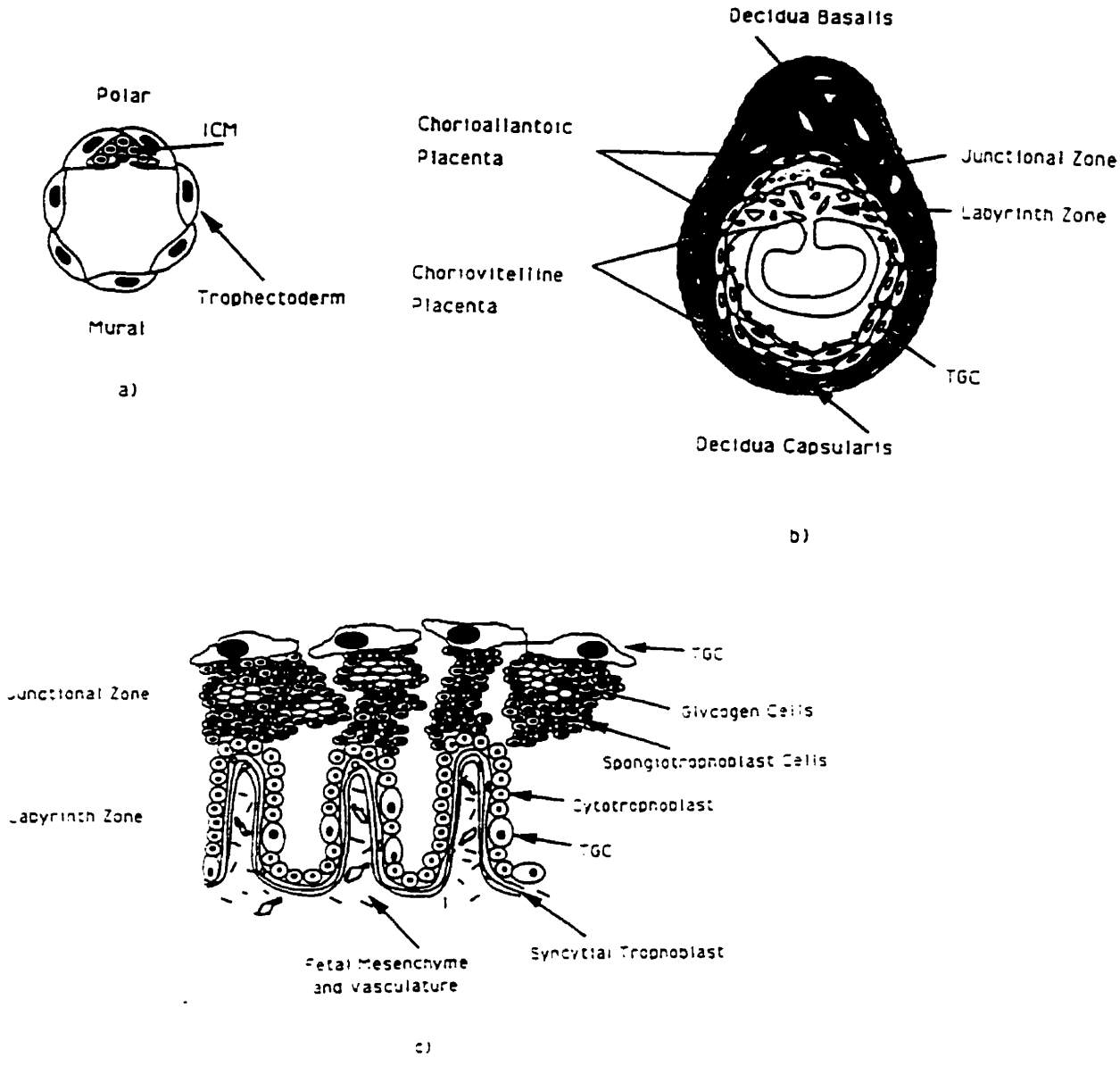


Fig.11 Schematic diagrams of the trophoblast cells at the various stages of pregnancy. a) blastocyst; b) midgestation; c) late gestation chorioallantoic placenta. ICM, inner cell mass; TGC, trophoblast giant cells. Diagram taken from Soares et al., 1991.

response. The giant trophoblast cells are mural trophoectoderm trophoblasts which have fused into giant cells. They are multinucleated and are the first barrier to maternal cells just after implantation. Spongiotrophoblasts are found beneath the giant trophoblast cells along with glycogen cells in the junctional placenta(Soares et al, 1991).

The chorion of the placenta forms the umbilical vein and arteries of the embryo and therefore is very important for nutrient exchange. Underneath the placenta lies the decidua basalis and the decidua in between the implantation sites are referred to as decidua vera. The placenta at the decidua basalis is referred to as the chorionic villi with its extensive projections into the uterine arterial sinuses(Jaffe et al, 1991).

The placenta is able to produce steroids such as progestins(most mammals), estrogens(subprimates and rodents), and glucocorticoids, human chorionic gonadotropin(hCG), oxytocin, vasopressin, corticotrophin releasing peptide(CRF), adrenocorticotrophin hormone(ACTH), enkephalins, dynorphins, lactogens(prolactin, placental lactogens etc.), prostaglandins, cytokines, interferons, inhibin/activin, human chorionic somatomammotropin(hCS), and growth hormone to mention a few(Tabibzadeh et al, 1991; Handwerger et al, 1992; Soares et al, 1991; Jaffe et al, 1991; Lefebvre et al, 1992; McLean et al, 1995).

1.3.5 Maternal Recognition of Pregnancy

Implantation, and placentation are only the first steps for viability of the embryo. The next and equally important step is suppression of luteolysis and prolongation of the maternal cycle. Again, within the eutherian subclass, different methods are employed to achieve the same beginning. Roberts et al.(1996) have reviewed these different approaches within various species.

In primates, the syncytiotrophoblast produces human chorionic gonadotropin(hCG) which is a functional analog of LH and which prevents luteolysis. The protein is 36-40kDA in size and its serum levels peaks in the first trimester and declines thereafter. The presence of hCG in serum

and its luteotrophic effect is an indication of maternal recognition of pregnancy.

In ruminants, the trophoblasts produces a type I interferon. Interferon-τ suppresses oxytocin receptor expression and the synthesis of the pulsatile luteolysin $PgF_{2\alpha}$(Roberts et al, 1992). The Interferon-τ is very similar to Interferon-α which has anti-viral effects and binds the same receptor.

In swine, injection of estrogen into a cycling animal will prolong the cycle, therefore estrogen is luteotrophic. It is believed since the embryo produces estrogen, that the fetal estrogen also has the same effects. The results remain inconclusive since there is disagreement in the literature.

1.36 Overview

Table II(Cross et al, 1994) summarizes the necessary and sequential steps of pregnancy.

Table II. CHECKPOINTS IN MOUSE INTRAUTERINE DEVELOPMENT

Days of gestation	Event
3.5	Blastocyst formation
4.25 to 4.5	Blastocyst activation
4.5 to 6	Implantation
6 to 8	Formation of the yolk sac and vitelline circulation
9 to 10	Development of the chorioallantoic placenta
8 to 18	Development of fetal vasculature
20 to 21	Labour or Parturition

CHAPTER 2

PARTURITION

2.1 Parturition

Parturition and labour are synonyms which describe the process of delivery when fetal development has reached completion; in humans this would be on average after 39-40 weeks of pregnancy(Okulicz et al, 1993). Usually, preparturient mothers experience in some cases days, in other cases hours, strong uterine contractions, weakening of fetal membranes and finally cervical dilatation just prior to delivery(Chan et al, 1992; Husslein et al, 1981). The duration of delivery per fetus may vary from hours in humans to ten minutes in rodents. After delivery, the fetal membranes such as the placenta become detached from the uterus and are excreted in humans or absorbed in rats.

In retrospect, when compared to the establishment of the agricultural economy in Mesopotamia dating back 11,000 yrs ago, the history of science and especially reproductive biology is relatively new. Given the great philosophers of antiquity and the mythology of Aphrodite and Zeus and Cleopatra and Ceasar, one might surmise every great civilization had an understanding of birth but only recently have we begun to understand this process in molecular terms.

Scientists undertaking this research determined the possible initiatory signals originated either from the maternal immune system or fetal and/or maternal endocrine derived factors and not the peripheral nervous system given the neural input to the uterus progressively diminished from conception onwards(Stjernquist et al, 1994).

The hypothesis that the endocrine system was the determinant factor in initiating parturition was strongly supported from studies done in

ruminants. Investigators found injection of CRH, ACTH, or cortisol into the fetal vasculature resulted in premature labour usually within 48hrs(Thornburn et al, 1979; Anderson et al, 1975). The manner in which fetal cortisol brings about these effects includes lowering the circulating progesterone concentrations by increasing its metabolism and increasing prostaglandin synthesis, the effects of which will be discussed later. Progesterone withdrawal usually leads to the abolition of the inhibitory mechanisms on uterine motility, increased gap junction formation and gating and synchronous contractions(Alexandrova et al, 1980). In addition, the cervix becomes dilated, the fetal membranes rupture and delivery follows(Huszar et al, 1986). Unfortunately, although placental CRH levels increase from the first trimester onwards and are a correlative indicator of premature labour(McLean et al, 1995), cortisol doesn't have the same effects in humans and furthermore, unlike in ruminants, serum progesterone levels don't decline(Yen, 1991).

In contrast to ruminants, parturition in primates seems to be much more complex. Current understanding points to a model where both release of inhibitory mechanisms and induction of activators must coincide. A threshold must be reached where activator function overseeds inhibitory capacity. Intertwined with these functions are the preparatory steps which gradually take hold during gestation. These include fetal membrane weakening, increase in smooth muscle numbers, size, membrane capacitance, and electromechanical coupling(Garfield, 1994).

Early results towards this model were mostly associative and derived from other species. Recently, within the last thirty years, more direct data have been obtained. For example, withdrawal of progesterone through receptor antagonism(Vogeto et al, 1992; Benhamou et al, 1992; Baulieu, 1989; Beaulieu, 1991), injection of prostaglandins(Mitchell, 1984), and injection of the peptide oxytocin(Green et al, 1974; Buckle et al, 1975), collectively induce premature delivery. Therefore both withdrawal of inhibition and availability of activators are needed. Although oxytocin and prostaglandins are both activators, differences do exist. Whereas prostaglandins are effective throughout pregnancy, oxytocin is effective only near term(Husslein et al, 1981; Topozoda et al, 1984; Owen et al, 1992).

Aside from the endocrine system, there is also associative data which implicates the immune system. For example, preparturient rupture of fetal membranes and its infection usually leads to premature labour(Romero et al, 1991). Additional and more direct data was obtained from mice. Injection of IL-1β into pregnant mice leads to premature labour and injection of its natural antagonist prolongs delivery(Romero et al, 1991; Romero et al, 1992; Romero et al, 1994). Prior to this work, it was known that certain syngeneic intercrosses in mice had high abortion rates, therefore the immune system was certainly involved(Gendron et al, 1992). In humans, a similar phenomenon exists where the maternal immune system rejects certain paternal antigens. If the mother is sensitized by vaccinating her with the paternal antigens, oddly enough, the embyro becomes viable indicating maternal immune suppression to the embryonic allograft must be an active process(Lala et al, 1986). Prior to interpreting the data, the following considerations should be noted: 1) MHCI and MHCII knockout mice are fertile and deliver normally(Grusby et al, 1993; Auchincloss et al, 1993), and, 2) mice deficient in NK cells, macrophages and lymphocytes are also fertile and deliver normally(Croy et al, 1990). Therefore, possibly the CD1 or other undefined minor histocompatibility antigens are involved. Evidence for this is derived from xenogeneic crosses done by Copp and Rossant(1978). When rat blastocysts were added to mice uteri, they implant and expand but the opposite effect fails to take place for mice blastocysts.

In all models examined so far, whether it is the primates, ruminants, or rodents, maternally derived oxtyocin and fetally and/or maternally derived prostaglandins have been shown to be involved in the process of parturition. Both factors are more or less conserved elements of labour. The following sections summarize the current molecular biology of both factors in the context of a simple model.

2.2 Uterine Oxytocin/Oxytocin Receptor Gene Expression

A excellent and current review of both OT and OTR has been published by us(Appendix A, Zingg et al, 1995). Topics not covered in the review will be presented in this section.

The oxytocin receptor has many effects on term myocytes. These include depolarizing the sarcolemmal membrane, increasing cytosolic IP_3 levels, inhibiting SR calcium pumps, and increasing the frequency of contractions(Kuriyama et al, 1976; Schrey et al, 1988; Wallace et al, 1993; Soloff et al, 1989). The net effect is breaking the fetal membranes and delivery of the fetus into an external environment. The OTR being a seven transmembrane domain receptor is thought to be coupled to G-proteins which in turn activate phospholipase C(PLC) and ion channels(Kimura et al; Moore et al, 1991). PLC releases IP_3 from phosphotidylinositol leaving diacylglycerol(DAG). DAG in turn accompanied with the increasing cytosolic calcium levels activates Protein Kinase C(PKC). As stated above, when OT is added to myocytes, it is able to depolarize the membrane. It not only increases the number of bursts but the number of spikes per burst. In general, the greater the number of spikes per burst amounts to larger tension values per contraction(Fuchs et al, 1976).

2.3.1 Prostaglandins/Leukotrienes

In 1930, Kurzrok and Lieb observed seminal fluid induced contractions in human uterus. From this early study, von Euler then proceeded to extract and purify the active components in 1934 and coined the term prostaglandin. Approximately twenty-six years later, Bergstrom and Sjovell(1960) crystallized PgE_2 and $PgF_{2\alpha}$ and four years later von Dorp and Bergstrom demonstrated arachidonic acid and di-homo-γ-linolenic acid could be used as substrates. Within the same decade in 1968 Karim and Wiqvist et al. initiated studies on the actions of prostaglandins on pregnant uterus as a possible means for fertility control. Many other studies in the early seventies showed prostaglandins in the first trimester induced abortions and, near term, it induced uterine contractions and cervical dilatation when administered. In terms of regulatory factors, estrogen was

shown to increase their levels within the rat uterus(Wilson et al, 1983; Wilson et al, 1987). In pace with these efforts, in 1971, Vane showed aspirin, a plant extract, blocked prostaglandin synthesis. From this finding, aspirin was shown to prolong premature labour but due to certain side effects such as maternal gastrointestinal ulceration and premature closure of the ductus arteriosus further studies were discontinued. Recent cloning of the rate limiting enzymes in prostaglandin synthesis has opened new doors for improved treatments.

Both prostaglandins and leukotrienes are derivatives of arachidonic acid metabolism. Arachidonic acid is an essential fatty acid which is stored in either plasma membrane esterified to the sn-2 position of phospholipids, bound to cholesterol or triacylglycerol found in vesicles(Irvine et al, 1982). Hydrolysis of arachidonic acid by phospholipase A_2(PLA_2) liberates it where it is utilized by either cyclooxygenase1 or 2 or lipooxygenase5,12,15 into prostaglandin or leukotriene products respectively(Romero et al, 1991).

2.3.2 PLA2

There are two forms of PLA_2 that have been characterized in mammalian tissues:1) a cytosolic PLA_2($cPLA_2$), and 2) a secretory PLA_2($sPLA_2$). Both enzymes liberate arachidonic acid from the sn-2 position of phospholipids but differences reside in substrate preferences, localization and Ca^{2+} requirements(Mayer et al, 1993; Dennis et al, 1994; Clark et al, 1991). The 14kDa $sPLA_2$ has the characteristic of being secreted into the interstitium where it functions, having no preference for the fatty acid in the sn-2 position of phospholipids, requires mM amounts of Ca^{2+} and utilizes the amino acid histidine within the active site(Fourcade et al, 1995). In contrast, the 85kDA $cPLA_2$ has the characteristic of being translocated to the plasma membrane where it becomes active, doesn't require Ca^{2+} for activity but for translocation, has the preference for arachidonic acid in the sn-2 position of phospholipids, and utilizes the amino acid serine within the active site(Clark et al, 1991). Furthermore, $sPLA_2$ is active in liberating arachidonic acid from the inner leaflet from phosphotidylserine and phosphotidylethanolamine but not from

phosphotidylcholine which is located in the outer leaflet(Mayer et al, 1993; Dennis et al, 1994).

Cytokines such as IL-1β and TNF-α, growth factors, and endocrine hormones such as vasopressin are able to activate PLA_2 activity(Bonventre et al, 1990; Margolis et al, 1988; Bry et al, 1992; Hanmin et al, 1994; Kolesnick et al, 1994). In some cases by transcriptional activation, which occurs for the $sPLA_2$, in other cases G-coupled mechanisms, and still in other cases by PKC phosphorylation(Nakazato et al, 1991; Axelrod et al, 1988; Parker et al, 1987). In contrast glucocorticoids such as cortisol and certain Gα subunits, $G_t\alpha$, inhibit its activity(Axelrod et al, 1988; Jelsema et al, 1987).

2.3.3 Cyclooxygenase

Prostaglandin dehydrogenase or cyclooxygenases are enzymes involved in the conversion of arachidonic acid into the precursor PgH_2(Fig.12). Two enzymes for this activity have been cloned, namely, cyclooxgenase1 and 2(Otto et al, 1994; Winona et al, 1991). Although both enzymes perform the same function, they do differ. They are encoded by separate genes, their expression patterns and sizes differ, and furthermore, there are differences in the N and C terminus of the proteins(Lee et al, 1992; Tsuji et al, 1995).

2.3.3.1 Cyclooxygenase Gene

The genes for the two isozymes have been mapped and localized to their respective chromosomes. The gene for Cox-1, which extends more than 22kb, is located on chromosome 2 and the gene for Cox-2 which is less than 8kb, is located on chromosome1 in mice(Fletcher et al, 1992). Both genes are quite similar in their exon-intron organizations with minor differences in the 5' and 3' regions. Whereas the Cox-2 gene has 10 exons and 9 introns, the Cox-1 gene has 11 exons and 10 introns. In the Cox-2 gene, there are 3 additional nucleotides which code for a proline that is absent in the Cox-1 protein. In contrast, the Cox-1 gene has an additional

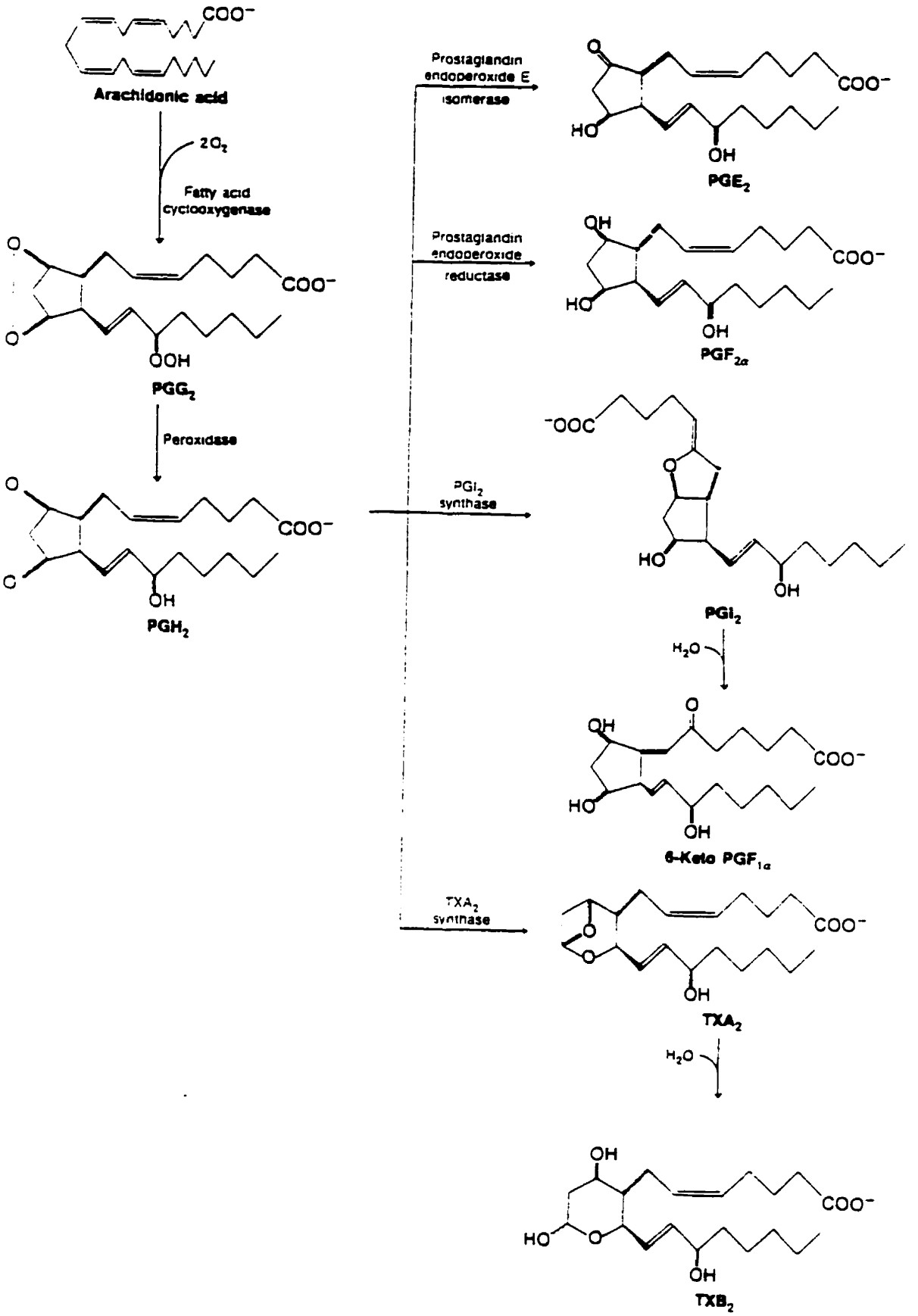


Fig.12 Schematic diagram of prostaglandin biosynthesis. Figure modified from Zubay, 1988.

exon, exon2, which codes for a hydrophobic leader sequence not present in the Cox-2 gene. Fig.13 summarizes these differences(Fletcher et al, 1992).

Both genes bear different promoters which is evident in their regulation. When examining Cox-1 gene expression, investigators have found most tissues constitutively express it(O'Neill et al, 1993). On the other hand, the Cox-2 gene is only expressed upon induction and has been defined as an immediate-early gene(Kujubu et al, 1991). Such factors as growth hormones, cytokines, and activators of PKA and PKC are able to induce it(Kujubu et al, 1991). The tissues that express it in relatively high levels include the prostate, uterus, and lungs(O'Neill et al, 1993).

2.3.3.2 Cyclooxygenase Protein

The ovine Cox-1 enzyme has been crystallized and its tertiary structure deduced. The protein contains 603 amino acids, it is 70kDa in size, functions as a dimer and is asparagine glycosylated. Amino acids 1-24 form the cleaved signal sequence. Amino acids 25-600 forms the mature protein. The tertiary structure of the mature protein is composed of three folding domains. The first domain, amino acids 34-72, is an EGF-like module linked to the main body of the enzyme by disulphide bridges. The second domain, amino acids 73-116, is composed of four amphipathic alpha helices A,B,C, and D which allow insertion into lipid bilayers. The third domain is the globular catalytic domain containing separate but intertwined cyclooxygenase and haem-containing peroxidase active sites(Picot et al, 1994; Picot et al, 1994).

The enzyme has an affinity for arachidonic acid of 5.2 μM, and its specific activity is approximately 68 μmoles oxygen consumed/mg protein per min(Percival et al, 1994; Barnet et al, 1994). It has a half-life of less than 10 minutes and furthermore, it irreversibly self-inactivates after 1500 substrate turnover(Maier et al, 1990). From immunohistochemical studies, it has been localized to endoplasmic reticulum and nuclear membranes. In contrast to simple reasoning, the enzyme has been shown to function within the lumen of the endoplasmic reticulum(Barnet et al, 1994). This would, therefore, indicate prostaglandin transporters must be

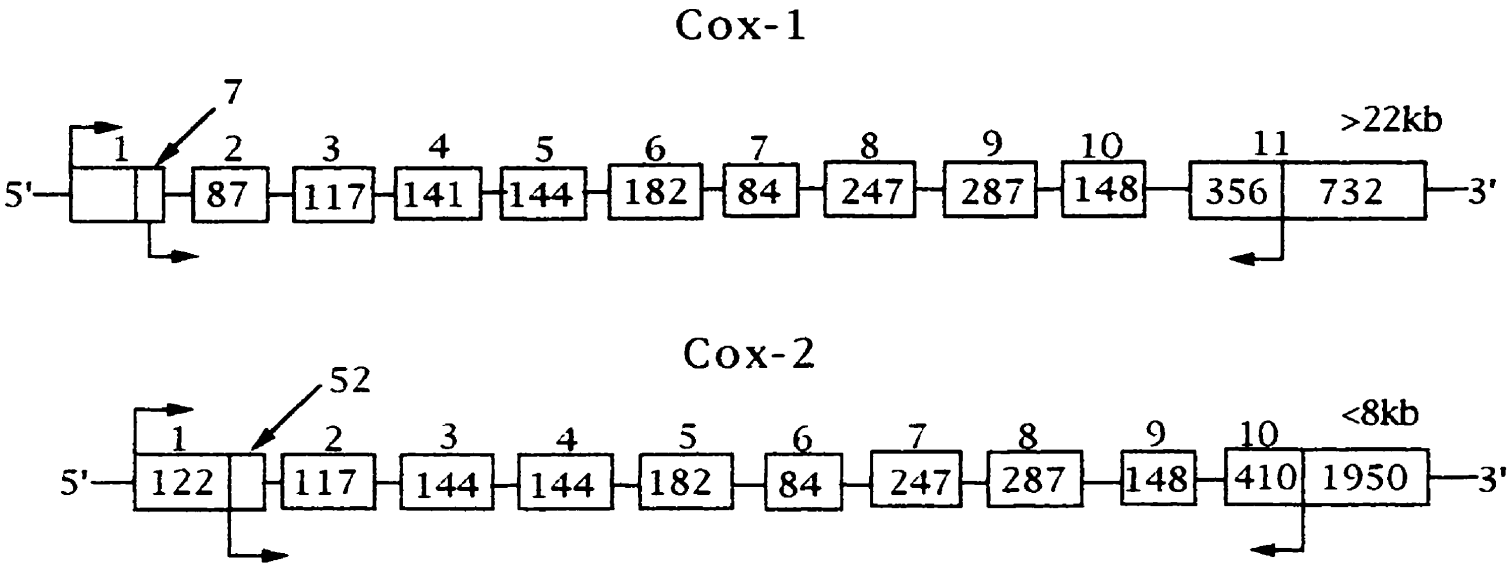


Fig.13 Schematic diagram of the Cox-1 and Cox-2 genes. The exon sizes are indicated within the squares. The bottom arrows indicate the coding region of the the transcript and the top arrow indicates the initiation of transcription. The size of the genes are as indicated. Figure obtained from Fletcher et al.,1992.

expressed on the endoplasmic reticulum or the produced prostaglandin precursor, PgH_2, must readily cross the ER membrane.

A comparison of the amino acid sequences of the isozymes Cox-1 and Cox-2 shows they are 60% identical and 75% similar(Barnet et al, 1994; Toh et al, 1992). The small differences reside in their N and C terminus and glycosylation. Cox-1 bears 17 amino acids present in its N terminus which is absent in Cox-2. In contrast, Cox-2 contains 18 amino acids in its C terminus which is absent in Cox-1. In addition, Cox-2 is differentially glycosylated to give apparent sizes of 72 and 74 kDA on a Western blot(Otto et al, 1994).

Immunohistochemical analysis of the Cox-1 enzyme during pregnancy has revealed expression in the placenta where its levels rise approaching the end of gestation, and in nonuniform amounts in the amnion, the chorion and decidua(Price et al, 1989; Wimsatt et al, 1995; Gibb et al, 1996; Boshier et al, 1991). Cox-2 expression has been shown in the placenta where, like Cox-1, it rises near term, and in the amnion, and decidua(Wimsatt et al, 1993; Teixera et al, 1994).

2.4 Prostaglandin Receptors

All cloned and sequenced receptors to date are seven transmembrane spanning domain receptors which are coupled to various secondary signals via G-proteins and which are glycosylated.

There are three reported receptors for PgE_2. Namely EP1, EP2, and EP3(Funk et al, 1993; Regan et al, 1994; Namba et al, 1993; Sugimoto et al, 1993; Breyer et al, 1994). EP3 has four splice variants EP3a,b,c,d which differ in the C terminus. Differences reside 10 amino acids after the seventh transmembrane domain. Furthermore, EP3a,d and EP3b,c are coupled to different secondary signals(Namba et al, 1993; Sugimoto et al, 1993; Breyer et al, 1994). It is believed EP3b,c increase cAMP levels by stimulating Gs and EPa,d increase IP_3 levels by stimulating Gi/Go and Gi/Gs/Gp respectively(Namba et al, 1993). The EP3 receptors have an affinity for PgE_2 ranging from 2.5-6.5 nM(Namba et al, 1993; Sugimoto et

al, 1993). The EP2 receptor is only coupled to cAMP. It has an affinity for PgE_2 of 11.2 nM(Honda et al, 1993; Regan et al, 1994). Lastly, the EP1 receptor is coupled to an increase in cytosolic Ca^{2+}. It has an affinity for PgE_2 of 1nM(249). EP1,2, and 3 are expressed in the uterus as judged from binding and Northern blots(Funk et al, 1993; Regan et al, 1994; Namba et al, 1993; Sugimoto et al, 1993; Breyer et al, 1994).

There is one reported receptor for $PgF_{2\alpha}$, FP, with an affinity of 8.3nm. It is highly expressed in the corpus luteum and moderately in the uterus. It is coupled to IP_3 turnover and increases cytosolic calcium levels(Sugimoto et al, 1994; Lake et al, 1994). Within the uterus, it is mostly expressed in the myometrium(Martel et al, 1985).

The receptor for prostacyclin is abundantly expressed in the myometrium and it has been shown to have a high and low affinity of 1 and 44 nM for the prostacyclin analog iloprost(since prostacyclin is unstable most binding studies are done with its analog) where it is coupled to the secondary signal cAMP. Functionally, it antagonizes the effects of prostaglandin receptors which are coupled to the secondary signal Ca^{2+}(Chegim et al, 1988; Omini et al, 1979; Boie et al, 1994).

Near parturition, PgE_2 and $PgF_{2\alpha}$ binding increases three fold(Molnar et al, 1990). In terms of PgE_2 binding, progesterone has been shown to increase the levels. Estrogen has no effect(Mitchell et al, 1993). An interesting phenomenon which occurs in the uterus is that PgE_2 can be converted to $PgF_{2\alpha}$ by the enzyme 9-keto reductase(Mitchell et al, 1993).

2.5 Prostaglandin Transporters

Prostaglandins are produced in the cytosol and released into the interstitial fluid where they act as autocrine, paracrine, or endocrine factors. Since the prostaglandins are anionic they can't readily cross the hydrophobic plasma membrane. They must be transported.

Recently, these transporters have been cloned, sequenced, and expressed in eukaryotic cells. They are thought to be ten transmembrane

spanning domain transporters which have a preference for certain prostaglandin types. Physiologically and biochemically, these transporters are important since prostaglandins have been shown to be released preferentially in the basolateral side of uterine epithelial cells(Jacobs et al, 1990). Not only are they released in morphologically distinct areas of the cell but the site of release is under regulation and could change. Therefore, most likely, the site of release, apical vs basolateral, is under regulation. In addition, the recently cloned transporter(Kanai et al, 1995) has a low Km for PgE_1, PgE_2, $PgF_{2\alpha}$, TxB_2(70-450nM) but a high Km for 6-keto $PgF_{1\alpha}$(7659 nM); and, it has been shown to be expressed in the uterus with approximately a 4kb transcript size.

2.6 15-hydroxy Prostaglandin Dehydrogenase

Prostaglandins are initially metabolized by the enzyme 15-hydroxy prostaglandin dehydrogenase to 15-keto prostaglandins, and then reduced by the NADH dependent enzyme 13-reductase. There are two isozymes for 15-hydroxy prostaglandin dehydrogenase. Type I requires the cofactor NAD^+, has a M.W. of 25-26 kDa, is thought to function as a homodimer, and type II requires the cofactor $NADP^+$(Hansen et al, 1976).

The NAD^+ dependent 15-hydroxy prostaglandin dehydrogenase has been shown to be highly expressed in the human chorion and placenta and in fair amounts in the decidua(Carminati et al, 1995; Cheung et al, 1990; Keirse et al, 1985). During pregnancy, its enzymatic activity increases and it has been shown to be regulated by progesterone(Jacobs et al, 1990; Carminati et al, 1995; Kelly et al, 1990). Interestingly, the enzyme metabolizes PgE_2 approximately five times greater than $PgF_{2\alpha}$(decidual and chorion protein extract). Measurements of its kinetic properties have shown during spontaneous labour, the Km and Vmax for PgE_2 and $PgF_{2\alpha}$ within the chorion are 155μmol/L and 15.2 pmol/μg protein.min. and 47.8 μmol/L and 3.2 pmol/μg protein.min. respectively(Jacobs et al, 1990).

2.7 Non-Steroidal Antiinflammatory Drugs(NSAID)

NSAID's are pharmaceutical compounds which lessen the effects of an inflammatory reaction. They reduce the amount of prostaglandin released at the inflamed site. Within an acute inflammation, prostaglandins vasodilate capillaries and expose extracellular collagen; vasodilation and exposure of the plasma to collagen activates plasmin which in turn cleaves C3 to C3a and activates the complement cascade and hence mast cell degranulation, bradykinin proteolysis and finally acute sensation of pain, nociception; therefore, prostaglandin inhibition decreases the sensation of pain and lessens the effects of an acute inflammation(Robbin et al, 1987; Guyton, 1986; Thompson, 1987).

These compounds range from irreversible inhibitors such as aspirin to time dependent reversible inhibitors such as indomethacin(Meade et al, 1993; Powell et al, 1982). Essentially they block prostaglandin dehyrogenase or cyclooxygenase activity. Within the last five years, since two isoforms of cyclooxygenase have been identified, these drugs have been reevualated. Since Cox-1 is constitutively expressed and has homeostatic function and Cox-2 expressed for the most part during but not exclusively in an inflammatory reaction, drugs which inhibit one and not the other would be more specific with fewer side effects. Pharmaceutical companies have realized the advantage of such drugs and invested heavily in their development. These new compounds have Ki in the nanomolar range for the Cox-2 enzyme with over 10,000 fold selectivity when compared to Cox-1(Chan et al, 1995; Reitz et al, 1994). Table III summarizes some of these compounds and their relative selectivity for the different isoforms. It's also very important to state that increased prostaglandin synthesis upon ligand activation is Cox-1 independent, therefore, selective inhibition is necessary(Reddy et al, 1994; Murakami et al, 1994).

TABLE III

PGH Synthase Inhibitors

Specificity	Inhibitor	ID_{50} for PGHS-1	ID_{50} for PGHS-2
		uM	uM
Equipotent	Flurbiprofen	0.46-0.50	2.1-3.4
	(S)-Ibuprofen	8.9-14	7.2-8.2
	Meclofenamic acid	2.0-2.5	13-18
	Decosahexaenoic acid	11	13-17
$PGHS_{mu}$-1	Piroxicam	9.0-24	70-240
	Indomethacin	4.9-8.1	130-160
	Sulindac Sulfide	0.3-0.5	11-14
$PGHS_{mu}$-2	6-MNA[a]	200-280	15-55
$PGHS_{hu}$-2	1,2 Diarly-cyclopentenes	>100	0.026
$PGHS_{hu}$-2	L-745,337[b]	>10	0.023

[a]6-Methoxy-2-napthyl acetic acid

[b]5-methanesulfonamido-6-(2,4-difluorothiophenyl)-1-indanone

2.8 Overview of Oxytocin and Prostaglandin Involvement in Parturition

Is the fetus the best judge of when it is ready for the external environment or the mother? Or do both systems interact for the timing?

Initially, as the embryo implants, it suppresses all luteolytic mechanisms and promotes luteotrophic and myometrial quiescent factors. At mid-pregnancy, the fetus has reached its peak in development and is stretching the uterus. Both the fetus and the mother are at a discomfort. It would be to the fetus' benefit to prolong pregnancy.

Without a doubt the fetus controls the onset of parturition for these reasons: 1) without luteotrophic factors or successful implantation the embryo becomes absorbed(Soares et al, 1987), 2) without progesterone or prolactin, especially, in the rat, pregnancy isn't maintained(Smith et al, 1975), 3) in some species progesterone is produced in the placenta itself(Jaffe, 1991), 4) in some models, such as ruminants, fetal cortisol itself can trigger parturition (Thorburn et al, 1979; Anderson et al, 1975).

Is the mother involved at all? Yes, but exactly how is the interesting question. For example, as previously stated, in sub-primates hysterectomy during diestrous prolongs the corpus luteum lifespan extending a period of full gestation, therefore, the signal ending pregnancy arises from a maternal tissue. Or stated otherwise the integration of maternal and fetal signals are occurring within the uterus.

What are these factors and how do they interact? I believe there is a link between the fetus and the contractile apparatus, fetal membrane weakening and cervical dilatation. If one studies any basic element of physiological function, whether it be reproductive or cardiovascular, there is always redundancy. Therefore there has to be more than one factor or system involved. What are they?

First, let me briefly state what has been stated before. The contractile apparatus whether be it the number of myocytes and their respective size or content of actin or myosin is positively stimulated by progesterone. In

corollary with this estrogen increases the initiatory mechanisms, for example, these include:1) induction of proteases such as collagenase and gelatinase(Salamonsen et al, 1996; Lei et al, 1995) and inhibition of TIMP1-3 which weaken the fetal membranes(Ito et al, 1988), 2) induction of receptors and associated factors such as ligands(Lefebvre et al, 1994; Larcher et al, 1995) and modulation of G proteins which are involved with the contractile response(Riemer et al, 1988), and 3) increasing the responsiveness of secondary signals for example the plasma membrane Ca^{2+} current(Mirronean et al, 1994), and gating(Puri et al, 1982), and 4) movement of cells out of and into the uterus(De et al, 1993). Although both steroids are myotrophic, myometrial contractions only occur near term because of the progesterone block and from endocrine factors that increase the myometrial cAMP content(Baulieu et al, 1989). Therefore, on the one hand, progesterone is increasing the uterus size, and on the other hand, it maintains quiescence. The onset of parturition occurs at the threshold when progesterone effects are withdrawn, and stimulatory effects reach a peak. The synchronized contractions produce enough pressure to break the fetal membranes and provide a rapid transit through the soft and compliant cervix(Fig.14). On a molecular basis it's when ligand activation is directly coupled to electromechanical force without or minimal inhibition.

Therefore, to resummarize, three modifications are needed to initiate parturition: 1) preparation of the uterus for contraction and cervical softening which involves the input of the immune system, 2) progesterone withdrawal whether at ligand or receptor level, and 3) activation of the uterus by such factors as oxytocin, PgE_2 and $PgF_{2\alpha}$, endothelins, angiotensin II, histamine, acetylcholine, and platelet-activating factor(PAF).

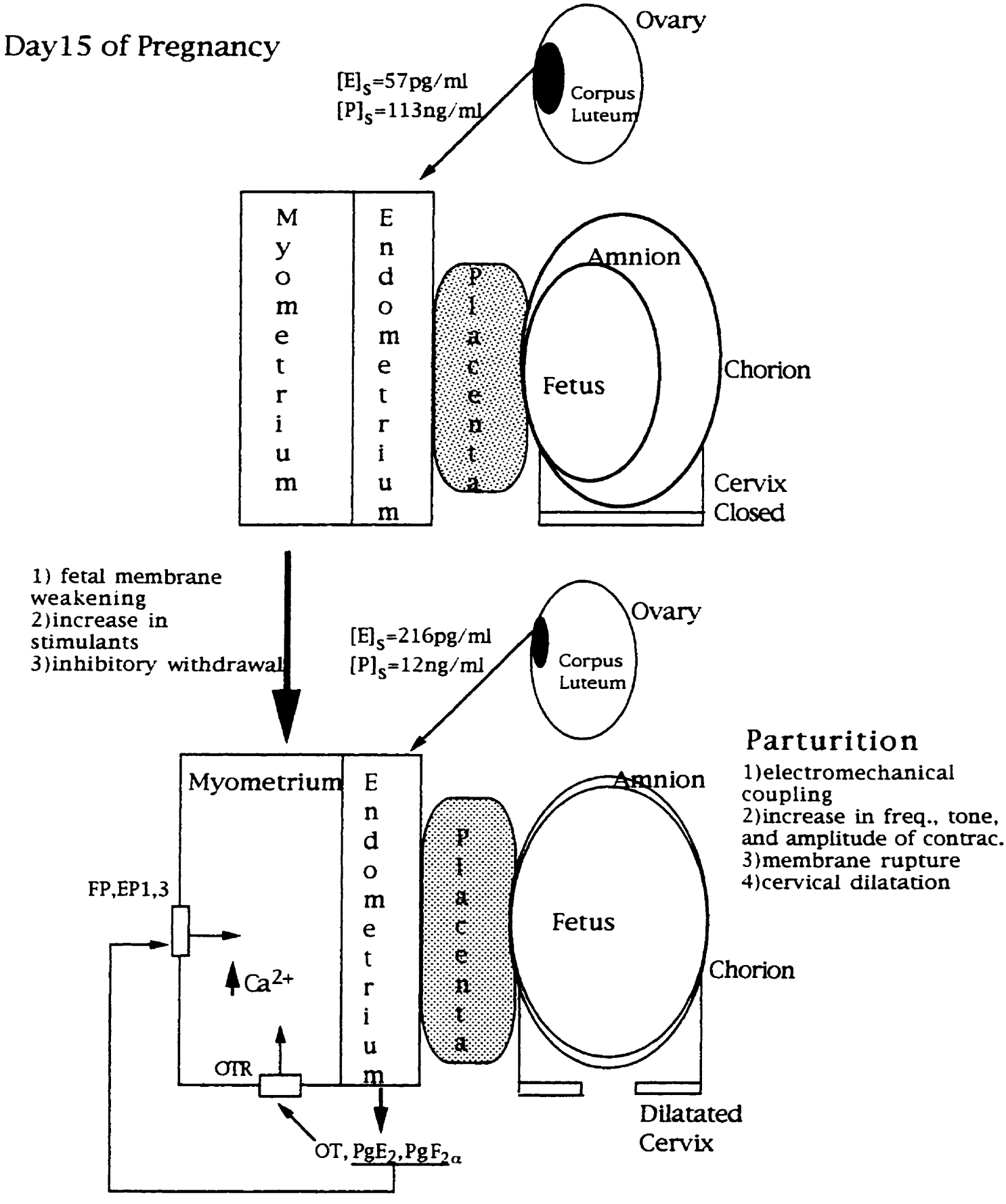


Fig.14 Overview of the major changes which occur during pregnancy. s=serum.

CHAPTER 3

Immortalization of Cell Lines

3.1 Immortalization of Cell Lines

Starting from a single a cell, the human organism evolves into a complex multicellular species. In the intervening time span many cell replications must occur not only to increase its size but the available cells to perform specialized functions. Exactly how a cell divides and its regulation became better understood within the last two decades.

3.1.1 Cell Cycle

The cell cycle may vary from 4-6 hrs, which occurs for epidermal cells during embryogenesis, up to a couple of days, as in the case of post-natal fibroblasts(Hogan et al, 1994; Arslan et al, 1995). Given both cells have the same DNA content, what regulatory mechanisms determine the differences?

The differences reside not in DNA replication or separation but the decision to enter the cell cycle. The cell cycle has been divided into different periods: G1 phase which is the most variable, S(6-7 hrs), G2(3-4hrs), and M(1hr)(Lewin, 1990). The G1 phase is when growth factors promote entry into the cell cycle. Once committed, the cell replicates its genome in the S phase. The G2 phase is when the cell is tetraploid and DNA repair and assessment and corrections of mutations are taking place. Mitosis is where the chromosomes are aligned and separated by mitotic spindles.

Aside from the obvious need of cell replication during development, continual cell division, differentiation and senescence is required for the adult organism. For example, in the immune system, granulocytes, monocytes, and lymphocytes are generated and destroyed daily(Veis et al, 1993; Chittenden et al, 1995; Kiefer et al, 1995; Moyotama et al, 1995;

Fukugama-Natanabe et al, 1992; Nicholson et al, 1995; Spangrude et al, 1988; Compton et al, 1992). The factors responsible for initiating cell division are growth factors. Different tissues have different growth factors. For example, IL-2 stimulates lymphocytes(Miyazaki et al, 1995), Colony Stimulating Factor-1(CSF-1) stimulates monocytes(De et al, 1993), Epidermal Growth Factor(EGF) stimulates epithelial cells(McDonnell et al, 1995), acidic and basic Fibroblast Growth Factors(FGF) stimulate fibroblasts(Klagsburn et al, 1991), Platelet Derived Growth Factor(PDGF) stimulates smooth muscle cells(Welsh-Claesson et al, 1994), Vascular Endothelial Cell Growth Factor(VEGF) stimulate endothelial cells(Charnock-Jones et al, 1994) and Nerve Growth Factor(NGF) stimulates sensory neurons(Davies et al, 1994). The common thread among these different growth factors is most of them activate in some fashion or another tyrosine kinases which are mitogenic and make the cells competent for entry into the S phase. A second signal which has been termed progression factors are also required to complete the process. Insulin or Insulin-like Growth Factor-I(IGF-I) have been identified as this second signal necessary for fibroblasts to start DNA synthesis(Aaronson et al, 1991).

Growth factors may activate tyrosine kinases by different means. In certain cases like the EGF or PDGF receptors, the receptors possess tyrosine kinase domains which are activated upon either hetero or homo dimerization and in many cases cross-phosphorylation of the receptor dimers takes place(Marshall et al, 1995; Pawson et al, 1995; Heldin et al, 1995; Pawson et al, 1995; Heldin et al, 1995, Rodrigues et al, 1993). These phosphorylated tyrosine residues serve as substrates for Src-homology domain 2(SH2) which are found on many tyrosine kinases such as src, lck, fyn and yes(Cooper et al, 1993; Koch et al, 1991). In addition they are found on PLC-γ, which directly increases IP3 turnover and indirectly activates PKC by making available DAG and increased Ca^{2+}, GTPase Activating Protein(GAP), ras activation, PI-3 Kinase, raf, GTP binding protein, and Phosphatase 1C(PTP1C)(Cohen et al, 1995; Pawson et al, 1992). Furthermore, they are also found on adaptor proteins such as SHC, NCK, GRB2, and ISGF3α(Cohen et al, 1995; Pawson et al, 1992). These adaptor proteins are important since it has been shown GRB-2 binds the protein SOS which activates the protooncogene ras(Fath et al, 1994; Bar-Sagi et al, 1994). Ras in turn activates the protein kinase raf which eventually leads

to phosphorylation of the Mitogen Activated Protein(MAP) Kinases, ERK-1,2(Hall et al, 1994; Settleman et al, 1992; Herskowitz et al, 1995)(Fig.15). These kinases activate transcription factors which promote mitogenesis(Hill et al, 1995). Other conserved domains found on these proteins include SH3 which bind proline rich stretches, and the "pleckstrin homology" domains(Cohen et al, 1995; Pawson et al, 1992; Sagi-Bar et al, 1993). For other cases like the Prolactin, Growth Hormone, and Erythropoietin Receptors, the Janus family of protein kinases, which were initially identified in the interferon receptors, are more prominent. These receptors phosphorylate JAK1,JAK2, Tyk2 which in turn has been shown to activate transcription factors such as the ISGF3α and therefore once again promote entery into the cell cycle by activating transcription factors(Muller et al, 1993; Darnell et al, 1994; Daly et al, 1994; Argetsinger et al, 1993). Last but not least, the T cell receptor activates tyrosine kinases such as lck and fyn, and transcription factors such as NF-κB, myc, fos and jun(Miyazaki et al, 1995; Klaus et al, 1984). NF-κB, which is activated by the cytokines IL-1 and TNF-α, induces the IL-2 receptor(Miyazaki et al, 1995).

The connecting factors which link growth factors to cell replication are proteins termed cyclins which regulate cyclin-dependent protein kinases(CDK's). Both factors determine proper timing and sequential coordination of events. In eukaryotes, seven CDK's have been cloned, sequenced and expressed (CDK1-CDK7) and at least seven cyclins have been cloned, sequenced and expressed (cyclinA,B1-2,D1-D3, E and H)(Lewin, 1990; Morgan, 1995; Heichmann et al, 1994; King et al, 1994; Hunt et al, 1991). Within the G1 phase, cyclinD expression is dependent on mitogen activation. Withdrawal of mitogens abrogates cyclinD expression (Toyoshima et al, 1994; Sherr et al, 1994). More specifically, it has been shown the transcription factor myc induces the cyclinD gene(Daksis et al, 1994). Using the yeast model and raising anti-sera to these factors has enabled a means to determine their roles within the cycle. CyclinD-CDK4 and CyclinD-CDK7 are important for the middle to late of the G1 phase. Entry into the S phase is dependent on active CyclinE-CDK2 complex formation(Lewin, 1990; Sherr et al, 1994). Completion of the S phase is dependent on a active CyclinA-CDK1 complex(Girard et al, 1991; Bandara et al, 1991). And finally entry from G2 to S is dependent on a active

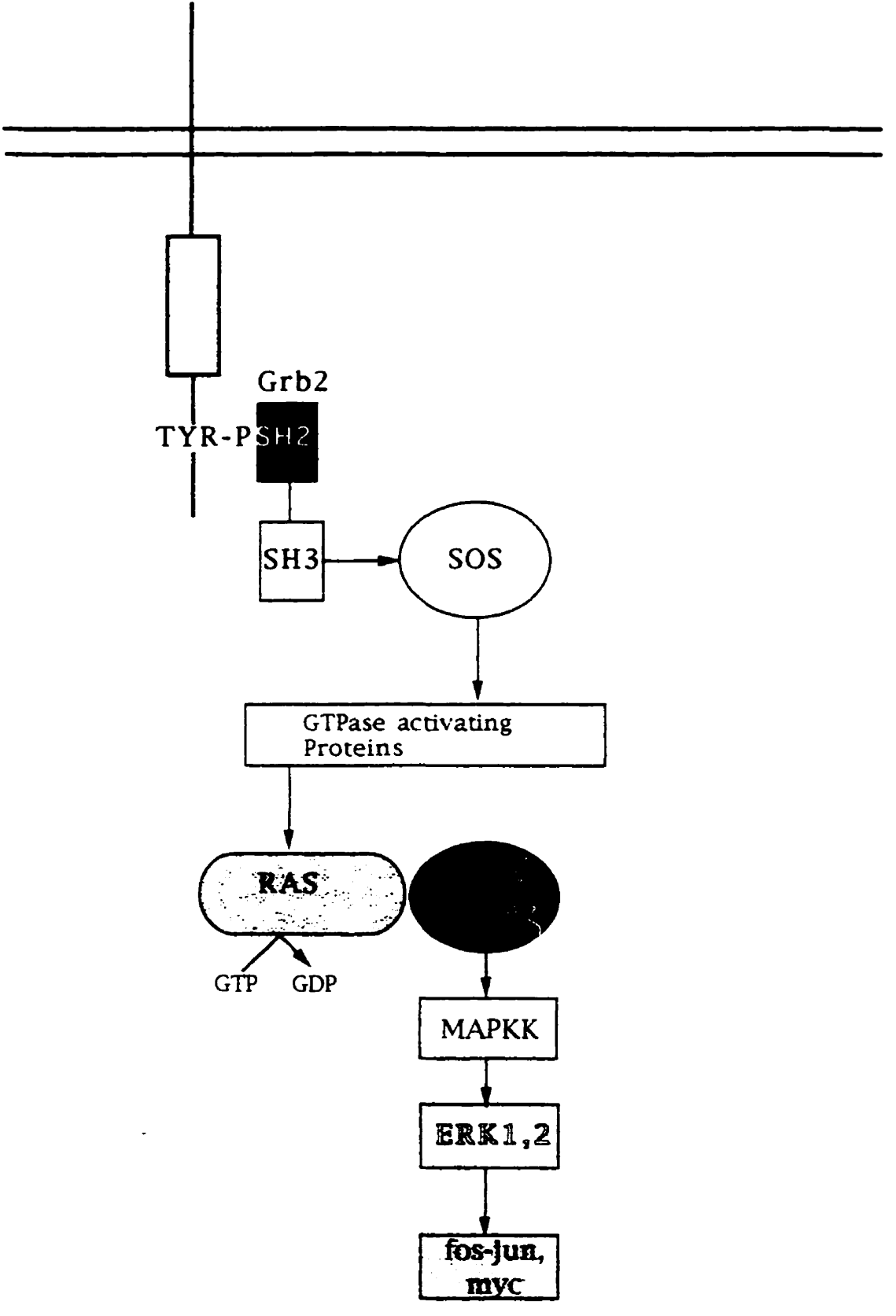


Fig.15 Overview of Receptor Tyrosine Kinase Signalling. Modified from Pawson and Gish, 1992.

CyclinB-CDK1 complex(Moreno et al, 1990; Moreno et al, 1994). CDK1, when phosphorylated on threonine14 and tryosine15 by the Wee1/Min1 kinases, remains inactive(King et al, 1994). The Wee1/Min1 kinases, in turn, require phosphorylation by the nutrient sensitive Nim1 kinase for increased activity(Morgan et al, 1995; Heichmann et al, 1994; King et al, 1994). At the G2/M transition, the phosphatase cdc25 removes the phosphates and the kinase then becomes active(Galaktinov et al, 1991; Enoch et al, 1991; Surana et al, 1991; Murray et al, 1992; Murray et al, 1992; Tyson et al, 1991). The cycle becomes complete at around anaphase when CyclinA and CyclinB become degraded by the ubiquination pathway and the cell reenters G1(Murray et al, 1992; Tyson et al, 1991).

As growth factors promote mitogenesis, other factors such as transforming growth factor(TGF)-β family which include members such as TGF-β1-5, activin, inhibin, Mullerian inhibiting substance, bone morphogenetic proteins2-7, and growth differentiation factor 3, inhibit it(Massague, 1990; Kingsley, 1994). Both type I and typeII TGF-β receptors contain a serine/threonine kinase domain which upon activation induces CDK inhibitors(CKIs)(Wrana et al, 1992; Koff et al, 1993). Specifically, it induces the $p15^{ink4B}$, which possesses four ankyrin repeats, and whose transcript levels increase greater than 30 fold upon stimulation in human keratinocytes(Hunter et al, 1994). Other members of this family include p15.5, $p16^{ink4}$, p18, p21, and $p27^{Kip1}$(Peter et al, 1994). Collectively when these proteins are expressed in high amounts, they inhibit CDK activity. The p15, and p16 proteins preferentially inhibit the CDK4 and CDK6-cyclin complexes and the p21 and p27 preferentially inhibit the CDK2 and CDK4-cyclin complexes(Peter et al, 1994; Polyak et al, 1994; Toyoshima et al, 1994). In addition, p21 has been shown to inhibit DNA polymerase δ by binding and inhibiting the proliferating cell nuclear antigen(PCNA) subunit(Hunter et al, 1994). Furthermore, there is an intimate relationship between tumour suppressor proteins and these factors. For example, the tumour suppressor protein p53 induces the p21 CKI protein and CDK4-CyclinD and CDK6-CyclinD complex phosphorylates the tumour suppressor protein Rb which permits disassocation of the E2F transcription factor(Peter et al, 1994). E2F activates genes required for DNA replication such as dihydrofolate reductase(Moran, 1991; Nevine, 1992).

3.1.2 Tumour Suppressor Genes

Within the cell cycle there are certain check points to assure that prior to cell division certain criterion's are met. For example assuring enough precursors for cell division, DNA integrity, and upregulation of the replication enzymes are just a few. Some of these proteins which perform these tasks have been cloned and well studied. In addition to the p53 and the Rb proteins mentioned above, the Wilm's Tumour protein and the VHL tumour suppressor protein, which inhibits transcription elongation, have been included in the list.

Many cancers, including those originating from hematopoietic, liver, brain, breast and lung, have been documented which carry mutations in the p53 alleles(Hollstein et al, 1991; Levine et al, 1991). Therefore, the p53 gene is widely expressed and active in many cell types. In terms of the quaternary structure, the protein is 393 amino acids in length and functions as homotetramer(Ullrich et al, 1992). Homozygous deletions or mutations of its gene predisposes to premature entry into the S phase(Ullrich et al, 1992). Recently, p53 has been shown to be involved in the apoptotic pathway. It induces the Bax gene which has been shown to promote apoptosis(Miyashita et al, 1995; Scharer et al, 1992). Although the Bax gene promotes apoptosis, it must be abundantly expressed and it must homodimerize since heterodimerization with Bcl-2 inhibits the programmed path(Oltvai et al, 1994).

The Rb gene is inactivated in retinoblastomas, osteosarcomas, small cell lung, bladder and breast carcinomas. It encodes a 105 kDa protein which binds transcription factors such as DRTF1, E2F, myc, E1A, SV40 LT, RB1 and RB2. In general, phosphorylation leads to inhibition and release of the transcription factors(Defeo-Jones et al, 1991; Ewen et al, 1992; Faha et al, 1992).

The absence of a functional Wilm's Tumour(WT) protein leads to familial and sporadic cancers in the kidney. The protein is 345 amino acids in size, is a transcription factor, and shares 60% amino acid sequence identity with the EGR-1 transcription factor. WT has been shown to be an antagonist of the strong transcription activator EGR-1 which is ubiquitously

expressed and turned on rapidly by serum stimulation(Cavenee et al, 1983; Horowitz et al, 1989; Weinberg et al, 1991).

The VHL(von Hippel-Lindau disease) gene encodes a 213 amino acid protein which when absent predisposes the individuals to clear-cell renal carcinoma, hemangioblastoma, and pheochromocytoma. The protein inhibits Elongin(SIII), which activates transcriptional elongation by RNA polymerase II, by binding to its regulatory subunits B and C(Duan et al, 1995). Many of the oncogenes and the immediate early genes such as myc and fos are regulated at the level of transcriptional elongation(Miller et al, 1989). Therefore dysregulation would lead to aberrant and uncontrolled expression of oncogenes.

3.1.3 Viral Oncogenes

Certain viruses express proteins which are able to induce cell replication. There are many, some of the more common viruses include the Adenoviruses-E1A, PappilomavirusesE6/E7, Epstein-Barr Virus, Retroviruses, and the Simian Virus 40(SV40)-Large T antigen(Hausen, 1991).

In our laboratory we used two vectors, a plasmid and a defective retrovirus expressing the temperature sensitive SV40 LT antigen, to immortalize epithelial and stromal cell lines. A human β-actin promoter expressed the SV40 LT in the plasmid and a Long Terminal Repeat(LTR) promoter expressed it for the retroviral vector.

The SV40 virus is a double stranded DNA virus approximately 5,243 bp in length(Jat et al, 1986; Cepko et al, 1984). Studies have shown the early genes of the virus are essential for its transformation of cells. The early genes code for the small and large T antigen which are 20kDa and 95kDa respectively(Jat et al, 1986). Functionally, the small T antigen binds to and alters the regulatory subunits A and C and not B or B' of phosphatase 2A(Hanmin et al, 1994) and the large T antigen has helicase and DNA binding activity and binds to a given number of cellular proteins(Paucha et al, 1986; SenGupta et al, 1992; Huynh et al, 1991; Endo et al, 1992;

Tegtmeyer et al, 1975; Bruggs et al, 1975; Mary et al, 1975). Deletion studies of the large T antigen have mapped its interaction with these proteins. Starting from the N-terminus, amino acid 105-114 has been shown necessary for Rb binding and amino acids 337-672 necessary for p53 binding(Prives et al, 1990). Within the G1 phase, it seems phosphorylation of certain serine/threonine residues can decrease its DNA binding activity(Prives et al, 1990).

Certain strains of papillomaviruses, such as HPV16, HPV18 and HPV33, are known to infect and cause benign squamous epithelial tumours(De Villiers, 1989; Lambert, 1991). A proposed mechanism has been by inhibition of tumour suppressor gene products. Data for such a model is derived from studies which have shown the E6 protein binding to p53 and increasing its degradation and the E7 protein binding and inhibiting the Rb gene product(Scheffner et al, 1990; Dyson et al, 1989; Phelps et al, 1988; Bandara et al, 1991).

Another large family of viruses, which are able to transform cells, are the Adenoviruses. Viral mapping has shown the early transcribed genes E1A and E1B as essential for transformation. Of the two proteins, the function of E1A has been better characterized. E1A is found in two major sizes from differential splicing of its primary transcript: a 243 amino acid size encoded by the 12S mRNA and 289 amino acid size encoded by the 13S mRNA(Yee et al, 1985; Harlow et al, 1986). Functionally, it alters transcription at various promoters by binding to basal and upstream transcription machinery such as CREB binding protein(CRB) and the p300 protein and altering their activity(Arany et al, 1995; Lundbland et al, 1995). In addition, it has been shown E1A binds to and alters Rb protein activity(Ewen et al, 1992; Faha et al, 1992).

3.2 Isolation, Plating and Growth of Endometrial Cells

Isolation and plating of primary tissue cells isn't a straightforward process. Certain steps need careful attention. First, a good sterile technique must be developed to isolate cells without damaging or altering their phenotype. For our studies, we were fortunate as Glasser and

McCormack(1980) had previously developed much of this work in the rat. Second, the growth media must be meticulously selected. Although most media contain the standard electrolyte, amino acid, vitamin, and buffer concentrations there are major differences in the amount of transitional metals, polyamines, and fatty acids. For dividing cells, polyamines have been shown to be essential(Davis, 1990). Third, since, in vivo, cell growth might be dependent on both autocrine and paracrine growth factors, the cells shouldn't be cultured in too much media as this will inhibit their growth. In most circumstances, these growth factors need to be added to the media and this requirement differs between tissues. For endometrial epithelial cells, we needed to add EGF, Insulin and 17-β Estradiol(Giudice et al, 1995). Fourth, we had selective growth conditions. This was achieved by using poly-lysine coated plates which hinders or stops fibroblast or stromal cell proliferation. Fifth, aside from the media content and growth factors, the type and amount of serum used is very important. Too much or not enough serum might select for the most transformed phenotypes. For example, transformed cells require less serum and, therefore, if the conditions are set for these cells, they will proliferate at the expense of immortalized cells. On the other hand, too much serum might alter the differentiation state of the cultured primary cells and the obtained cell lines. For our epithelial cells, we cultured them in 5% NuSerum which contains 1.25% heat inactivated Fetal Bovine Serum and a defined supplement of growth factors and steroids. And last, the plating density is very important. If the cells are plated at a too low density, they won't replicate.

3.3 Transfection and Clonal Selection

Primary cells are quite difficult to transfect. Usually, they need to be dividing for efficient transfection. Depending on the cell type, the choice of the employed transfection technique may or may not be important. At present these range from Electroporation, Lipofection, Calcium Precipitation and Retroviral Infection. For our laboratory, we prefer Calcium Precipitation and Retroviral Infection.

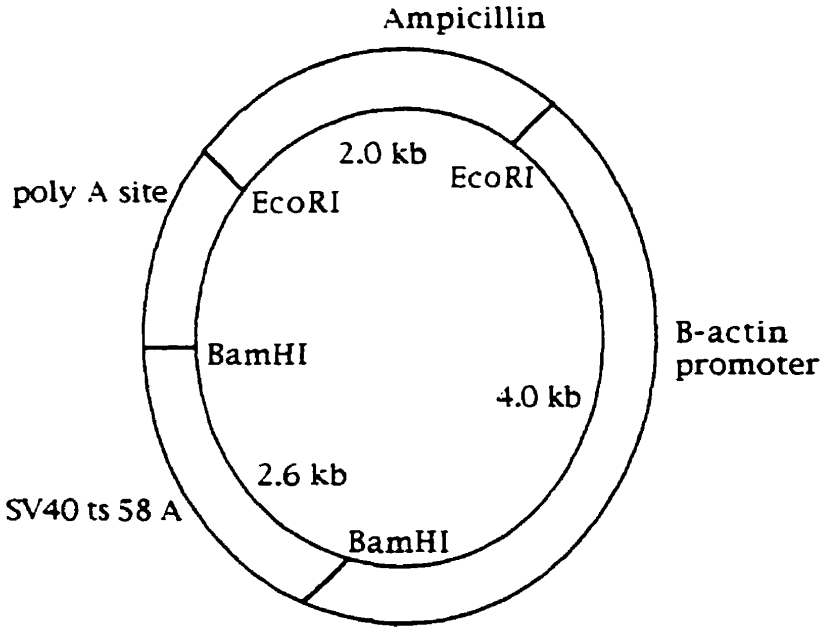
Two plasmids were used for the Calcium Precipitate Transfection. The first, as mentioned above, was a plasmid encoding for a temperature sensitive SV40, pBAPSV40TtsA58, and the second was a plasmid encoding a neomycin marker, pSV2neo(Fig.16). The plasmids were added at a ratio of 5 to 1 in favor of pBAPSV40TtsA58. To obtain small and manageable precipitates for the cells, the plasmids need to be quite pure. An Optical Density ratio of 260nm/280nm of 1.8 is desireable.

The Retroviral Transfection is quite straight forward. The retrovirus is added at a high titer with polybrene to increase viral and cell interaction and after a certain period of incubation the media is changed and the cells are incubated overnight to allow retroviral integration and expression of its gene products. The limiting factors in this technique is the titer of the virus and this requires predetermination for success.

After the cells have been transfected, the following morning neomycin or its analog, G418 is added. If the cells are expressing both markers, they will grow and remain viable. It's very important not to add to much neomycin as this could over a two week period lead to amplification of the locus where the plasmids or retrovirus has integrated(Schimke et al, 1984; Schurr et al, 1994; Chin et al, 1992; Solomon et al, 1991). We used 100μg/ml. After a two week period under neomycin selection, usually a few colonies are observed which are expanding radially in the case of epithelial cells and spirally in the case of stromal cells.

The manner that we utilized to isolate the colonies were as follows. We used circular filters soaked in trypsin and placed them directly on the colonies. After a 2 minute incubation at room temperature, the filters were removed and placed in 12 well plates. From the 12 well plates, they were allowed to grow and progressively transferred to 6 well plates and finally 10 ml dishes. These steps are very important as too a low a density, the cells won't grow and you will lose the cell lines.

A)



B)

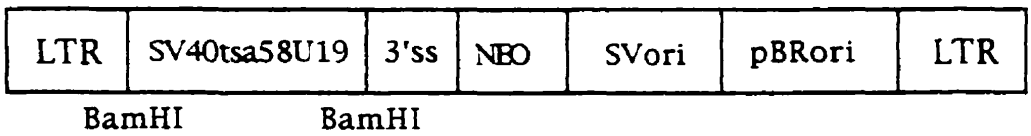


Fig.16 Schematic diagram of the vectors used for immortalizing endometrial cell lines. A) plasmid pBAPSV40T; B) retrovirus shuttle vector SV40U19tsA58.

3.4 Analysis of Differentiation Markers

Although we used stringent conditions to culture primary epithelial cells, stromal cell contamination was unavoidable. This complicated matters since stromal cells are able to attain an epitheloid like morphology, therefore, a careful assessment of the established cell lines was necessary. We used various markers to differentiate between epithelial and stromal cell lines.

In terms of identifying different cell types, intermediate filaments are an excellent choice to readily distinguish between stromal and epithelial cells (Steinert et al, 1985). During the estrous cycle, epithelial cells have been shown only to express cytokeratin, stromal cells have been shown to only express vimentin (smooth muscle cells express desmin)(Glasser et al, 1988; Mani et al, 1992). We used anti-sera against both to assess if the endometrial cell line was epithelial or stromal in origin. Furthermore, we assessed the expression of the adhesion molecule E-cadherin which is only found in epithelial cells(Takeichi et al, 1990, Blaschuk et al, 1990; Geiger et al, 1992; Schubert, 1992). And finally, aside from morphology and duplication times of the cell lines, the final marker we checked was alkaline phosphatase activity. Alkaline phosphatase is expressed in high amounts in epithelial cells in the cycling rat and in high amounts in stromal cells at day7 of pregnancy(Schubert, 1992; Pritchard, 1947; Finn et al, 1964; Murdoch et al, 1978). Collectively, from our experience, at least two markers should be utilized not only for identifying the cell type but the differentiation state.

PART II

EXPERIMENTAL RESULTS

CHAPTER 1

Characterization and Co-Culture of Novel Nontransformed Cell Lines Derived From Rat Endometrial Epithelium and Stroma

PREFACE

The discovery of the rat uterine endometrium as a major site of prostaglandin synthesis and peripheral location of oxytocin expression prompted us to develop cell lines to better understand premature labour. The following chapter details how they were obtained.

SUMMARY

Normal and neoplastic growth of epithelial cells depends on mutual interactions between epithelial and stromal cells. As a tool for the study of the underlying molecular mechanisms, we have developed temperature-sensitive, nontransformed cell lines derived from rat uterine epithelium and stroma by transfecting primary cultures with a temperature-sensitive mutant of the SV40 large T antigen. The epithelial and stromal cell lines obtained shared relevant morphological characteristics with the primary cells from which they were derived. Immunocytochemical analysis showed that the epithelial cell lines expressed the intermediate filament cytokeratin, whereas the stromal cell lines expressed the intermediate filament vimentin. Alkaline phosphatase activity was present in all cell lines examined. All cell lines were anchorage dependent and did not form foci. One epithelial cell line expressed oxytocin mRNA, a gene product recently shown to be highly expressed in vivo in the uterine epithelium at term. If grown on Matrigel, this cell line formed domelike structures, a further characteristic of its differentiated phenotype.

In an attempt to reconstitute an endometrium in vitro, epithelial cells were seeded on top of a layer of stromal cells. Paraffin cross sections

showed that this in vitro system consisted of a bilayer structure. Four to five cuboidal epithelial cells were typically anchored atop one stromal cell, forming an endometrium like tissue. The present in vitro system should provide a useful model for further studies on endometrial functions and epithelial/stromal cell interactions at a molecular level.

INTRODUCTION

The endometrium plays an important role in reproduction. Not only does it provide the environment for the successful implantation and development of the blastocyst, but it also plays an important role in parturition. As shown recently, the endometrium is a site of oxytocin gene expression during late pregnancy(4,24). The endometrium is a dynamic tisssue where gene expression, extracellular matrix, immune cell composition, and cellular differentiation state depend on the specific stage of the reproductive cycle(7,9,11,13,27,30). The above-mentioned changes are, in large part, hormonally induced by ovarian steroids. In addition, locally produced factors and cell-to-cell interactions are also important determinants for endometrial function and differentiation. For example, stromal cells affect epithelial cell function through turnover of extracellular matrix and the release of soluble factors(9,13,30). Epithelial cells, in turn, affect stromal cell function through the release of soluble factors and, in some cases, through direct cell-to-cell contact(21,25,37). Without these interactions, proper differentiation of the cells does not occur. In addition to epithelial-stroma interactions, locally produced hormones such as β-endorphin(26), prolactin(16), oxytocin(4,24), and cytokines, such as IL-1, IL-6, TNFα, TGF-β1, TGF-β2, CSF-1, and GM-CSF(5,6,21,35,36), probably also play important roles in the regulation of endometrial proliferation, secretion, and differentiation. Finally, products of local steroid metabolism might affect endometrial gene expression(15).

Due to the complexity of these interactions, a precise dissection of the mechanisms involved is difficult in vivo. On the other hand, transformed cell lines grown in isolation may lack essential determinants present in the intact cells in the original in vivo context. Therefore, in an attempt to provide a model for the study of epithelial/stromal interaction in a

controlled in vitro environment, we have established nontransformed cell lines derived from both epithelial and stromal uterine cells. This paper describes the feasibility and the potential applications of this approach.

MATERIALS and METHODS

Isolation of Uterine Epithelial and Stromal Cells

Uterine epithelial and stromal cells were isolated using a combination of mechanical and enzymatic dispersion techniques according to the method of McCormack and Glasser(29). Twenty-one to 23 day old immature Sprague-Dawley rats were purchased from Charles River Canada(St. Constant, Quebec, Canada) and injected subcutaneously overnight with 5μg 17β-estradiol(Sigma, St. Louis, MO) in 0.1ml of oil. The following morning, uteri were excised, sliced into smaller parts, and placed in 3ml of pancreatin(Gibco/BRL, Burlington, Canada) containing 14.4 mg of dispase typeII(Boehringer-Mannheim, Laval, Canada) under sterile conditions. The digestion was carried out 1h on ice, 1h at room temperature, and 10 min. at 37C. The digestion mix was decanted and replaced with 3ml of Hanks' balanced salt solution(HBSS, GIBCO). The mixture was vortexed briefly and allowed to settle. The supernatant(now containing dispersed epithelial cells) was transferred to a new tube. The sediment was placed into 3ml of HBSS and kept on ice for stromal cell isolation(see below). The dispersed epithelial cells were pelleted by centrifugation for 5 min. at 1000Xg, resuspended in 3ml HBSS, and allowed to sediment for 10 min. The supernatant was removed and the sediment cells were suspended in culture medium and seeded in Primaria culture plates(Falcon, Oxnard,CA) at a density of at least 500 cells/mm^2.

The sediment kept on ice from the previous step was used for isolation of stromal cells. The sediment was treated with the pancreatin/dispase mixture described above for 15 min. at 37C. Undigested tissue fragments were allowed to settle, and the dispersed cells present in the supernatant were concentrated by centrifugation(5 min. at 1000Xg) and resuspended in HBSS. Contaminating epithelial cells were allowed to settle for 10 min. and

the cells present in the supernatant were again concentrated by centrifugation, resuspended in medium, and plated.

Plasmid Transfection and Retroviral Infection

For immortalization of primary epithelial and stromal cells, two different methods were used.

Transfection with plasmid pBAPSV40T. The plasmid pBAPSV40TtsA58 was kindly provided by L. Chalifour, Biotechnological Research Institute, Montreal. This plasmid contains the coding region of the SV40 large T antigen placed under the control of human β-actin promoter and a 0.75 kb SV40-polyadenylation sequence. The plasmid pSV2neo contains the neomycin-resistance gene linked to an SV40 early promoter(34). Adherent epithelial cells were contransfected with 2.5μg of linearized plasmid pBAPSV40TtsA58 and 0.5μg of the linearized plasmid pSV2neo. After 4h, the cells were washed with phosphate buffered saline(PBS) and exposed to 15% glycerol for 2 minutes. Following two washes with PBS, cells were cultured overnight at 37C. The culture medium consisted of a 1:1 mixture of F10 medium and Dulbecco's Modified Eagle's Medium(DMEM) supplemented with 27ng/ml estradiol, 10μg/ml insulin(Sigma), 100U/ml penicillin(BRL, Burlington, Ontario, Canada), 50μg/ml streptomycin(BRL), and 5% NuSerum(Collaborative Research, Bedford, MA).

Infection with retroviral shuttle vector SV40U19tsA58.

Recombinant retrovirus SV40U19tsA58 was collected from the cell line psi2-pZiptsa58U19(1). This retrovirus encodes both the SV40 large-T and small-T antigen, along with a neomycin resistance marker. For infection, medium was replaced with medium carrying the virus (titer10^4-10^5 CFU/ml) and polybrene(8μg/ml;Sigma) was added to each dish. After incubation at 33C for 2-3h, virus-containing medium was removed and replaced with fresh medium consisting of F10/DMEM supplemented with 27ng/ml estradiol, 10ug/ml insulin(Sigma), 100U/ml penicillin(BRL), 50μg/ml streptomycin(BRL), and 5% NuSerum, as above, plus 5% fetal bovine serum(FBS)(P.A. Biologicals Co., Sydney, Australia).

G418 Selection

Starting the following morning, the drug G418 was added to the medium(100μg/ml) for a period of 2 wk. Resistant colonies were isolated and expanded. Briefly, the colonies were washed twice with PBS-citrate and isolated with small circular sterile filter papers impregnated with 0.1% trypsin(Sigma). The trypsin filter paper was placed directly on the colony and incubated for 1-2 min. The filter paper was briefly pressed on the cells, picked up, and placed into a well of a 24-well plate(Falcon). After 2 weeks, the cells were confluent and ready for trypsinization and further replication. Epithelial and stromal cell lines were maintained and grown at 37C in F10/DMEM media supplemented with 100U/ml penicillin, 50μg/ml streptomycin, and 5% NuSerum (epithelial cell cultures) or 5% NuSerum and 5% FBS(for stromal cell cultures).

Alkaline Phosphatase Assay

Following enzymatic dispersion by brief trypsinization (0.1% trypsin, 5mM EDTA), an equal amount of FBS was added and cells were harvested and centrifuged in round-bottom polystyrene tubes(Falcon) for 10 min. at 1000Xg. The cell pellet was resuspended in culture medium, stained with 0.2% Trypan Blue, 5mM ethylene-diaminetetraacetic acid, and counted using a hemacytometer; 500,000 cells were placed in a microcentrifuge tube and suspended in 1 ml PBS. Next, the cells were fractured by repeated freezing; they were frozen on dry ice and thawed at 37C. Following centrifugation for 10 min. at 10,000 Xg, the pellet was washed twice with PBS and 1ml of the substrate for alkaline phosphatase(3mM para-nitrophenylphosphate, 50mM $NaCO_3$, 50μM $MgCl_2$, pH11.0) was added. The pellet was resuspended and incubated at room temperature overnight. After centrifugation at 10,000 Xg for 1min., 500μl of the supernatant was used to measure absorbance at 405 nm. Each assay was done in triplicate.

Southern Blot Analysis

DNA was obtained from cultured cells according to published protocols(2). Twenty μg of the digested DNA was electrophoresed through a 1% agarose gel and transfected to a nitrocellulose membrane overnight.

A 2.6 kb restriction fragment of the plasmid pBAPSV40TtsA58 coding for the large-T antigen was labeled to a specific activity of $>10^8$ cpm/μg by random primer labeling(10). The membrane was prehybridized for 4h at 42C in ECL gene detection system hybridization buffer containing 0.5M NaCl(Amersham Canada, Oakville, Ont., product-RPN 2102). Denatured probe was added at a final concentration of 500,000 cpm/ml and hybridization was carried out overnight. The membrane was washed twice for 20 min. with primary wash buffer (6M Urea, 0.5%SDS, 0.5XSSC) at 42C, and twice with secondary wash buffer (2XSSC) at 42C, for 5min. each. The membrane was exposed to x-ray film for 24 h.

Immunocytochemistry

The stromal cell lines were cultured on tissue slide chambers (GIBCO) alone, whereas the epithelial cell lines were cultured on tissue slide chambers coated with Matrigel(Collaborative Research) diluted 10-fold with culture media. A sufficient amount of Matrigel was added to cover each slide, followed by immediate aspiration and incubation at 37C for 30 min. to allow sufficient binding of Matrigel to the chamber slides. The cells were cultured for a period of 48h at 39C to allow attachment and differentiation, prior to immunohistochemical staining. Cells were fixed in 70% ethanol for 30 min., dipped eight times into cold water and two times into PBS for rehydration. Excess PBS was removed by blotting. Primary antibody was added, and the cells were incubated at room temperature for 1h and washed three times for 5 min. with PBS. The secondary antibody was added next and the cells were again incubated for 1h at room temperature and rewashed three times with PBS. The cells were rapidly dipped in water twice and mounted with IMMUMOUNT(Shandon, Pittsburgh,PA). The primary antibodies used were rabbit anti-pan-cytokeratin IgG(Dako Corp., Carpinteria, CA), and monoclonal mouse anti-vimentin IgG(clone no. V9, Sigma). The secondary antibodies were anti-mouse IgG/FITC conjugate raised in goat(Sigma), and anti-rabbit IgG/FITC (Dako) raised in swine. The pan-cytokeratin IgG used for the staining was diluted 100-fold, the vimentin IgG was diluted 40-fold, anti-mouse IgG was diluted 16-fold, and anti-rabbit IgG was diluted 20-fold. The solution used for dilution was 10% FBS, 0.01% Triton in PBS, sterilized by filtration.

Detection of Oxytocin mRNA by Reverse Transcriptase/Polymerase Chain Reaction(RT-PCR)

The RT-PCR was performed according to Lefebvre et al.(24). Briefly, 3μg of total RNA was reverse transcribed, incubated at 90C for 5min. to heat inactivate reverse transcriptase, precipitated with 2M ammonium acetate, and dissolved in 30ul of 10mM Tris(pH 8.0), 5mM EDTA. One-sixth of the reverse transcribed RNA reaction was used for the PCR in a total volume of 50μl, and run for 35 cycles. Ten μl of the PCR reaction was electrophoresed in a 1% agarose/1% low-melting agarose gel and stained with ethidium bromide. The primer pairs used to amplify the rat oxytocin mRNA were described by Lefebvre et al.(4,24).

Anchorage Independent Growth

To test for anchorage-independent growth, cells were suspended in 0.3% agar. One ml of 0.8% agar in culture media(Agar Noble, Difco Lab., Detroit, MI) was used to cover the bottom of six-well culture plates(Falcon). For each cell line, 30,000 cells were dissolved in 10ml of 0.3% agar made up in F10/DMEM supplemented with 5% NuSerum, 5% FBS. Five ml or 15,000 cells were added per well in duplicate. The cells were cultured at either 33C, 37C, or 39C. For a period of 2 weeks, the cells were monitored for growth.

Preparation of Collagen Gels

Collagen gels were formed according to the method of Elsdale and Bard(8). Collagen fibers obtained from rat tails were washed in 1.5M KCL and rinsed in distilled water. The fibers were allowed to dry, weighted, and suspended in 0.1% acetic acid at 200ml per g of collagen fibers. The mixture was stirred for 48h at 4C and then centrifuged at 27,000 Xg for 1h at 4C. The supernatant was stored in aliquots at 4C.

Eight volumes of the collagen solution was added to 1 volume 10X Waymouth's medium(Gibco) and 1 volume 0.34 M NaOH. The solution was mixed gently and immediately added to culture dishes. The matrix was

allowed to gel at 37C for 1h. The gel was washed twice with medium for 1h at 37C prior to use.

Preparation of Stromal/Epithelial Co-Cultures

The stromal cell line CUS-V2 was added at high density to the collagen gel with its respective medium. Upon reaching confluence, usually within 2days, the culture medium was changed and the epithelial cell line CUE-P was added, at high density as well. Under light microscopy, the rapid attachment of the epithelial cells to the confluent layer of stromal cells was visualized. After 4h of incubation at 37C, the collagen gel was released from the dish and allowed to float. The following day, the collagen gel was removed from the petri dish, fixed in ethanol and prepared for paraffin sectioning and hematoxylin-eosin staining.

RESULTS

Morphology of the Cell Lines

A single epithelial cell line, CUE-P(Cemal Uterus Epithelial Cells-Plasmid transfected), was obtained with the pBAPSV40TtsA58 plasmid after 2 weeks under G418 selection. Four stromal and two epithelial cell lines were obtained following infection with the retroviral shuttle vector, again after 2 weeks under G418 selection. Two of the stromal cell lines, CUS-V2(Cemal Uterus Stromal cell-retroViral transfected, clone2) and CUS-V4, and one of the two epithelial cell lines, CUE-V1, were further expanded and characterized.

The morphology of the cell lines is illustrated in Fig.1. Panels A and C depict cultures of primary epithelial and stromal cells, respectively. Panel B shows the epithelial cell line CUE-P and panel D the stromal cell line CUS-V2. On the one hand, the two cell lines differ markedly with respect to their morphology and, on the other hand, each cell line shares several important features with the corresponding primary cell type from which it is derived. Epithelial primary cells as well as the CUE-P cells have a cuboidal morphology. By contrast, primary stromal cells as well as CUS-V2

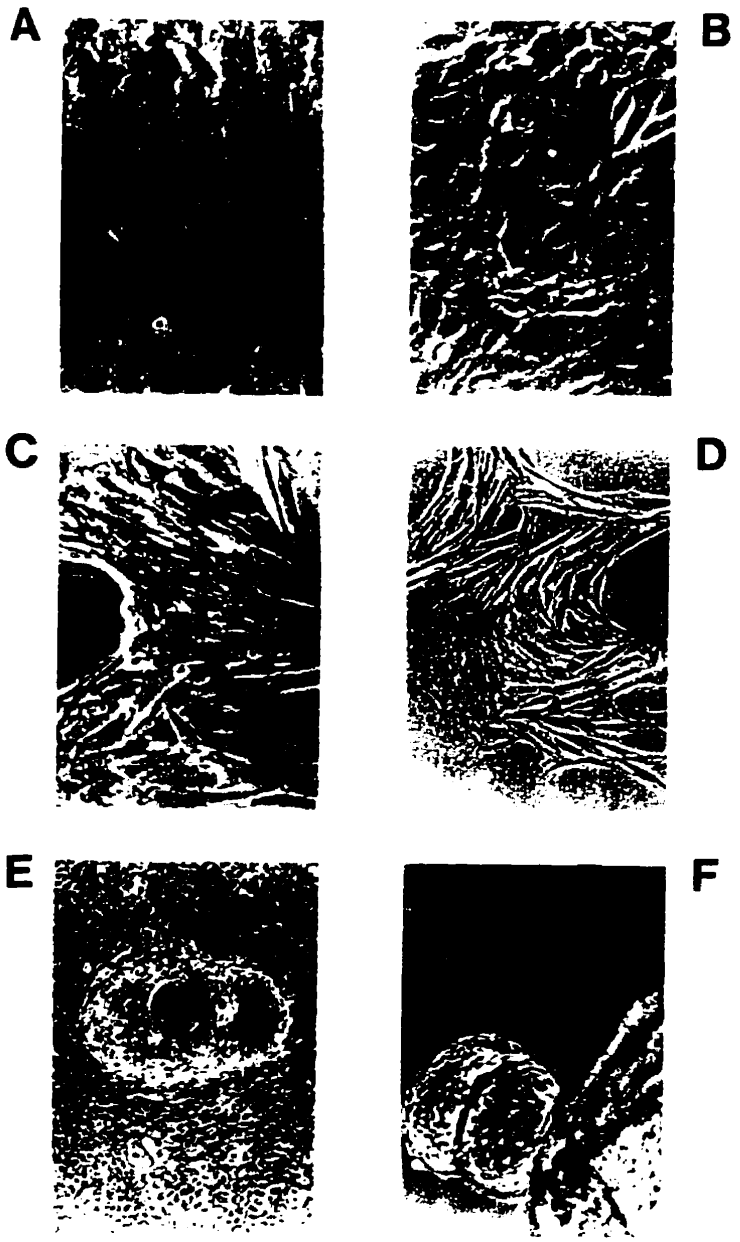


Fig. 1 Morphology of primary and immortalized uterine cells. A, primary epithelial cells; B, epithelial cell-derived cell line CUE-P; C, primary stromal cells; D, stromal cell-derived cell line CUS-V2. E and F, formation of a domelike structure by CUE-P cells grown on Matrigel(E, top view; F, side view). Magnification:A and B: X250; C,D,E, and F: X100.

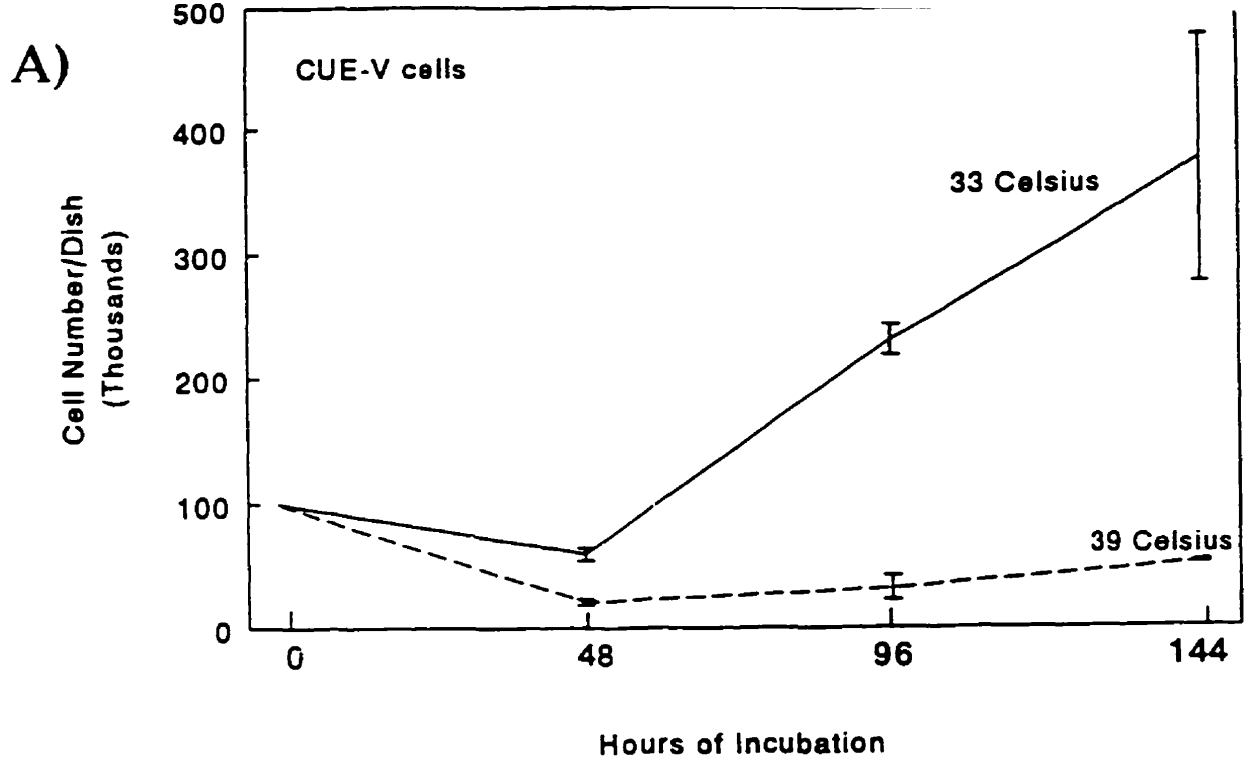
cells resemble fibroblasts and exhibit a spindlelike morphology. Both the epithelial and stromal cell lines have maintained their respective morphologies to date, after at least 150 passages.

Because the matrix on which epithelial cells are grown influences their morphological development, we tested the effect of culturing CUE-P cells on Matrigel, a rich mixture of extracellular matrix proteins whose major component is laminin followed by collagen type IV, heparan sulfate proteoglycan, entactin, and nidogen(as indicated by the supplier). If cultured on this matrix, CUE-P cells form domelike structures, as observed in panels E and F of Fig.1. Panel F depicts a side view of a domelike structure taken from a layer of cells peeling off the plate.

Growth Characteristics

Fig. 2A illustrates the growth properties of the epithelial cell line CUE-V1. This cell line is temperature sensitive in its growth. At the nonpermissive temperature of 39C, the cells undergo a noticeable change in morphology and grow slowly, whereas at the permissive temperature of 33C, the cells grow more vigorously and double every 24h. The plating efficiency of these cells is quite low, which is typical for epithelial cells(12,14). Some 100,000 cells were plated and, after 2 days, the cells hadn't reached the original plating density.

Fig. 2B shows the growth properties of the stromal cell line CUS-V2. This cell line is also temperature sensitive in its growth. It grows quite readily at the permissive temperature of 33C, doubling every 48h and, at the nonpermissive temperature, the cells also undergo a noticeable change in morphology and double at a slower rate. The plating efficiency for the CUS-V2 cells is quite high, as expected for cells with a fibroblast/stromal phenotype. After 48h, the cells almost reach the plating density of 200,000 cell per plate. In both cell lines, the doubling rate appears dependent on density. This trend is observed in the growth curves, since, at higher densities, the cells double faster, CUE-P and CUS-V2 cells are not temperature sensitive in their growth. CUE-P cells double every 17h and CUS-V4 cells double every 24h when incubated at 37C.



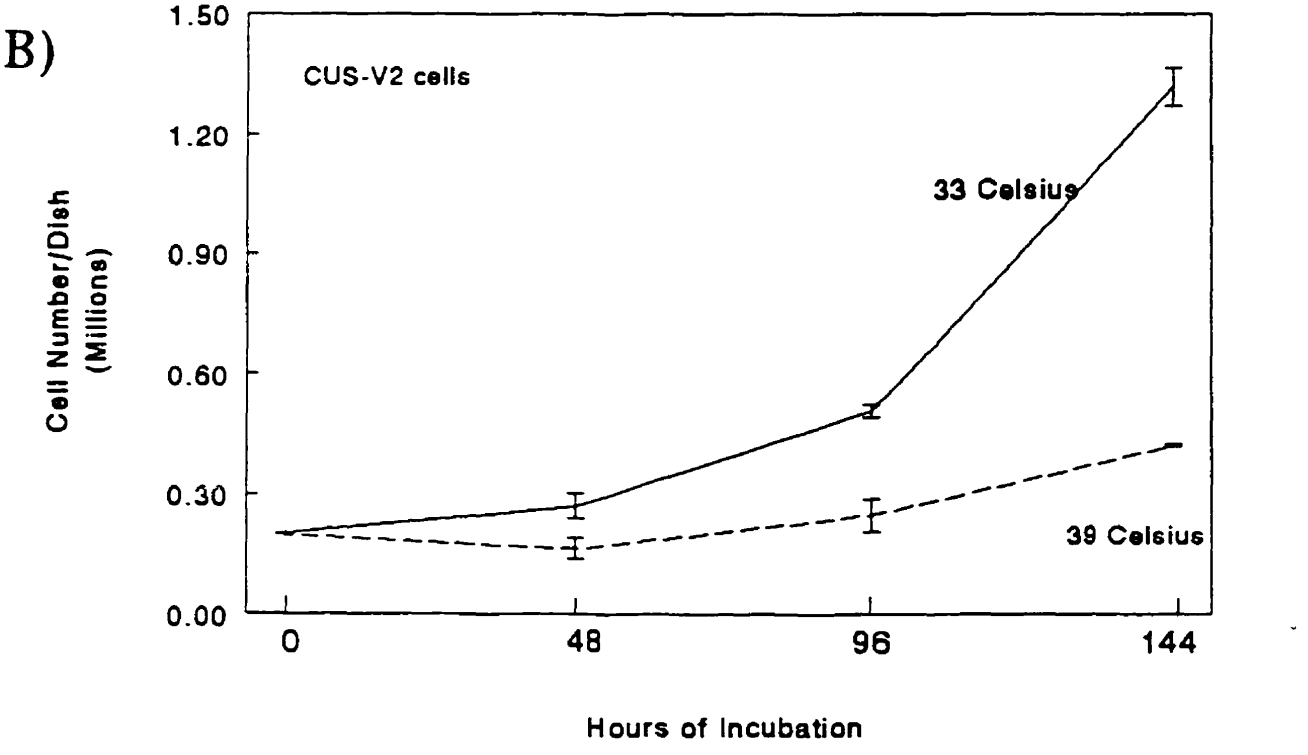


Fig.2 Growth properties of CUE-V1 cells(A) and CUS-V2 cells(B). 100,000 cells (A) or 200,000 cells (B) were added per dish, incubated at either 33C or 39C and monitored for growth every second day in duplicate dishes for up to 6 days. The cells were trypsinized, stained with Trypan Blue, and counted using a hemocytometer.

Alkaline Phosphatase Assay

Alkaline phosphatase is an enzyme present in differentiated epithelial, as well as stromal cells of the rat endometrium(11). As a marker for differentiation, we assessed the capacity of our different cell lines to synthesize this enzyme. The substrate for alkaline phosphatase, para-nitrophenylphosphate, was metabolized by all four cell lines to para-nitrophenyl(Fig.3), indicating that all four cell lines express the enzyme. The level of activity differed among the cell lines. The highest activity level was found in the CUE-P cell line, followed by CUS-V2, CUE-V1, and CUS-V4. In contrast to the epithelial cell lines, some variability in alkaline phosphatase levels was observed in stromal cell lines. This may be accounted for by differences in culturing conditions such as duration of trypsinization during passaging or steroid levels in the FBS, which have been shown to modulate alkaline phosphatase activity(18).

Intermediate Filament Expression

Upon decidualization, stromal cells can adopt an epitheliallike morphology and express certain markers typical for epithelial cells. This fact represents an added difficulty for determining if a given cell line is epithelial or stromal in origin. However, the types of intermediate filaments that are expressed by a cell line represent a useful indicator for the cell type from which it is derived. Stromal cells express vimentin and, upon decidualization, they also express desmin(28,30). In contrast, epithelial cells typically express the intermediate filament cytokeratin(14). Fig.4 shows the immunocytochemical detection of cytokeratin- and vimentinlike immunoreactivity in the cell lines CUE-P and CUS-V2. The stromal cell line CUS-V2 expresses vimentin only, whereas the epithelial cell line CUE-P expresses cytokeratin. The fluorescence for vimentin expression in CUE-P cells appears to be mainly nonspecific, because there is no specific cytoplasmic staining and the secondary antibody cross-reacts with other cellular proteins. This cross-reactivity is only observed in the epithelial cell lines and not in the stromal cell lines. Table 1 summarizes the relative levels of intermediate filament expression in all four cell lines.

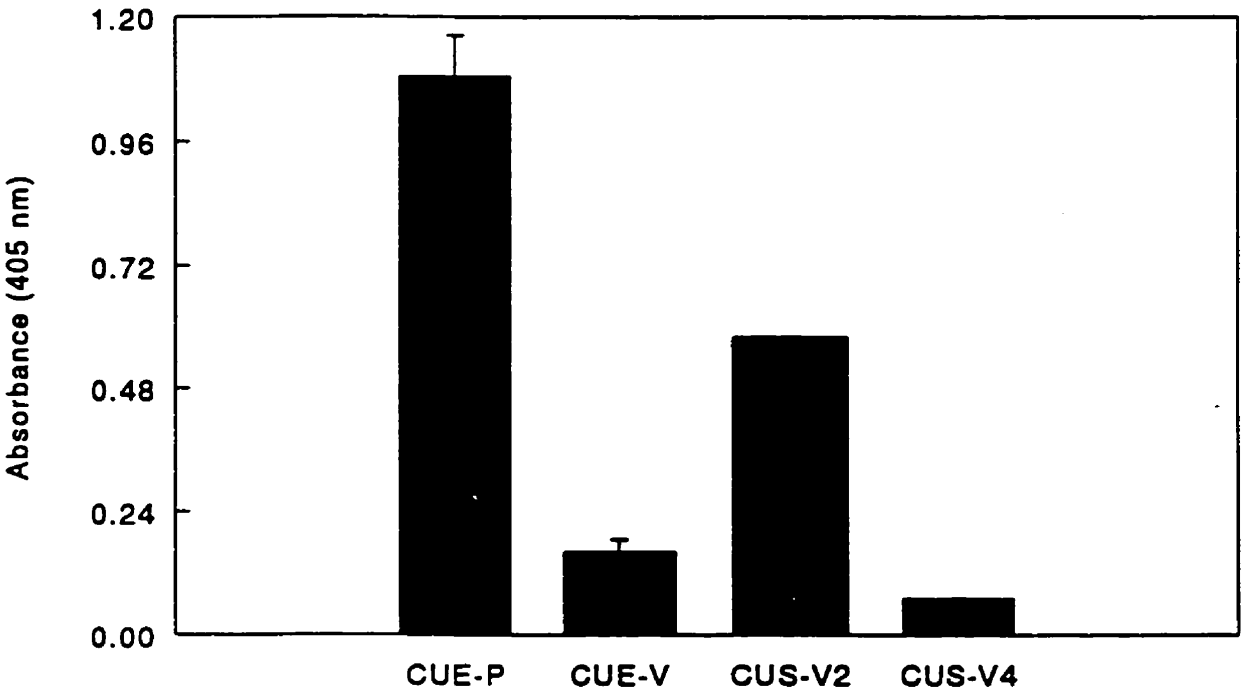


Fig.3 Alkaline phosphatase activity present in the cell lines. For each assay, membranes were prepared from $5X10^5$ cells and alkaline phosphatase activity was determined as described in "Materials and Methods". For each cell line, the absorbance measurements were done in triplicates.

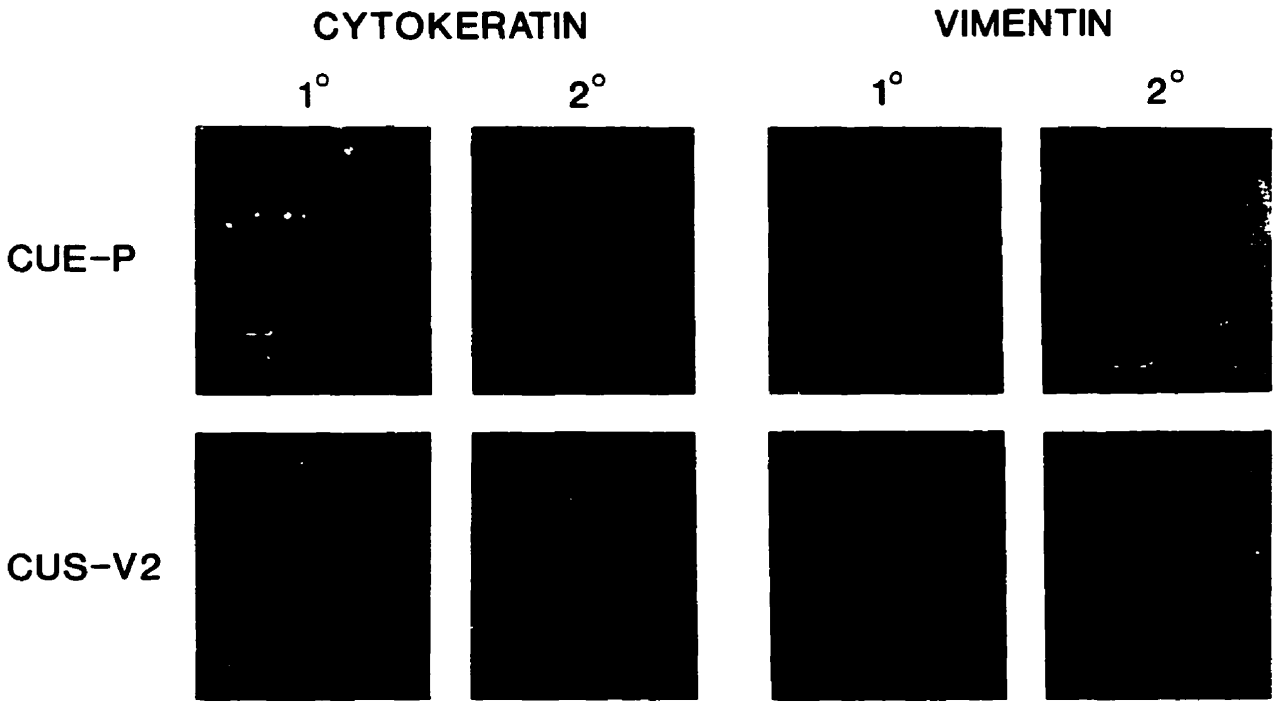


Fig.4 Intermediate filament expression in CUE-P and CUS-V2 cell lines. The cells were fixed, rehydrated, and immunostained either with anti-vimentin or anti-cytokeratin antibodies. After removing excess primary antibody, the cells were stained with FITC-conjugated secondary antibody(left column). To assess the specificity of the fluorescence, the cells were stained with the FITC-conjugated secondary antibody only(right column).

Table I **Relative intermediate filament expression by different cell lines.**

Cell Line	Cytokeratin	Vimentin
epithelial cell lines:		
CUE-P	++*	+
CUE-V1	+	++
stromal cell lines:		
CUS-V2	–	++++
CUS-V4	–	+++

* " ++++ " : highest levels; " - ": no observable immunostaining

Southern Blot Analysis

Recent primary cells when cultured on plastic culture plates have been known to become established spontaneously. Therefore, a Southern blot analysis was performed to assess if the vectors coding for SV40 large-T had integrated into the genome of the cell lines(Fig.5). The data shows that the vectors were inserted into the cell genomes and the expected EcoRI fragments were obtained(29,30), 2.3kb for the cell lines infected with the recombinant retrovirus(CUE-V1, CUS-V2, CUS-V4 in lanes 1,3,4,respectively), and 2.6kb for the cell line transfected with the plasmid(CUE-P in lane2). The additional bands seen in lane2 are likely incomplete digestion products that disappeared upon prolonged digestion with EcoRI. The approximate number of insert copies was determined using a standard curve obtained from a blot containing different copy numbers of the SV40 insert and EcoRI digested DNA from each cell line(not shown). From this analysis, the insert copy number per haploid genome was estimated to be 3-4 for the CUS-V4 and CUS-V2 cell lines and >10 for both epithelial cell lines.

Anchorage Independent Growth and Focus Formation

To test for anchorage independent growth and focus formation, CUE-P, CUE-V1, CUS-V2, and CUS-V4 cells were suspended in 0.3% agar and observed for a period of 2 weeks. Under these conditions, none of the cell lines grew. Therefore, all the cell lines are anchorage dependent in their growth. Furthermore, the cell lines do not form foci when kept in a confluent state for a period of 6 days. Both these observations support the notion that the cell lines examined are immortalized but not transformed.

Oxytocin Gene Expression

In vivo, rat endometrial cells express the oxytocin gene with a maximum at proestrous and estrous, as well as during the last few days of pregnancy(4,24). To determine if the epithelial cell line CUE-P was similarly capable of expressing the oxytocin gene, RT-PCR was performed using primers specific for exons A, B, and C of the rat oxytocin gene in three different combinations, as described earlier. The RT-PCR products

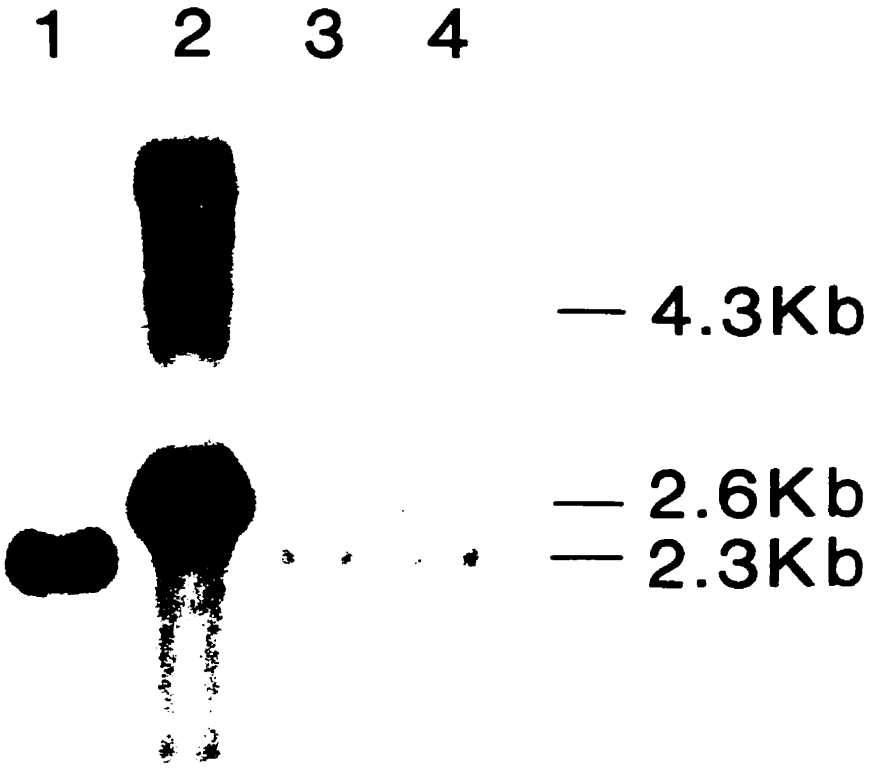


Fig.5 Southern blot to assess genomic integration of expression vectors for SV40 large T antigen in different cell lines. Lanes1-4: CUE-V1, CUE-P, CUS-V2, and CUS-V4 cells. Twenty ug of DNA obtained from the respective cell lines was digested with EcoRI endonuclease, separated on a 1% agarose gel, blotted, and hybridized with a 2.6kb SV40 large T antigen probe labeled with ^{32}P. Exposure to film:24h.

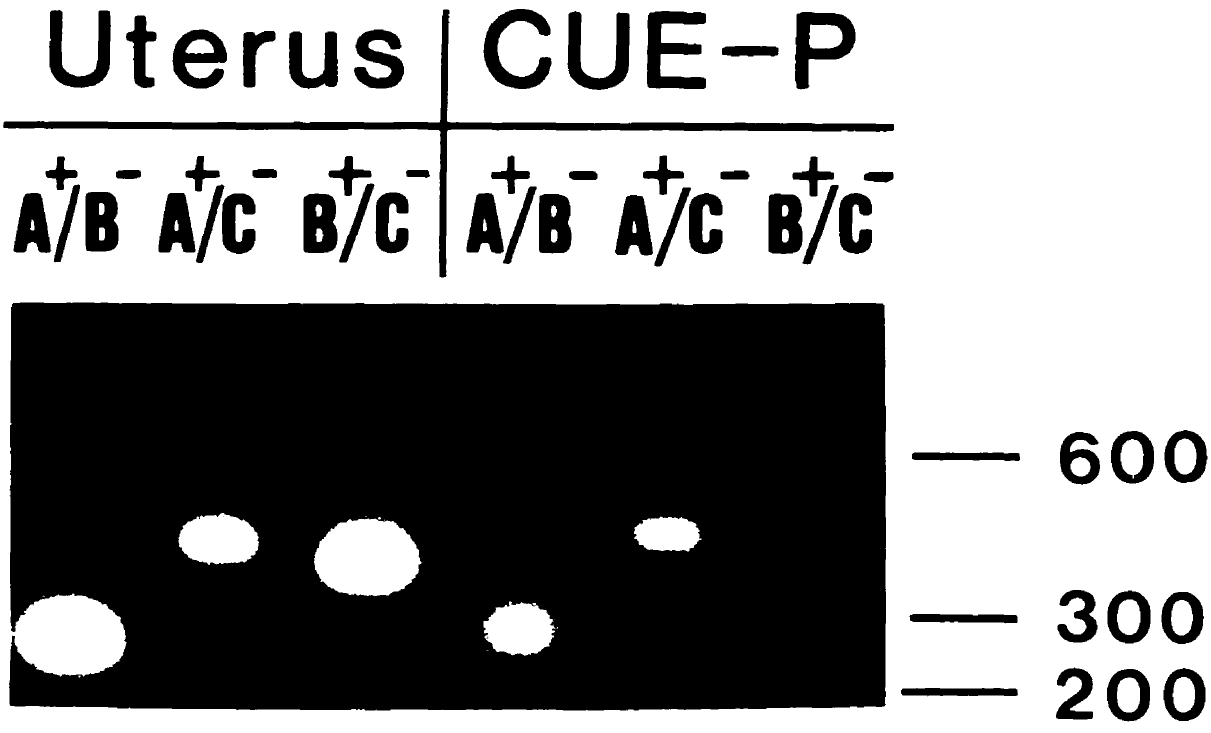


Fig.6 Demonstration by RT-PCR of oxytocin gene expression by CUE-P cells. Three ug of total RNA was reverse transcribed and one-sixth of the reaction product was used for amplification with three different primer combinations, each specific for the oxytocin cDNA. A,B,C correspond to exon1-, exon2-, exon3-specific primers, respectively. Orientation of primers:"+", sense; "-", anti-sense.

obtained with RNA extracted from a rat uterus at Day 21 of pregnancy and from CUE-P cells are shown in Fig.6. The data demonstrate that the CUE-P cells express the oxytocin gene, albeit at lower levels than does the pregnant rat uterus.

Co-Culture of Stromal and Epithelial Cell Lines

The differentiation of the endometrium is a very complex process that is, in part, dependent on stromal and epithelial cell interaction and, in part, on blood born factors, such as estrogen and progesterone. CUS-V2 cells were grown to form a monolayer on a type I collagen gel, and CUE-P cells were plated on top. The CUE-P cells attached on top of the stromal cells and grow to confluence forming a bi-layer cell culture resembling the endometrial tissue. Fig.7B is an enlargement of the co-culture observed in Fig.7A. Stromal cells are under the small cuboidal epithelial cells. They are larger and, as can be seen from the photomicrograph, four to five epithelial cells lie just above one stromal cell.

DISCUSSION

To develop an in vitro model system of rat endometrium, stroma and epithelium-derived cell lines were established by transfer of a gene encoding the temperature-sensitive SV40 large-T antigen into primary uterine cells. The SV40 large-T antigen is a member of a group of oncogenes, which includes myc and adenovirus E1A proteins, that are able to immortalize but not transform cell lines(22 and references therein, 39). Indeed, several observations suggest that the cell lines obtained are immortalized, but not transformed, a) The cell lines have retained morphological features that are characteristic of the cell types from which they are derived. The stromal cell line display a spindle shape and the epithelial cell lines exhibit a cuboidal shape. These shapes are characteristic for stromal and epithelial cells, respectively(12,28). Growing CUS-V2 cells arrange themselves in a vortexlike structure as this is the case in primary stromal cell cultures. The "cobblestone pavement" morphology displayed by confluent CUE-P cultures is characteristic for confluent epithelial cell layers(12,14). b) The cells are unable to grow in

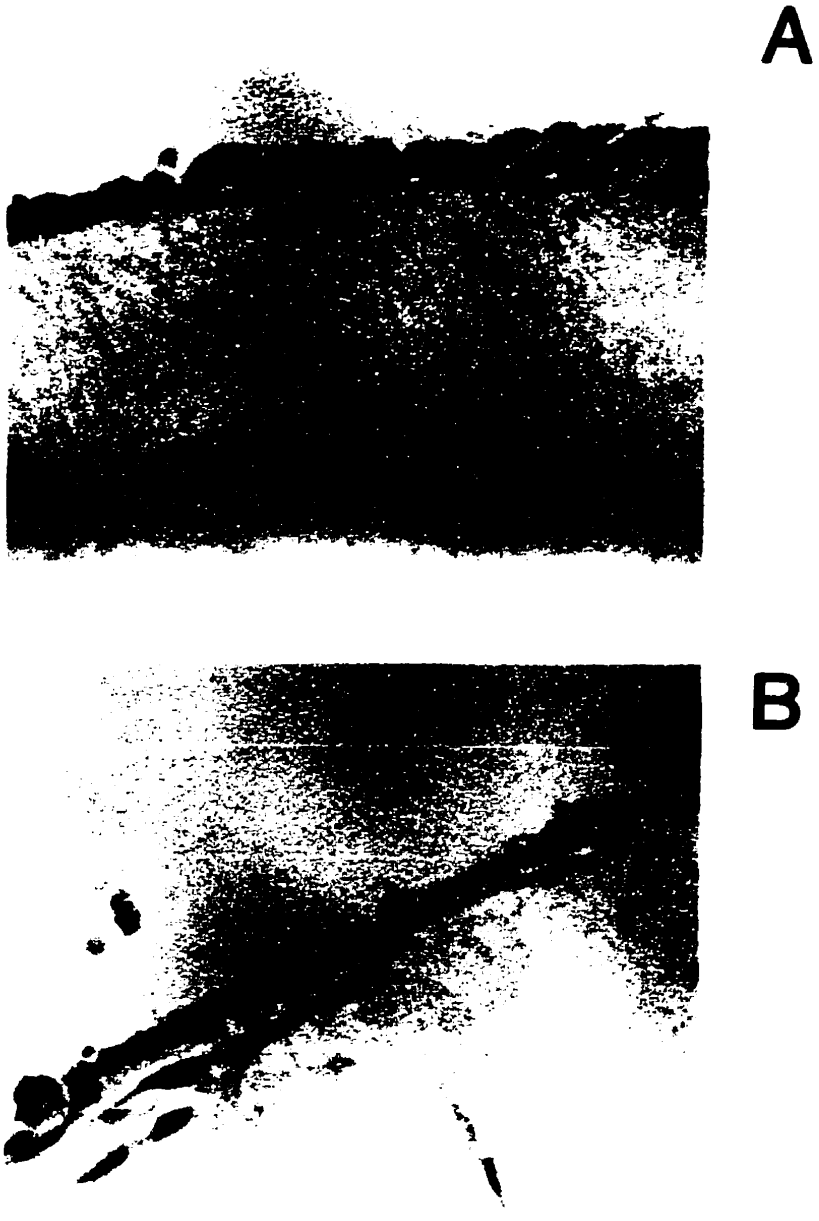


Fig.7 Paraffin section of a co-culture of stromal cells with epithelial cells stained with hematoxylin and eosin. Magnification: A: X100; B: X450. CUE-P cells were seeded on top of a confluent layer of CUS-V2 cells grown on a collagen gel and cells were fixed in ethanol 18h later. A compact layer of small cuboidal epithelial (CUE-P) cells spreads on top of a more loosely associated layer of stromal CUS-V2 cells. Four to five epithelial cells lie above one stromal cell.

an anchorage independent fashion. c) The cells do not form foci upon reaching confluence. Anchorage independent growth in soft agar and foci formation are both well-accepted criteria for a transformed cellular phenotype(22). d) The cells are capable of expressing specific genes that are typical of a differentiated phenotype. Among the proteins expressed by differentiated rat stromal cell in vivo are the intermediate filament vimentin(28) and the enzyme alkaline phosphatase(11). These genes were strongly expressed in both our stromal cell lines. In vivo, alkaline phosphatase is only expressed by stromal cells upon decidualization(11). The fact that this gene is also expressed by our stromal cell lines in vitro may indicate that they too have also undergone a decidualization process. This phenomenon is well known to occur in primary stromal cells upon in vitro culturing(28). On the other hand, the intermediate filament cytokeratin represents a marker for differentiated epithelial cells(14). Moreover, upon polarization, epithelial cells also express alkaline phosphatase, typically at the apical brush border(11). These two marker proteins were expressed by the epithelial cell lines CUE-V1 and CUE-P. To what extent these cells may also express vimentin remains unclear, at present. Low levels of vimentin expression have also been observed in other endometrium epithelial cell lines(39). Finally, CUE-P cells are capable of expressing low levels of oxytocin mRNA, a gene recently shown to be expressed specifically in uterine epithelial cells(4,24).

An additional feature of a differentiated phenotype displayed by CUE-P cells is that they are capable of forming domelike structures when cultured on Matrigel. Dome formation has been observed in other differentiated epithelial cell lines(32). These domes probably result from the tendency of epithelial cells to form glandular- or cryptlike structures in vitro(33). Indeed, in vivo, endometrial epithelial cells typically form glands upon decidualization and during pregnancy. This morphological transformation may be induced by specific matrix components, since Matrigel resembles quite closely the extracellular matrix formed by the uterine stroma upon decidualization and during pregnancy(30). The common extracellular matrix components include laminin, entactin, collagen typeIV, and heparan sulfate proteoglycan.

Cell lines with various phenotypes have also been derived from rat uterus by other investigators. Wiehle et al.(39) have obtained immortalized and transformed uterine epithelial cell lines and Helftenbein et al.(17) a temperature-sensitive transformed stromal cell line. However, in contrast to the cell lines described here, the immortalized epithelial cell lines obtained by Wiehle et al.(39) do not express alkaline phosphatase or cytokeratin. Li et al.(26) have obtained a transformed temperature sensitive rabbit endometrial cell line. The precise phenotype of this line is, however, unknown and the cells lack contact inhibition. Other cell lines exist that have been derived from various uterine cancers, including the frequently used Ishikawa cells(18). However, due to their origin, they are by definition transformed. To our knowledge, none of the epithelial uterine cell lines described so far combines the characteristics of immortalization, temperature sensitivity, and expression of differentiation-specific markers.

Previous investigators studying the endometrium have realized the importance of stromal and epithelial interaction. Epithelial morphology, basement membrane formation, stromal decidualization and proliferation seem to depend on this interaction (3,12,14,20,25,38). In an attempt to achieve some of these in vivo effects, investigators have co-cultured both primary epithelial and stromal cells(37). The techniques usually involved culturing the cells together in the same dish(37) but rarely, if at all, culturing epithelial cells on top of endometrial stromal cells(19). In our simple in vitro model, we are able to form an endometriumlike tissue by forming a distinct layer of epithelium on top of stromal cells. The potential for this model is quite exciting because it can be used to study in vitro blastocyst implantation and hatching, trophoblast/endometrium interactions, epithelial/stromal interactions(specifically epithelial proliferation and its modulation by stromal cells), and endometrial basement membrane formation.

Clearly, one of the important applications of these cell lines involves extending our understanding of the proliferative processes in the endometrium. Several distinct stimuli can induce epithelial and stromal cells to proliferate. Epithelial cells proliferate in response to estrogen(20,23,29,31) and stromal cells upon decidualization(11).

Understanding the basic principles that are involved in regulating epithelial and stromal cell proliferation should foster better therapies towards uterine cancers. There is growing evidence that the proliferative effect of estrogen on the epithelial cells is indirect(3,20) and involves steroid-induced secretion of growth regulators by epithelial and stromal cells, including TGFα and EGF(31). The present in vitro system should provide a novel and powerful model to address these questions in an attempt to further understand the mechanisms underlying growth, differentiation, and specific gene expression in uterine epithelial and stromal cells. A similar model was also developed in Mink(Appendix B).

Acknowledgements

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CHAPTER 2

Regulation of Cox-2 Gene Expression in Rat Uterus in Vivo and in Vitro

PREFACE

Once the cells were characterized, we decided to test various factors which would modulate prostaglandin production. Since the rate limiting enzymes for prostaglandin synthesis have been defined, the research was simplified. The following chapter examines the expression and regulation of COX-1 and COX-2 genes in the pregnant uterus and in these cell lines.

SUMMARY

Prostaglandins are involved in mediating several important processes in mammalian reproduction, including the initiation of parturition. In the present study, we examined the expression in rat uterus of two-rate limiting enzymes involved in prostaglandin production, cyclooxygenase(COX) 1 and 2. Expression of the Cox-2 gene in the pregnant rat uterus gave rise to a single mRNA transcript of approximately 4.4 kb. Cox-2 mRNA levels increased 3.5 fold between day 7 of pregnancy and the onset of parturition on day22. In contrast, COX-1 mRNA levels remained constant during the same period. To investigate factors involved in mediating the regulation of COX-1 and COX-2 gene expression, rat endometrial stromal and epithelial cell lines, were used. In the stroma-derived cell line, CUS-V2, COX-2 gene expression was demonstrated by reverse transcriptase/polymerase chain reaction(RT-PCR) and by immunocytochemistry. In these cells, COX-2 gene expression was inducible by the cytokines interleukin-1β and tumour necrosis factor α, but not by interleukin-6. The two former cytokines also induced prostaglandin $F_{2\alpha}$ production. In contrast, COX-1 gene expression was constitutive in this cell line. In the endometrial epithelium-derived cell line, CUE-P, both Cox-1 and Cox-2 genes were expressed in a constitutive fashion. In conclusion, the present in vivo and in vitro data indicate that decidual COX-2, but not

COX-1, gene expression is regulated during pregnancy and implicate specific cytokines as possible inducers within the decidua.

INTRODUCTION

Prostaglandins play important roles in the mediation of several key processes during pregnancy and parturition. These include induction of ovulation, implantation, decidualization, immunosuppression, dilatation of uterine arteries and initiation of parturition(1-5). In subprimates, increases in plasma $PGF_{2\alpha}$ levels induce luteolysis(6,7), and in primates, administration of $PGF_{2\alpha}$ results in cervical dilatation and myometrial contractions(5,8). Furthermore, inhibition of PG synthesis by non-steroidal anti-inflammatory drugs prolongs labour(5).

PG synthesis is controlled by two rate limiting enzymes, phospholipase A_2(PLA_2) and cyclooxygenase(COX), also referred to as PGH_2 synthetase. Whereas the cytosolic form of PLA_2 is involved in receptor-coupled arachidonic acid liberation(9,10,11), COX cyclizes and oxidizes arachidonic acid to yield PGG_2 and PGH_2, the precursor for PGI_2, $PGF_{2\alpha}$, PGE_2, PGD_2, and thromboxane A_2(1,5,8,12-14).

Two distinct forms of COX have been identified, which are encoded by two different genes(15). COX-1 is expressed ubiquitously, whereas COX-2 has a more restricted expression pattern(16). Furthermore, in contrast to COX-1, COX-2 is highly inducible. Specifically, COX-2 is induced as part of the inflammatory response together with other immediate early response genes. Specific inducers of COX-2 include phorbol esters, mitogens, cytokines, forskolin and serum(17-19). COX-1 and COX-2 are both glycoproteins which function as homodimers(20-22). Amino acid sequence analysis indicates a 61% identity between the two molecules. Specific differences include a stretch of 17 amino acids present in the N-terminus of COX-1, which is absent in COX-2 and a segment of 18 amino acids which is present in the C-terminus of COX-2, but not in COX-1.

During pregnancy, uterine prostaglandin synthesis is regulated by cytokines such as IL-1α, IL-1β, TNF-α and IL-6. These cytokines are

produced locally in uterine tissues, specifically during pregnancy, from the implantation period until parturition(23,24). Sites of production are bone marrow derived cells, particularly macrophages(25), as well as endometrial epithelial and decidual cells(23). Cytokine-induced prostaglandin production is also a likely contributory factor in abortion induced by amniotic infection(5), and in IL-1β induced abortions in mice(26,27). Finally, prostaglandin production can be induced in vitro in cultured decidual cells by stimulation with cytokines(28,29). In vivo, there is a rise in decidual prostaglandin production during the latter half of pregnancy(30,31), without an increase in PLA_2 activity(32).

The objective of this study was 1) to examine in vivo, if COX-1 or COX-2 mRNA expression during pregnancy are differentially regulated with respect to gestational age, 2) to determine in vitro, if cytokines IL-1β and TNF-α regulate COX-1 or COX-2 mRNA expression, and finally, 3) using our rat endometrial cell lines(33), to examine whether COX-1 and COX-2 gene expression and regulation differs between uterine stromal or uterine epithelial cells.

MATERIAL and METHODS

Animal Care and Tissue Preparation

Timed-pregnant Sprague Dawley rats were purchased from Charles River Laboratories (St. Constant, PQ, Canada). They were fed rat chow ad libitum and kept in a temperature and humidity-controlled environment. Animals were sacrificed by decapitation under light ether anesthesia. The procedures were approved by the Royal Victoria Hospital Animal Ethics Committee. Animals were sacrificed in the various stages of the estrous cycle and at days 7(n=3), 13-14, 17-18 and 21(n=3) of gestation and during parturition(n=3). Dissected non-pregnant uteri and pregnant uteri stripped of fetal tissue were frozen in liquid nitrogen and stored at -70C.

Cell Culture

Establishment and characterization of the uterine stromal-derived cell line CUS-V2 and the uterine epithelial cell-derived cell line CUE-P was described earlier(33). Both cell lines were cultured in DMEM/F10 medium(Gibco) supplemented either with 7% Fetal Bovine Serum(CUS-V2) or 5% NuSerum type IV(Collaborative Research, Bedford,MA).

Cells were grown to confluency at 37C, followed by a 48 h incubation at 39C(non-permissive temperature) to induce differentiation. Twenty-four hours before treatment with various test substances, serum concentration was decreased to 0.5%. Cells were treated with the following compounds: 1,000U/ml human IL-1β(Immunex, Seattle, Washington,$1X10^9$U/mg), 1,000U/ml murine TNFα(Genzyme, Cambridge,MA,$1X10^{10}$U/mg), or 100U/ml of human IL-6(Immunex,$1X10^7$U/mg). RNA from cultured cells was extracted as described below. Media were removed, freed of contaminating cells by brief centrifugation and stored at -20C. $PGF_{2\alpha}$ concentration in the media was assayed using a commercial ELISA assay(Cayman Chemicals Co., Ann Arbor,MI) and analyzed by the program Logprog, developed by Rodbard and Hutt(34).

Reverse Transcriptase/Polymerase Chain Reaction(RT/PCR)

RNA was extracted in 4M guanidine thiocyanate and purified by ultracentrifugation through 5.7M CsCl as described(35). First strand cDNA was synthesized using Avian Myeloblastosis Virus Reverse Transcriptase(Life Sciences, St. Petersburg, FL.)(36) primed with random hexamers(Gibco/BRL). The cDNA products were dissolved in 30μl of TE and of which 5μl was used for the PCR amplification. Unless stated otherwise, PCR was performed for 35 cycles with the following parameters: 94C, 90s; 65C, 90s;72C, 2minutes. Following electrophoresis on 2% agarose, the PCR products were transferred to nitrocellulose and hybridized to a labeled oligonucleotide probe(see below). Autoradiograms were obtained and the intensity of autoradiographic bands was quantitated by densitometry(37) or by phosphorimager analysis(Fujix Bio-Imaging Analyzer BAS1000). The quantified COX-2 transcript values were normalized to the GAPDH transcript values where indicated. As uterine GAPDH mRNA increases

slightly during gestation, the in vivo results were presented relative to day7.

The following primer pairs were used: for glyceraldehyde phosphodehydrogenase(GAPDH) amplication(38): 5'CCCTTCATTGACCTCA ACTACATGGT3'(forward), and 5'GAGGGGCCATCCACAGTCTTCTG3'(reverse). The amplified product being 470 base pairs(bp) in length. For COX-1(39): 5'TGCATGTGGCTGTGGATGTCATCAA3'(forward), and 5'CACTAAGACAGA CCCGTCATCTCCA3'(reverse). The amplified product being 450 bp in length. For COX-2(39): 5'GAGTGGGGTGATGAGCAACTATTCC3'(forward), and 5'TTTGATTAGTACTGTAGGGTTAATG3'(reverse). An additional reverse primer was also used in some experiments (5'CTGTAGGGTTAATGTCATCTA GTCT3'). The annealing temperature used for the former primer was 55C and 65C for the latter primer. The use of the first primer pair resulted in an amplification product of 872 bp, the second primer pair yielded an amplification product of 861bp. Both reverse primers used were complementary to a cDNA region which encodes the 18 amino acids C-terminus of COX-2 which is absent from COX-1. All primer pairs were designed such that the primers hybridized to regions encoded by separate exons. By this strategy, amplification of contaminating genomic DNA was avoided or resulted in bands of different sizes. Furthermore, the COX-2 amplified product was cloned using the Invitrogen TA Cloning Kit(Invitrogen Co., San Diego,CA, U.S.A) and sequenced which demonstrated that the amplified product was indeed the COX-2 cDNA. Following transfer to nitrocellulose, the PCR amplification products were hybridized to the following internal anti-sense oligonucleotide probes:5'CTGTAGGGTTAATG TCATCTAGTCT3'(COX2-AS1)or5'ATCTAGTCTGGAGTGGGAGGCACTTG CATT3'(COX2-AS2) for the amplification products obtained with the COX-2-specific primer pair 1 or 2 respectively and 5'-GTCATGGATGACCTTGGCCA GGGG-3' for the GAPDH amplification product.

Northern Blot Analysis

RNA was enriched for poly(A)$^+$ RNA by absorption to oligo(dT)(40) and 5μg was denatured in 1M glyoxal, 50% dimethylsulfoxide(DMSO), and 10mM sodium phosphate buffer (pH6.5) for 60 min. at 50C. Following electrophoresis in 1.5% agarose, the RNA was transferred to Hybond N

membranes(Amersham) and hybridized(5XSSPE, 5XDenhardt, 0.2%Sodium Dodecly Sulphate(SDS), 200ug/ml tRNA, 10% dextran sulphate) to a radiolabelled oligonucleotide probe complementary to COX-2 mRNA, (5'ATCTAGTCTGGAGTGGGGAGGCACTTGCATT-3'). The probe was 5' kinase labeled with γ-ATP. The blot was washed(12mM NaCl, 0.12% SDS) at 55C for 2hr and exposed to Kodak film for 16 days. Next, the blot was stripped and then hybridized to a GAPDH probe(as above).

Immunocytochemistry

The primary antibody used was a polyclonal rabbit anti-COX-2 antibody, kindly provided by Dr. Daniel Hwang(41). The secondary antibody was an Ig anti-rabbit IgG antibody coupled to FITC(Dako Corporation, Carpenteria,CA). Both antibodies were used at a dilution of 1:20. Cultured cells were fixed in 70% ethanol for 30 min., dipped eight times into cold water and two times into phosphate buffered saline(PBS) for rehydration. Following incubation with primary antibody for 1h at room temperature, cells were washed three times with PBS and secondary antibody was added for 1h at room temperature. Following washing with PBS(three times) and water(twice), the cell preparations were mounted with Immumount(Shandon, Pittsburgh,PA) and viewed under Nikon fluorescence microscope.

Statistics

Where the results were expressed as means +/- SE(n=3, separate experiments), differences were examined with one way analysis of variance (ANOVA) and individual comparisons between the treatments was assessed by the Tukey's Test, if there was a significant difference, $P<0.01$. For other result values, which were derived from two separate data points, linear regression analysis was done.

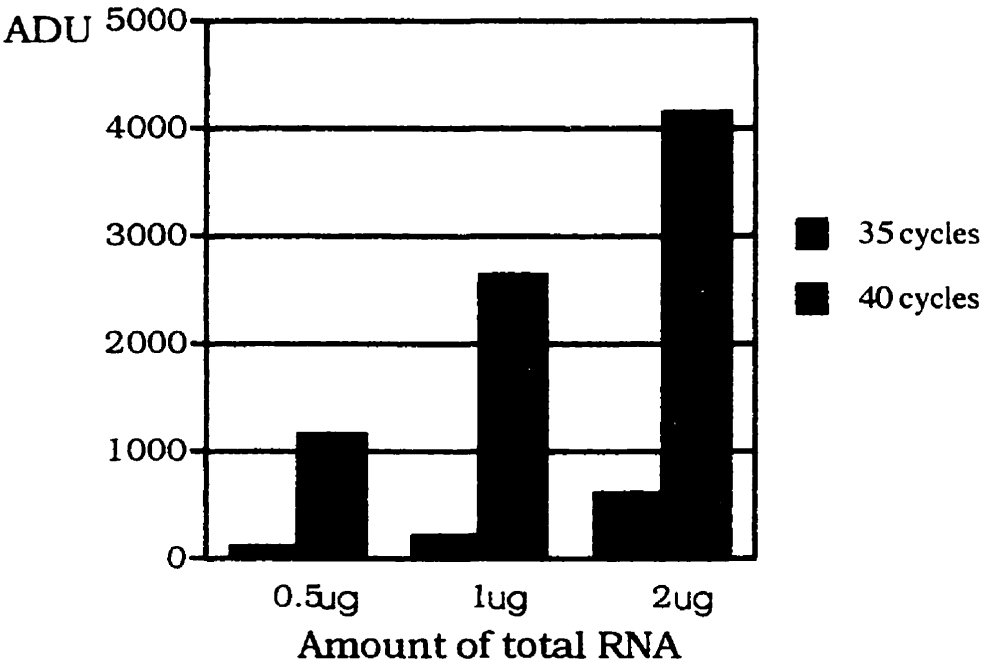


Fig.1 Dose-response relationship of different amounts of input total RNA from a parturient uterus and the intensity of autoradiographic bands obtained by Southern blot analysis of RT-PCR products. Each data point corresponds to the mean of two independent experiments. Linear regression analysis of the input total RNA used for the RT-PCR versus the amount of COX-2 transcript amplified, at 35 cycles, gave us a coefficient of determination of 0.761. ADU:arbitrary densitometric units.

RESULTS

Uterine COX-1 and COX-2 Gene Expression In Vivo

To determine whether the increase in uterine prostaglandin production during the second half of pregnancy was associated with an increase in COX-1 and/or COX-2 gene expression, we assessed the levels of uterine COX-1 and COX-2 transcripts using RT/PCR.

To validate the COX-2 RT/PCR assay used, a dose response relationship was determined using different amounts of input RNA(0.5μg-2μg) and using two different cycle numbers(35 and 40 cycles). The results show that the dose range used falls within the linear input/output relationship of the system and that with 35 cycles, the amplification capacity of the system has not been exceeded(Figure1). At 40 cycles we were reaching the plateau phase.

As shown in Fig2a, Cox-2 transcript levels were low during the estrous phase and day7 of pregnancy, and increased during the latter half of pregnancy. This is in contrast to GAPDH and COX-1 transcript levels(Fig.2a and b) which showed only minor changes during this period.

To quantitate the increases in COX-2 transcript levels, Southern blots of the RT/PCR reaction products were hybridized to an internal primer and the intensities of the resulting bands were quantitated by phosphor-imager analysis. As shown in Fig.3, COX-2 mRNA levels increased 3.5-fold between day7 of pregnancy and the time of parturition. Specifically, a significant increase occurred between day21 and the onset of parturition(normally occurring on day22).

Uterine COX-2 transcripts were further characterized by Northern blot analysis. As shown in Fig.4(upper panel), COX-2 mRNA expression increases at day21 of gestation relative to day7. The transcript size was determined to be approximately 4.4 kb as assessed from this and other blots(data not shown). At day21, we also observe a faster migrating transcript which hybridizes with the COX-2 probe. In contrast to the COX-2

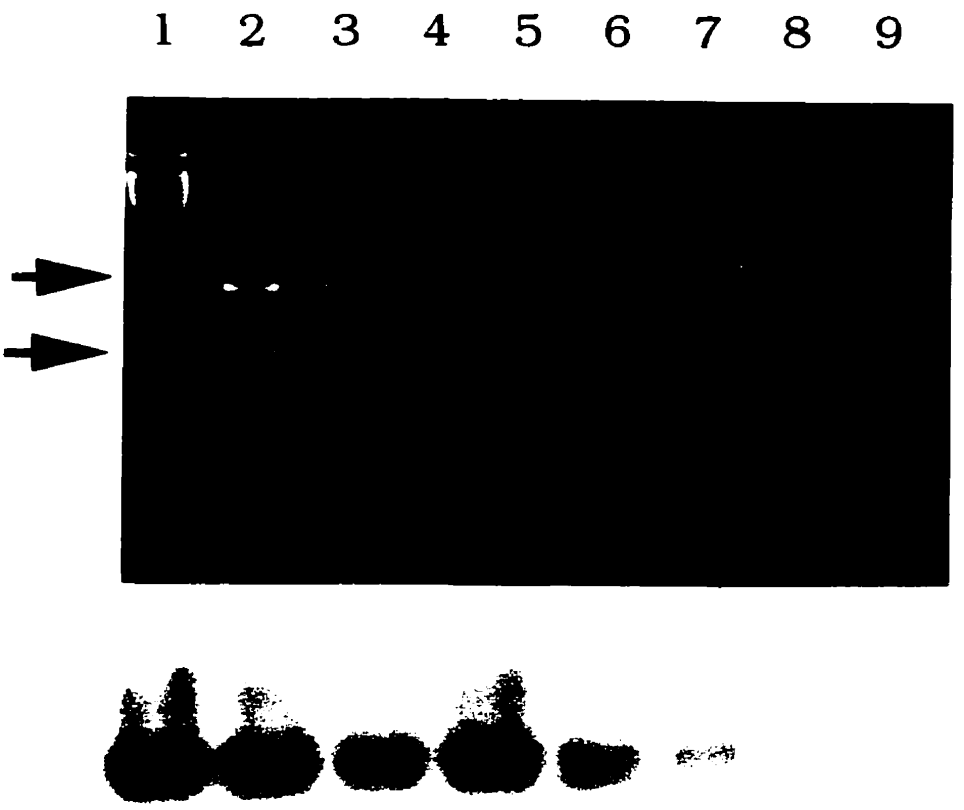


Fig.2A Uterine expression of the COX-2 and GAPDH genes assessed by RT-PCR. Upper panel: RT-PCR analysis of COX-2 mRNA (upper arrow) and GAPDH mRNA(lower arrow). PCR products were electrophoresed in 2% agarose and stained with ethidium bromide. In each RT-PCR assay, 2ug of total uterine RNA was used. Animals were in the following stages: parturition(lane2), day21 of pregnancy (lane3), day17 of pregnancy(lane6), day 13 of pregnancy(lane5), day7 of pregnancy(lane6), estrous(lane7), proestrous(lane8), diestrous(lane9). Lane1 contains DNA markers. Lower panel: Southern blot analysis of the agarose gel shown in the upper panel. The blot probed with a labelled oligonucleotide probe specific for the COX-2 gene(COX-2 AS1). Lanes 1 to 8 correspond to lanes 2 to 9 of the upper panel. The total RNA obtained from each stage of pregnancy was RT-PCR analysed at least twice.

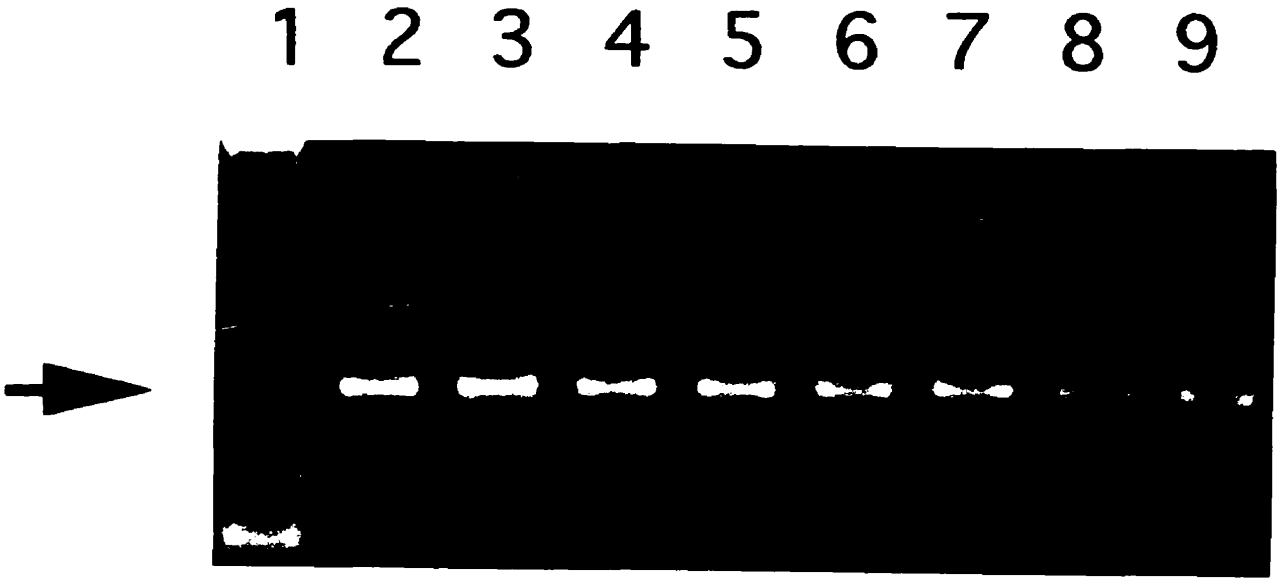


Fig.2B Uterine expression of the Cox-1 gene assessed by RT-PCR. Animals were in the following stages: parturition(lane2), day21 of pregnancy (lane3), day17 of pregnancy(lane6), day 13 of pregnancy(lane5), day7 of pregnancy(lane6), estrous(lane7), proestrous(lane8), diestrous(lane9). Lane1 contains DNA markers. The total RNA obtained from each stage of pregnancy was RT-PCR analysed at least twice.

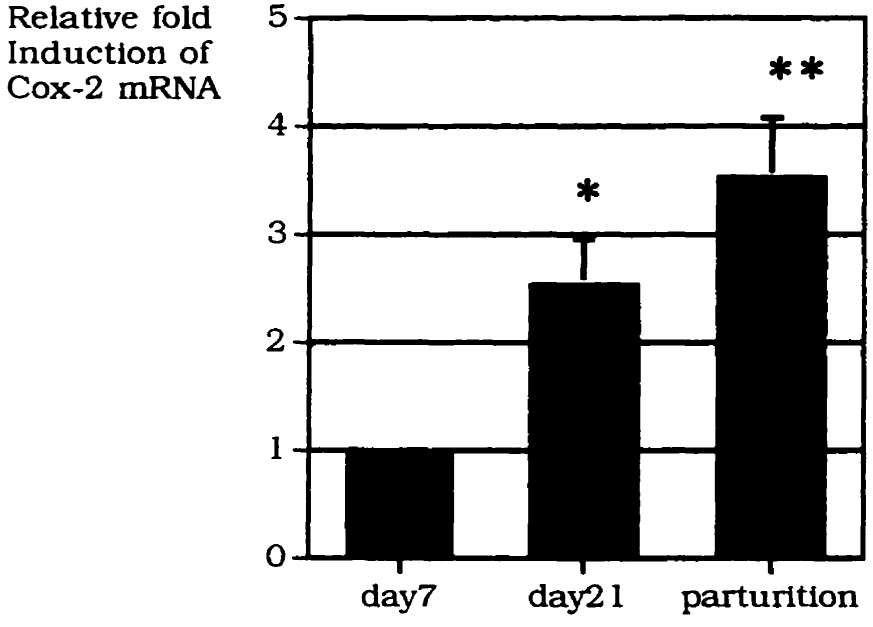


Fig.3 Semi-quantitative analysis of uterine COX-2 mRNA levels at days 7 and 21 of pregnancy and during parturition. Southern blots of RT-PCR reactions, as shown in Figure 2A, were scanned densitometrically. Each bar represents the mean +/- S.E.(n=3 pregnant rats). Statistically significant differences vs. day7 are indicated by asterisks(*,P<0.01;**,P<0.05).

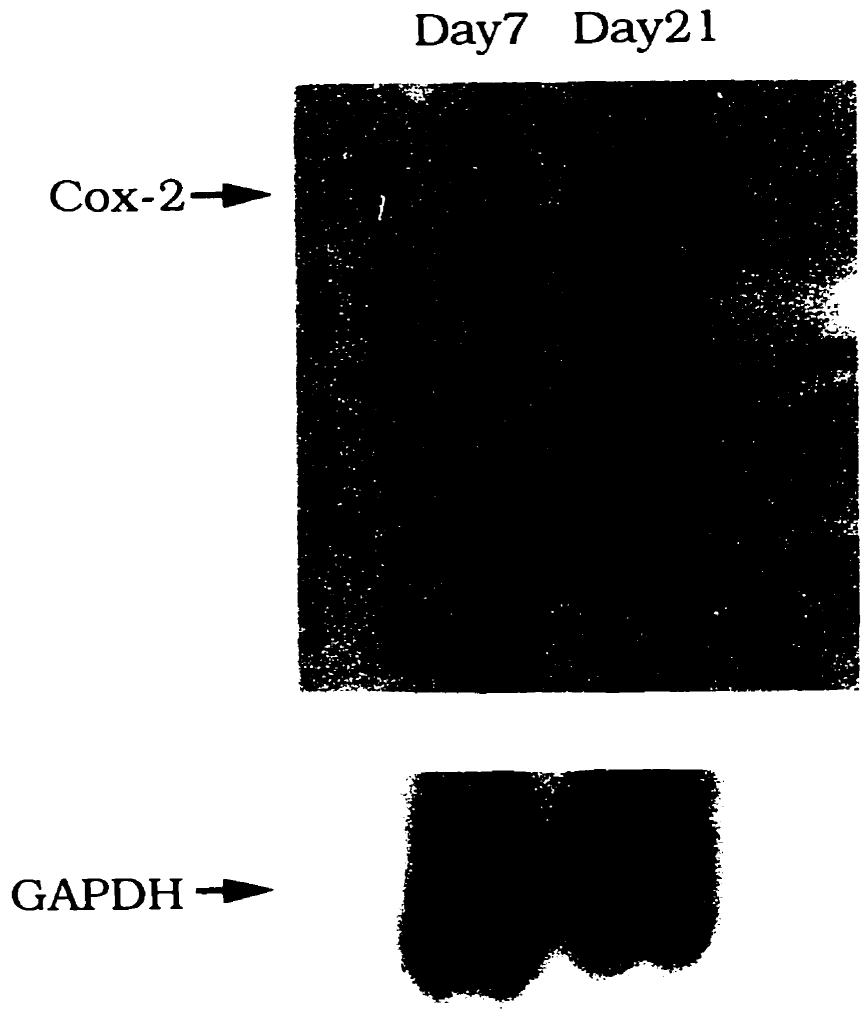


Fig.4 Northern blot analysis of COX-2 mRNA extracted from the uterus. 5ug of polyA enriched RNA was electrophoresed in a 1.5% Agarose gel and then transferred to a nitrocellulose blot which was probed with a labelled oligonucleotide probe specific for the COX-2 mRNA (upper panel). Exposure time: 16 days, with intensifying screen. The blot was stripped and reprobed for the GAPDH mRNA(lower panel). Exposure time: 24hrs with intensifying screen.

transcript, the level of the GAPDH mRNA expression remained relatively unchanged(lower panel), similar to the RT-PCR results.

Cox-1 and Cox-2 Gene Expression in CUS-V2 cells

To study potential factors involved in the regulation of COX gene expression, a cell line derived from rat endometrial stroma was used. As described previously(33), this cell line was developed by retrovirus-mediated transfection of DNA encoding the temperature-sensitive mutant of the viral SV40 large T antigen(tsT-Ag). The cells for this initial experiment were cultured in DMEM/F10 supplemented with 7%FBS. Test substances were added for 4 hours following a 24 hour incubation at non-permissive temperatures(39C). As shown in Figure5a(upper panel), the cytokines IL-1β and TNFα alone or combined, had a strong inducing effect on COX-2 gene expression, whereas the cytokine IL-6 remained without effect. By contrast, both GAPDH and COX-1 transcript levels remained unaffected by the cytokine treatments(Figure5a upper panel and 5b).

In order to characterize further the effect of IL-1β and TNF-α on COX-2 gene expression, cells were grown for a period of 24 hours at 39C in 0.5% FBS prior to a four-hour exposure to the tested cytokine without a fresh change of media. These culturing conditions enabled us to dissociate the cytokine response from the serum response. As shown in Figure 6a, a dose response curve for IL-1β, a maximal response is observed at 1000U/ml. As an internal control, GAPDH levels were also assessed and shown to be unaffected by this treatment. Quantitative evaluation of autoradiographic bands resulting from Southern blot analysis and normalization to the GAPDH transcript showed that IL-1β treatment resulted in a 5.3-fold increase in COX-2 transcripts($P<0.01$;Figure 6b). Addition of actinomycinD(2μg/ml) abolished the IL-1β-induced increase in COX-2 transcript levels, indicating that the interleukin effect involved a transcriptional event.

The dose response relationship of COX-2 induction by TNFα was also investigated. As shown in Figure7, when quantitated and normalized to the GAPDH transcript levels, a 2-fold increase of COX-2 mRNA levels was obtained at a dose of 1.2U/ml of TNFα. Higher doses did not lead to a

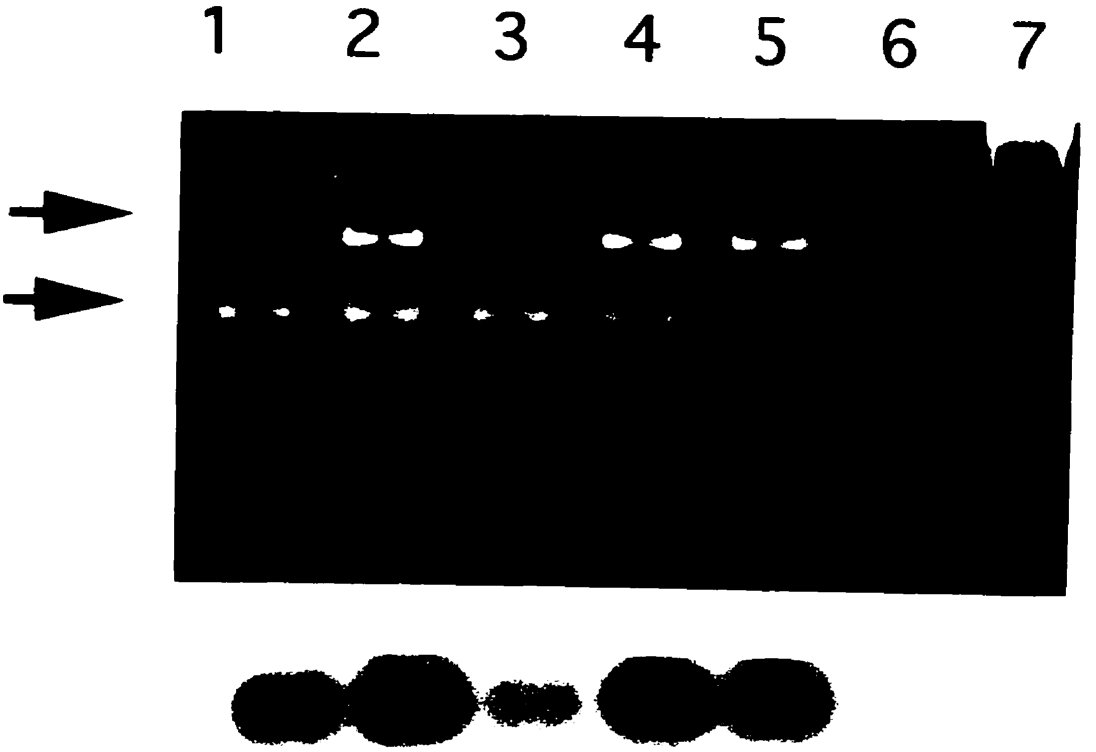


Fig.5A COX-2 and GAPDH gene expression in the rat endometrial stromal cell line CUS-V2 which were cultured in 7%FBS, DMEM/F10, at 39C. Upper panel: RT-PCR amplification products of COX-2 transcripts (upper arrow) and GAPDH transcripts (lower arrow). Cells were treated for 4 hours with the following agents: 10,000 U/ml TNFα and 10,000 U/ml IL-1β(lane2), 1,000U/ml IL-6(lane3), 10,000 U/ml TNFα(lane4), 10,000U/ml IL-1β(lane5). Lane6: no cytokine addition. Lane7: DNA markers. Lane1: RNA extracted from a rat uterus at day21 of pregnancy. Lower panel: Southern blot analysis of the gel shown in the upper panel, performed as in Figure2a.

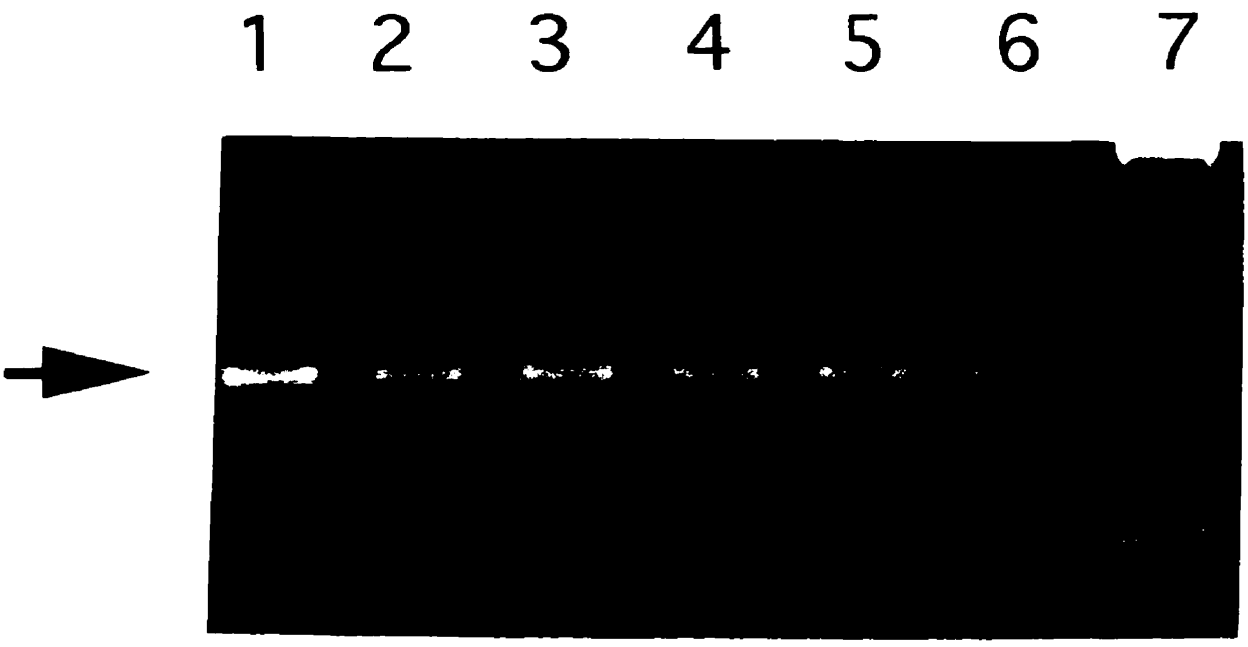


Fig.5B COX-1 gene expression in the rat endometrial stromal cell line CUS-V2 which were cultured in 7%FBS, DMEM/F10, at 39C. RT-PCR amplification products of COX-1 mRNA. Aliquots from the same RNA extracts used in 5A were assessed. Cells were treated for 4 hours with the following agents: 10,000 U/ml TNFα and 10,000 U/ml IL-1β(lane2), 1,000U/ml IL-6(lane3), 10,000 U/ml TNFα(lane4), 10,000U/ml IL-1β(lane5). Lane6: no cytokine addition. Lane7: DNA markers. Lane1: RNA extracted from a rat uterus at day21 of pregnancy.

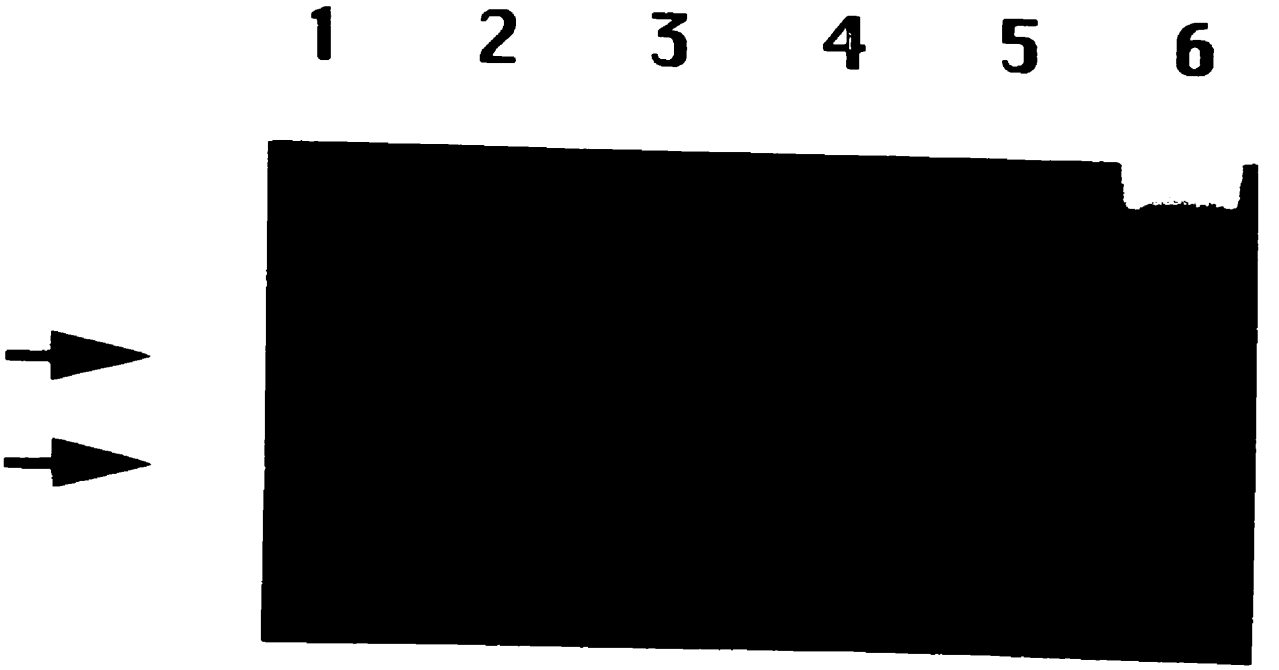


Fig.6A Dose-response of the Cox-2 gene in CUS-V2 cells to IL-1β. CUS-V2 cells, which were cultured in 0.5% FBS, DMEM/F10, at 39C, were treated for 4h with buffer alone(lane1) or different concentrations of IL-1β. Lane2: 100U/ml; lane3: 1,000U/ml; lane4:10,000 U/ml; lane5: 100,000U/ml. Cox-2 transcript amplication(upper arrow); GAPDH transcript amplification(lower arrow).

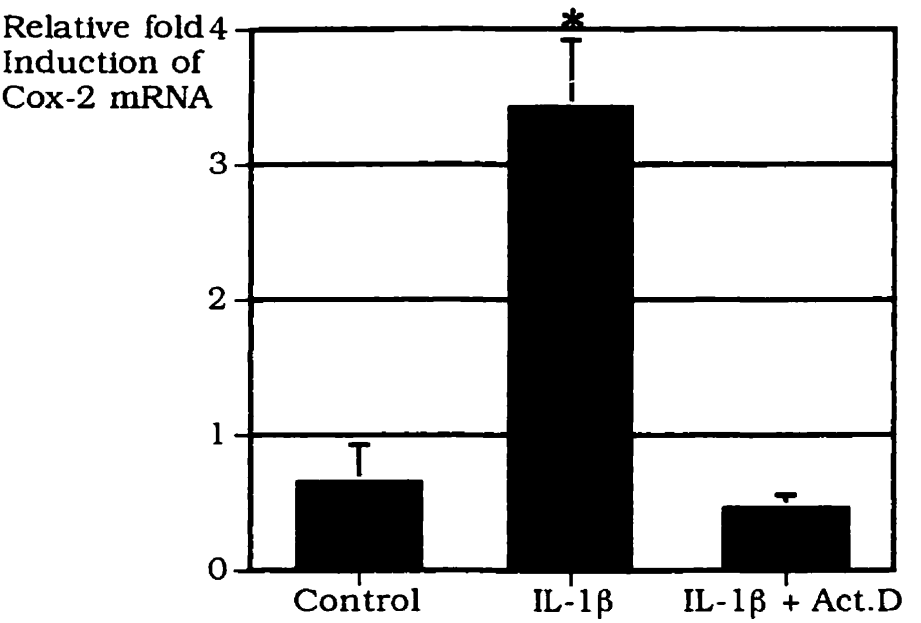


Fig.6B Effect of Actinomycin D on Cox-2 gene expression. Cells were treated with buffer(bar1) or 1,000U/ml IL-1β in the absence(bar2) or presence of 2ug/ml Actinomycin D(bar3). mRNA was quantitated by RT-PCR followed by Southern blot analysis and quantitation of the autoradiographic band levels. Each bar represents the mean +/- S.E. of three independent experiments. The value significantly different from control is denoted by an asterisks($P<0.05$).

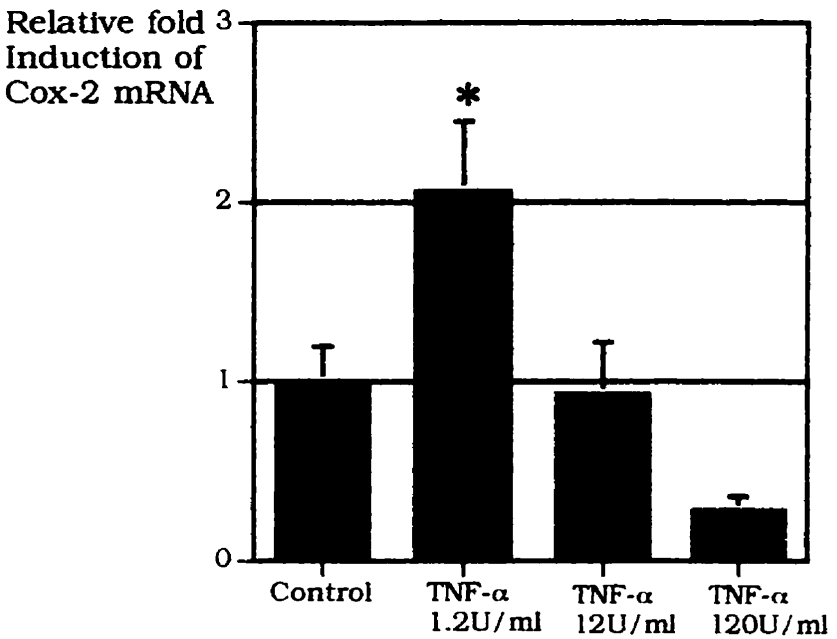


Fig.7 Effects of TNFα on COX-2 gene expression. CUS-V2 cells, which were cultured in low serum, were treated with different concentrations of TNFα or buffer alone. TNFα mRNA levels were determined semi-quantitatively by RT-PCR and normalized as in Fig.6. The value significantly differently from control is denoted by an asterisks($P<0.05$).

further increase in COX-2 gene expression. The cells seem to have a lower tolerance for TNFα without serum. With serum, one can induce the gene at much higher doses of TNFα. Perhaps, α-2-macroglobulin(42), which binds TNFα, and/or the plentiful serine proteases may contribute to the increased tolerance at these higher concentrations.

Prostaglandin $F_{2\alpha}$ release

To determine whether the induction of COX-2 gene expression was associated with increased prostaglandin production and release in CUS-V2 cells, the levels of $PGF_{2\alpha}$ was determined in the cell culture media following induction by cytokines. As shown in Fig.8, both IL-1β as well as TNF-α treatment induced a significant increase in $PGF_{2\alpha}$ levels present in the culture media following a 24 hour incubation period.

Immunocytochemistry

To determine whether the COX-2 protein was expressed and detectable in the CUS-V2 cell line used, the COX-2 protein was localized by immunocytochemistry in CUS-V2 cells, which were cultured in 20%FBS, using a polyclonal anti-Cox-2 antibody. As shown in Fig.9, COX-2-like immunoreactivity was clearly detectable in the cytoplasm of CUS-V2 cells, specifically in the perinuclear regions.

Cox-1 and Cox-2 Gene Expression in CUE-P cells

As demonstrated in Fig.10, COX-1 as well as COX-2 transcripts were readily detected in unstimulated CUE-P cells. In contrast to the stroma-derived CUS-V2 cells, both mRNA were found in relatively high amounts without prior stimulation of their respective genes.

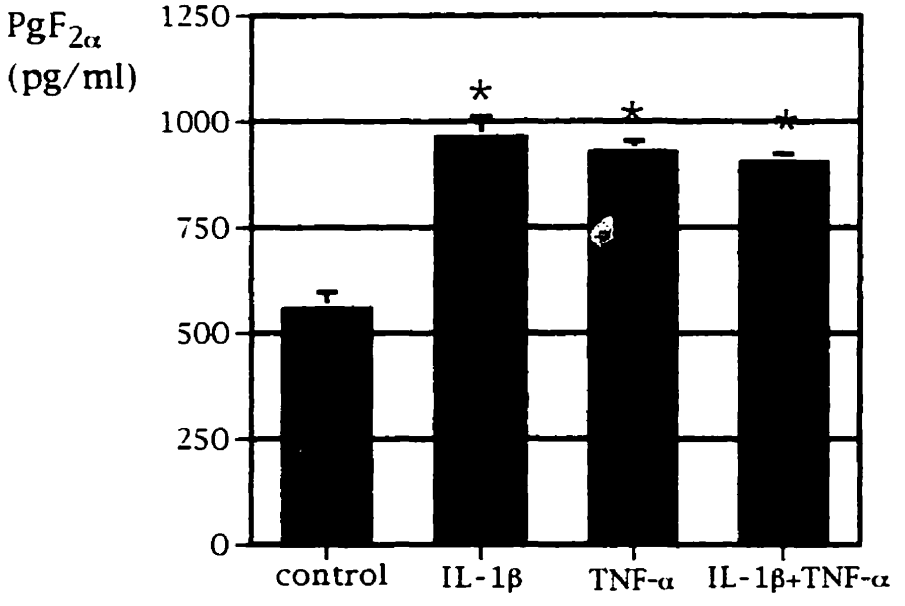
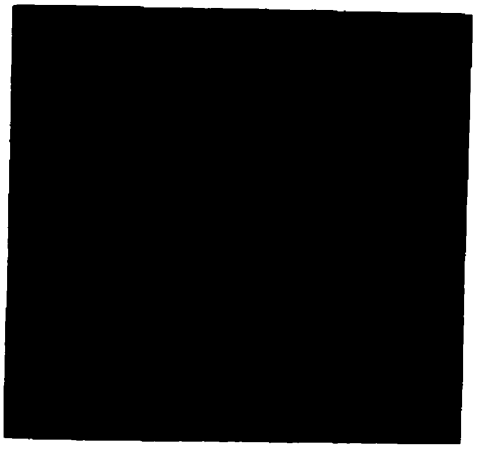


Fig.8 Prostaglandin release from endometrial stromal CUS-V2 cells in response to cytokine treatment. Cells were treated for 24 hours with the following cytokines:1,000 U/ml IL-1β, 1,000 U/ml TNFα or both. Values are expressed as means +/- S.E.(n=3). Values that are significantly different from control($P<0.01$) are indicated by an asterisks.

A)



B)

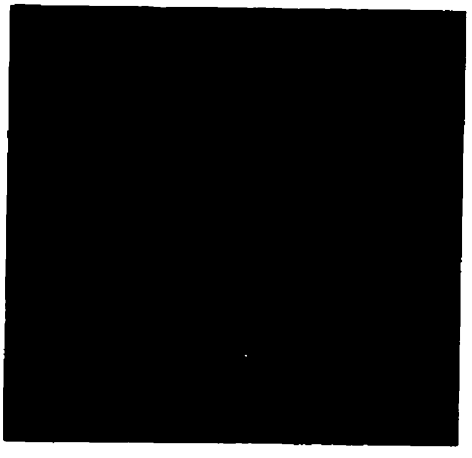


Fig.9 Immunocytochemical detection of the COX-2 protein in CUS-V2 cells. A: immunocytochemical staining with a polyclonal rabbit anti-COX-2 antibody(primary antibody) and a goat FITC-conjugated anti-rabbit antibody(secondary antibody). B: as in A, but the first antibody was omitted from the staining reaction. Enlargement: 400x.

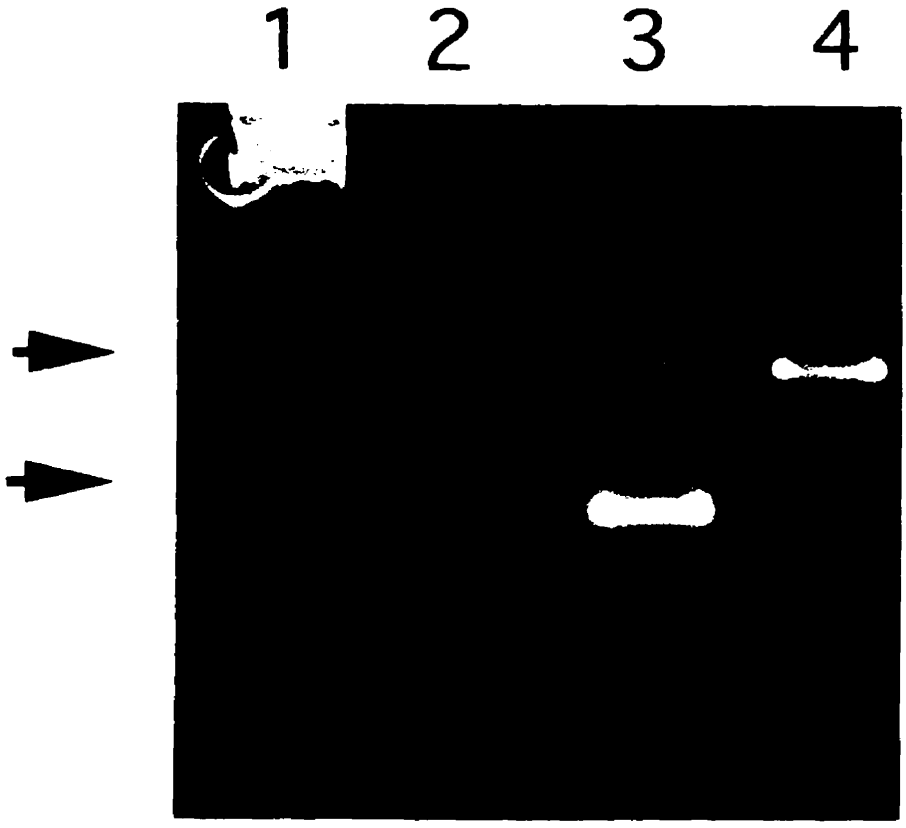


Fig.10 RT-PCR analysis of COX-2 gene expression in the uterine epithelial cell line CUE-P. Two ug of total RNA extracted from CUE-P cells was analyzed by RT-PCR as in Fig.5. Lane1: DNA markers. Lane2: no RNA added. Lane3: amplification of COX-1 mRNA. Lane4: amplification of COX-2 mRNA.

DISCUSSION

The present study was undertaken to address which of the cyclooxygenase isoforms regulates the increase in uterine prostaglandin levels seen near parturition. Both in vivo and in vitro techniques were utilized.

The results from the present study support the hypothesis that a specific increase in uterine COX-2 gene expression towards term may represent an important factor mediating increased uterine $PGF_{2\alpha}$ production and, in turn, luteolysis and parturition:1) In vivo, uterine COX-2 transcript levels increase in the second half of pregnancy, whereas COX-1 transcript levels remain unchanged; 2) COX-2 gene expression is increased by IL-1β and TNFα in an established endometrial stromal cell line in vitro; 3) The cytokine-induced increase in COX-2 gene expression is associated with an increase in in vitro $PGF_{2\alpha}$ release.

To quantitate the increase seen in vivo of the uterine COX-2 transcript we used the semi-quantitative approach of RT-PCR Southern blotting. Prior to being blotted, the PCR products were analyzed by ethidium bromide staining. Whereas the COX-2 transcript levels were seen to rise substantially from the cycling stages to parturition, the COX-1 and GAPDH transcripts only showed a modest increase. Given both these transcripts are in most cells constitutively expressed, a small rise may be reflective of the hypertrophic uterus near term. Therefore, when quantitating the COX-2 transcript levels the in vivo data were normalized to day7 to show that there is a rise in uterine COX-2 expression approaching labour. In contrast in the CUS-V2 cells, since the GAPDH transcript levels remained constant, normalizing the COX-2 values to GAPDH was possible and logical.

Two approaches were used to critically assure us that the amplified product was indeed that of the COX-2 gene. Firstly, an internal complementary primer when used in Southern blots hybridized to the PCR product. And, secondly, the amplified product was cloned and sequenced. Analysis and comparison of the resultant sequence to previously published rat COX-2 sequences showed no differences. Therefore, we are quite confident of the specificity of our PCR technique.

The results from several other studies are compatible with the present findings and are in support of the above hypothesis. Teixeira et al.(43) demonstrated an increase in COX activity in human amnion during labor. In ovine cotyledonary tissues, Wimsatt et al.(44) demonstrated an induction of COX-2 protein near term. However, no COX-2 protein expression was found in the amnion and the allantochorion. The authors, therefore, suggested that the increase in COX activity is due to an increase in placental COX-2 gene expression. The chorion, placenta and decidua express several enzymes capable of metabolizing prostaglandins, and the activity of these enzymes increases towards term(45-48). These enzymes include the nicotinamide-adenine dinucleotide(NAD^+)-dependent 15-hydroxy prostaglandin dehydrogenase and NADH-dependent 13-reductase. These enzymes are likely involved in the degradation of prostaglandins produced by the amnion. Due to the proximity to the uterine vasculature and the myometrium, as well as due to the high content of bone marrow-derived cells, the uterine decidua is likely to represent a physiologically relevant source of prostaglandins, specifically with respect to luteolysis and the triggering of parturition(2,4,32 and references therein, 49).

Specific increases in COX-2 protein levels in stromal cells during the pre-implantation period has been demonstrated by two independent groups(50,51). Jacobs et al.(50) observed increased prostaglandin production and COX-2 protein levels in pure cultures of mice uterine stromal cells upon addition of IL-1α. Similarly, Bany et al.(51) observed an increase in COX activity and $PGF_{2\alpha}$ and PGE_2 release in cultured rat endometrial stromal cells following treatment with IL-1α. In both cases, the observed effects were greatly reduced or abolished following addition of actinomycinD or cycloheximide, indicating that both transcriptional and translational events are involved in the mediation of the observed cytokine effects. Our present findings indicate that a specific increase in COX-2 mRNA accumulation underlies, at least in part, the previously observed induction of COX activity.

In the present study, we have specifically studied the effect of the cytokines IL-1β and TNFα. Both cytokines induced $PGF_{2\alpha}$ release and COX-2

mRNA expression in uterine stromal cells in vitro. No additive effects were observed when both cytokines were added together. This may be due to a post-receptor convergence on the same secondary signal pathways. Indeed, Osbourne et al. have shown that the TNFα and IL-1 induced stimulation of the human immunodeficiency virus enhancer occurs by activation of NF-κB(52). It is possible that a similar or the same signalling pathway is involved in activating COX-2 gene expression in the present system.

In contrast to the highly regulated expression of the COX-2 gene in stromal cells, we observed high levels of COX-2 gene expression in unstimulated uterine epithelial cells. This could be due to an autocrine induction of COX-2 gene expression. As Jacobs et al. have shown, mouse endometrial epithelial cells constitutively express the cytokine IL-1α which, in turn, is capable of stimulating COX-2 gene expression(53). Moreover, a stimulatory effect of specific growth factors(e.g. epidermal growth factor) present in the NuSerum used for culturing the cells cannot be excluded.

The precise identity of the cells which mediate the increase in uterine COX-2 gene expression in vivo before term remains to be determined. Several previous studies underscored the importance of decidual cells in uterine prostaglandin production(1,2,5,54). Moreover, the present studies demonstrated a highly regulated expression of the COX-2 gene in decidual cells(33 and references therein). Therefore, they are the most likely candidates for the mediation of the observed increase in COX-2 gene expression.

Uterine prostaglandin production is induced by a variety of factors in addition to cytokines. These include oxytocin(54), growth factors(51), as well as cortisol(55) and placental CRH(56). It is possible that these different signalling pathways converge on the COX-2 gene which may thus serve as an integrator of immune, endocrine and fetal signals.

In humans, pregnancy can be prolonged by inhibition of prostaglandin synthesis(8). However, the pharmacological usefulness of this approach has been hampered so far by side effects on fetal development, including

premature closure of the ductus arteriosus(5). If indeed the activation of COX-2 enzyme expression and activity is an essential step in the triggering of parturition, specific inhibitors of COX-2, such as diarylcyclopentenes (57,58) should represent promising tools for the treatment of premature labor. The recently obtained COX-1 deficient mice(59) add further indirect support for the involvement of COX-2 in parturition, since in the absence of maternal and/or fetal COX-1, parturition is not compromised.

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CHAPTER 3

Cox-2 Induction by Oxytocin

PREFACE

Oxytocin and prostaglandins are uterotonic agents that have been used to induce labour in the third trimester. This chapter examines the link between the two at a molecular level and helps define new areas of potential therapeutic value for premature labour.

SUMMARY

Having previously determined that the uterine Cox-2 gene was upregulated during pregnancy in the rat, in this manuscript we study if the oxytocin receptor(OTR) is involved in this induction. Our initial approach was to test if both proteins were co-expressed at mid-gestation and at parturition in the same cells. Immunohistochemical analysis revealed that at days 13-14 of pregnancy, both the epithelium and myometrial cells stained positive for OTR and Cox-2. Approaching parturition, the staining for both decreased in the epithelium and increased in the myometrium. Interestingly, we also found strong Cox-2 expression in the sub-epithelial stroma at parturition but not at days 13-14 of pregnancy.

Since both proteins were co-expressed, next, we used CHO cells, which were stably transfected with a vector expressing the OTR, to ascertain if OT stimulated PgE_2 release and induced the Cox-2 gene. The addition of 1μM OT to the cells resulted in a 107 fold increase in PgE_2 released in a 24 hour period relative to unstimulated cells. This effect was substantially reduced with the co-addition of 3μM OT antagonist. Western blots probed with specific polyclonal antibodies against Cox-2 revealed no expression in control cells and induction in OT stimulated cells. Similar to previously published results, we observed two bands of approximately 74 and 66 kDa.

Since prostaglandins are uterotonic, an increase by OT is suggestive of an autocrine mediated positive feedback loop.

Introduction

Oxytocin, a cyclic, C-terminally amidated nonapeptide, belongs to a small group of hormones involved in mediating parturition. The classic site of OT gene expression has been in the hypothalamic paraventricular and supraoptic nuclei where its transcript is translated and cleaved from neurophysin I, stored in vesicles and eventually released at the posterior pituitary. Near term and as well after labour, oxytocin is not only involved in initiating uterine contractions but during lactation, it releases milk from the mammary gland. Both myometrial and myoepithelial cells respond and contract when stimulated by it(reviewed in ref.1). Aside from the hypothalamus, recently, our laboratory has shown high levels of OT gene expression within the rat uterus(2). Both peptide and transcripts were shown to be expressed in epithelial cells. The OT mRNA levels dramatically rises approaching term. At day21 of pregnancy, the level of OT mRNA expressed in the uterus equals approximately 140 times the level of OT mRNA that is found in the hypothalamus.

As stated above, oxytocin induces uterine myometrial contractions by binding to its receptor. The receptor is expressed mainly in the myometrium as judged from ligand binding studies. Cloning and expression have shown it to be a seven transmembrane domain Gprotein-coupled receptor which is regulated by estrogen and progesterone(1, Appendix C). Upon activation, it increases cytoplasmic Ca^{2+} levels(3). The rising Ca^{2+} levels induce myosin light chain kinase activation and myosin phosphorylation which permits binding to actin and induces the contractile response(4).

In addition to oxytocin, prostaglandins E_2 and $F_{2\alpha}$ have also been shown to induce myometrial contractions. Their levels, like oxytocin, rise approaching term(5,6). And again, similarly, the prostaglandin receptors are also coupled to Ca^{2+} in myometrial cells(7,8). Although the above

prostaglandins are mainly produced in the endometrium, a considerable amount is synthesized in the myometrium(10).

For cells to produce prostaglandins, they need the substrate arachidonic acid. This unsaturated fatty acid is found within lipid bilayers where it is hydrolyzed by phospholipase A_2 and then converted to PgH_2 by cyclooxygenase(COX)1 or 2(11,12). In our last manuscript, we showed the Cox-2 expression levels increase in the pregnant uterus approaching delivery(13).

Previous manuscripts have suggested that myometrial contractions involve an interaction of the prostaglandin and oxytocin effects(5,6). The present manuscript shows that this interaction does occur and as well, delineates some of the key steps involved.

Materials and Methods

Animal Care and Tissue Preparation

Time-pregnant Sprague Dawley rats were purchased from Charles River Laboratories(St. Constant, Quebec, Canada). They were fed rat chow ad libitum and kept in a temperature and humidity-controlled environment. Animals were sacrificed by decapitation under light ether anesthesia. The procedure were approved by the Royal Victoria Hospital Animal Ethics Committee. Animals were sacrificed at day13-14 of pregnancy, and at parturition. Dissected uteri stripped of fetal tissue were rapidly frozen in liquid nitrogen cooled isopentane and stored at -70C.

Cell Culture

Chinese Hamster Ovary(CHO) cells expressing the recombinant rat oxytocin receptor(CHO-OTR) were kindly provided by Dr. Steven Lolait, Laboratory of Cell Biology, NIH. They were grown in 10% Fetal Bovine Serum(Gibco/BRL, Burlington, Ont.) in Dulbecco's Modified Medium (Gibco/BRL). The media was changed just prior to addition of oxytocin

and/or its antagonist([d(CH_2)$_5$,Tyr(OMe)^2,Thr^4,Tyr^9-NH_2]OVT,Peninsula Laboratories, Belmont, CA).

Immunohistochemistry

The tissues were brought to -20C in a cryostat whereupon they were cut into 6μm sections and mounted onto Superfrost slides(Promega, Madison,WI). The slides were stored at -70C prior to their use.

The primary antibodies used were a polyclonal rabbit anti-Cox-2 antibody, kindly provided by Merck Frost Canada Inc. and a polyclonal anti-OTR antibody obtained from Dr. J. Verbalis, Georgetown University,Washington. The former antibody was used at a 1:5 dilution and the latter was used at a 1:500 dilution. Mounted tissue slides were washed with phosphate buffered saline(PBS) for 5minutes, and then incubated with primary antibody for 1h at room temperature. The sections were then rewashed three times with PBS and the secondary antibody was added for 1h at 1:20 dilution at room temperature. Following washing with PBS(3X) and two rapid dips into distilled water, the cell preparations were mounted with Immumount(Shandon, Pittsburgh,PA) and viewed under a Nikon fluorescence microscope(Nikon Instruments Group, Melville, NY). The solution used for the dilutions were 10% FBS, and 0.1% Triton in PBS, sterilized by filtration.

Elisa Immunoassay

OT and/or OT antagonist was added to CHO cells at different concentrations and after a 24h incubation period, the media was collected, centrifuged to remove the cellular debris and stored at -70C.

The PgE_2 assay was performed as stated by the supplier(Cayman Chemical Company, Ann Arbor, MI). The intra- and interassay variance of the assay is <10%.

Western Blot

The western blot was performed as given in Current Protocols in Molecular Biology(14). Briefly, after the CHO cells were stimulated with OT overnight, the cells were twice washed with PBS and then lysed into hot(85C) 1XSDS gel loading buffer(50mM Tris-Cl(pH6.8), 100mM dithiothreitol, 2%SDS, 0.1%bromophenol blue and 10% gylcerol). The samples were then boiled for 10 minutes, centrifuged at 10,000g, and the resultant supernatant transferred to a new tube and stored at -70C. The amount of protein in each sample was determined using the Bradford Method. 100μg of protein from each tube was loaded unto a 10% Sodium Dodecyl Sulphate-Polyacrylamide Gel. Protein markers were also loaded alongside(Prestained SDS-Page Standards, Gibco/BRL). After electrophoretic separation, the proteins were transferred unto a nitrocellulose membrane and blocked using 10% dried milk powder. The blot was then incubated overnight with primary anti-Cox-2 antibody(1:1000 dilution) which at this dilution has been shown to have no cross-reactivity to Cox-1(Merck Frost, Chan et al,1995) and then with alkaline-phosphatase conjugated anti-rabbit secondary antibody(1:1000 dilution). The substrate for alkaline phosphatase(5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium) were added next and the resultant bands visualized after a brief incubation at room temperature. Protein markers were used to estimate the size of the migrating bands.

Statistics

Results were expressed as means +/- SE(n=3, separate experiments). Differences were examined with one way analysis of variance (ANOVA) and individual comparisons between the treatments was assessed by the Tukey's Test, if there was a significant difference, $P<0.01$.

RESULTS

Uterine OTR Staining

Fig.1 shows immunohistochemical staining of OTR in the rat uterus at day13-14 of pregnancy. One observes positive immunofluorescence in the epithelium(Fig.1A,B) and myometrium(Fig.1C) collectively but none to very small amounts in the endometrial stroma, and in the myometrial interstitium and stroma. A similar pattern of staining is observed during parturition(Fig.2). In the myometrium, the intensity of fluorescence increases and, in contrast, in the epithelium it decreases. Table I summarizes the aforementioned changes.

Uterine COX-1,2 Staining

Fig.3 represents immunofluorescence resulting from polyclonal cyclooxygenase antibody binding to different uterine cells during the course of parturition. Not all cells express COX-2. Positive and specific fluorescence is seen in the sub-epithelial stroma(Fig.3A) and, in the myometrium(Fig.3B). For COX-1, which is constitutively expressed, both endometrium and myometrium stain positively. Within the endometrium, epithelial cells stain to a greater extent than stromal cells(Fig.3C). TableII summarizes the observed changes for COX-2 expression on day13-14 and parturition.

PgE_2 Release

CHO cells expressing OTR were used to observe prostaglandin release in response to oxytocin addition and incubation overnight. Oxytocin stimulation at 0.1μM and 1.0μM concentrations led to 528 ± 132 pg/ml and 1,718 ± 474 pg/ml, respectively, of PgE_2 released into the medium. When stimulated with 1.0μM oxytocin in the presence of 3.0μM oxytocin antagonist, only 128 ± 28 pg/ml of PgE_2 was released. Control plates contained 16 ± 5 pg/ml. Therefore oxytocin led to a 33 fold and 107 fold increase in the amount of PgE_2 produced and released. A 3 fold excess of antagonist substantially reduced this increase indicating direct binding to OTR. These results are depicted graphically in Fig.4.

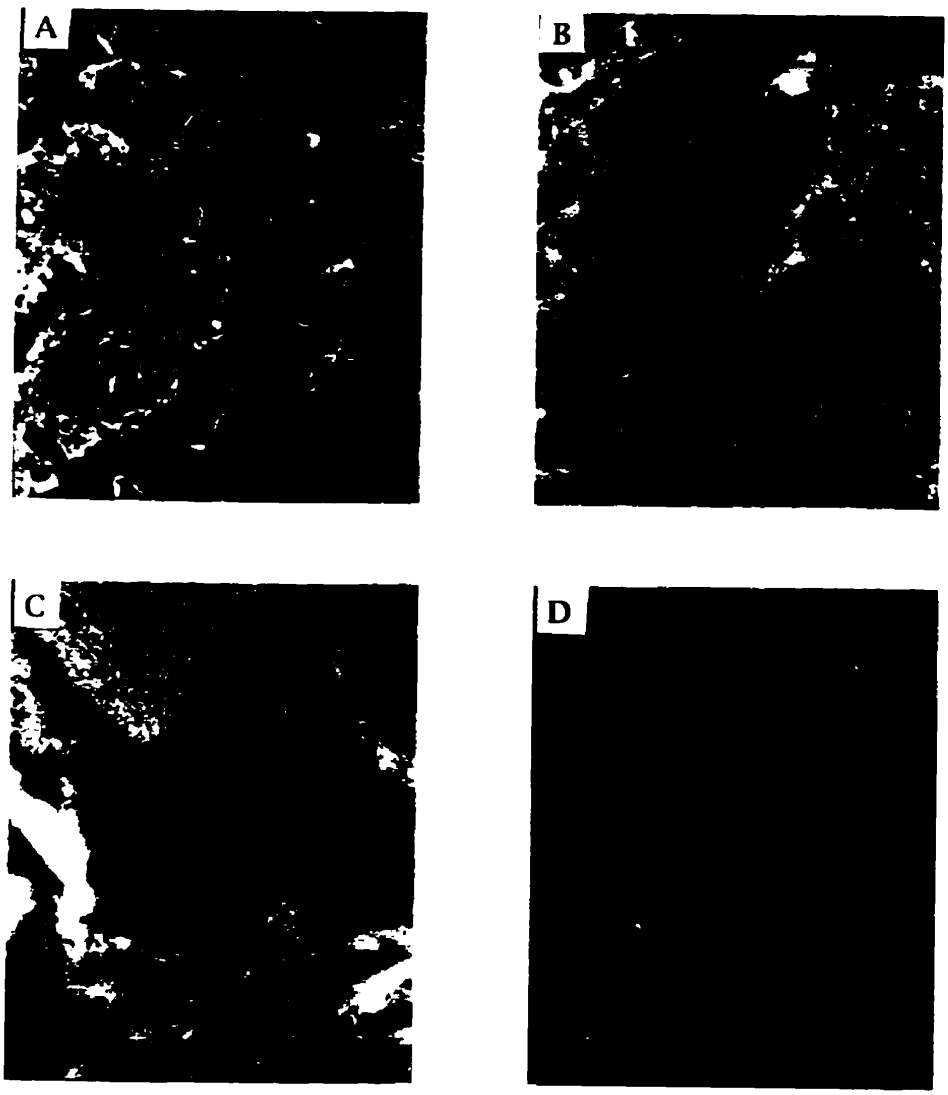

Fig. 1 Uterine OTR expression at day13-14 of pregnancy. Uterine sections were washed with PBS, and immunostained with primary and secondary antibody. The primary antibody was rabbit anti-OTR polyclonal antibody. The secondary antibody was swine anti-rabbit IgG conjugated to FITC. A) endometrium, 200X enlargement; B) endometrium, 400X enlargement; C) myometrium, 200X enlargement; D) staining without primary antibody.

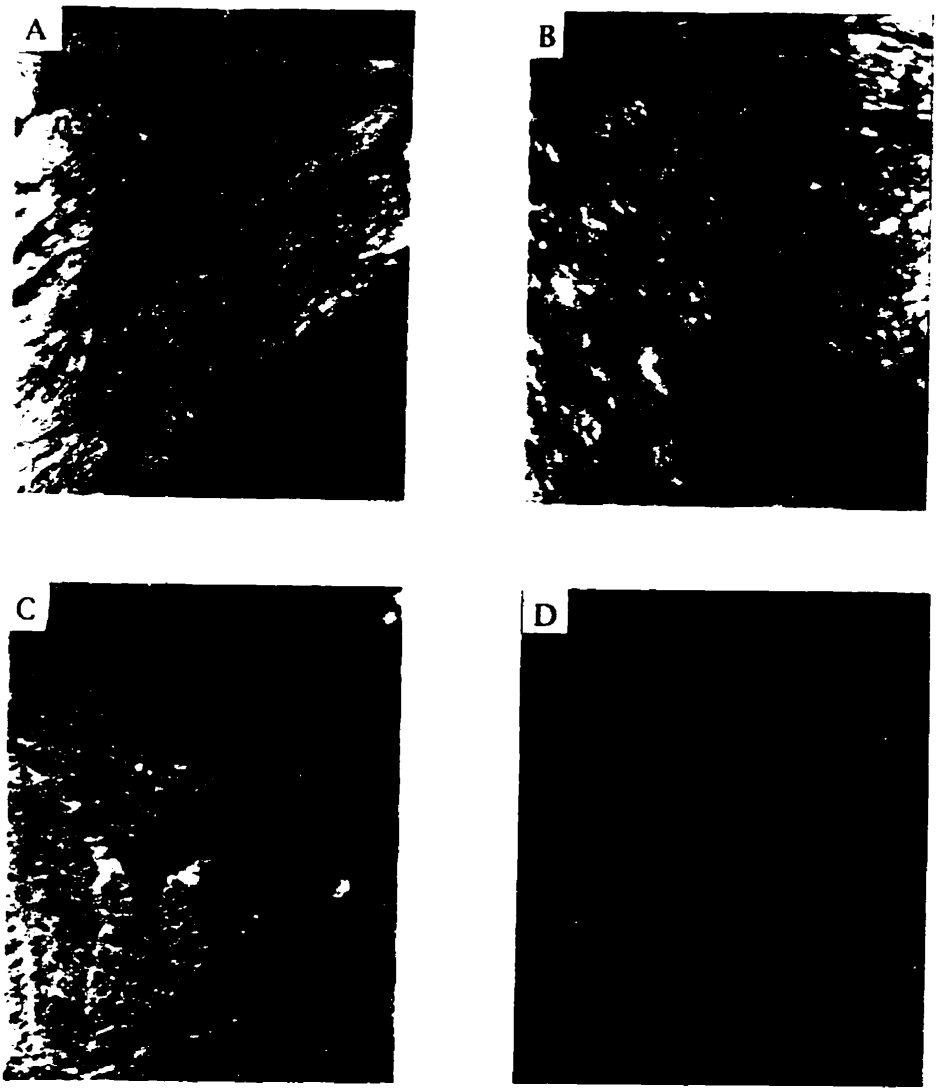

Fig.2 Uterine OTR expression at parturition. Uterine cryosections were handled as in Figure1. A) circular myometrium + endometrium(lower right), 400X enlargement; B) circular myometrium + longitudinal myometrium, 400X enlargement; C) endometrium, enlargement 400X; D) staining without primary antibody.

Table I

Uterine OTR Expression[a]

	day 13-14	parturition
epithelial cells	++	+
stroma cells	–	–
circ. myometrium	++	++++
long. myometrium	++	++++

[a]Pregnant uterine tissue sections were stained using a polyclonal rabbit anti-oxytocin receptor(OTR) antibody and viewed under a fluorescence microscope. Fluorescence intensity was graded visually:"++++":highest levels;"-":no observable immunostaining.

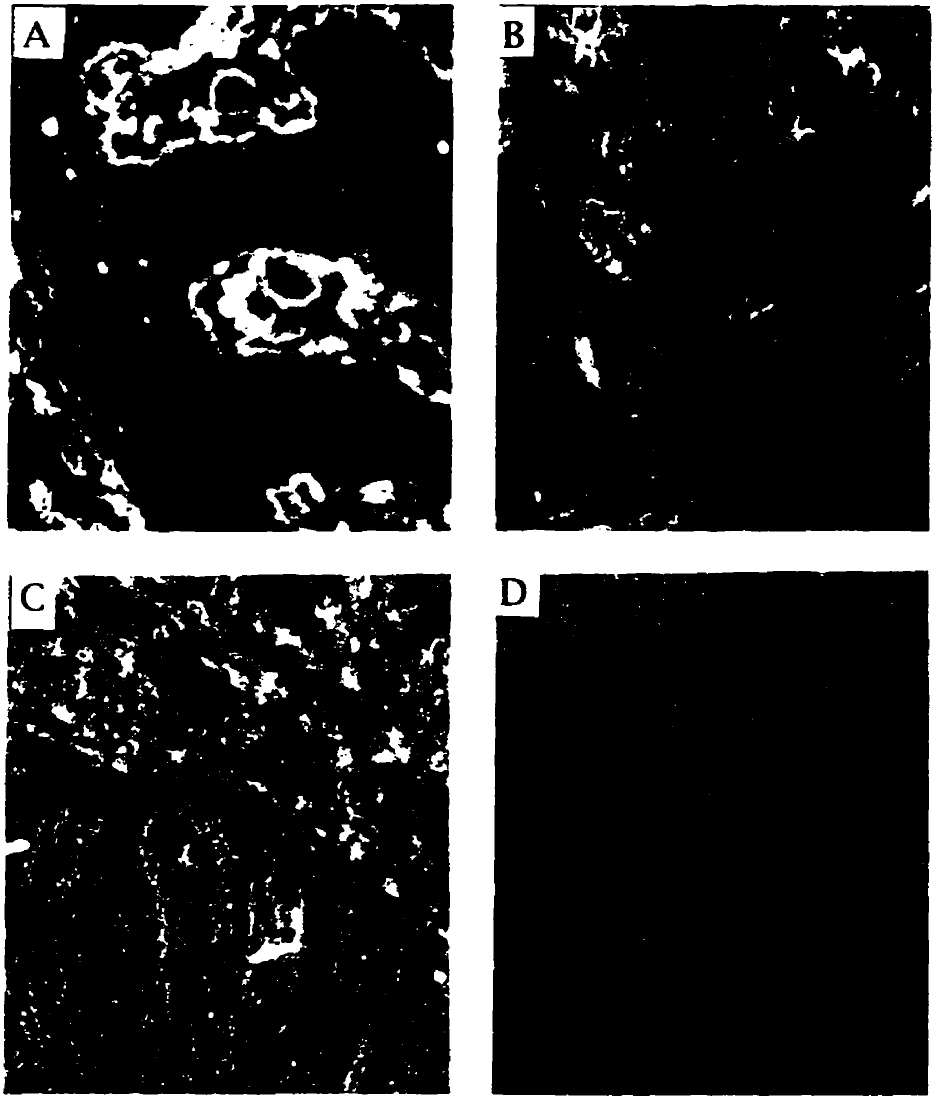

Fig.3 COX-1 and COX-2 expression at parturition. Uterine sections were washed with PBS and then immunostained with first rabbit anti-COX-2 followed with swine anti-rabbit antibodies. A) COX-2 expression in endometrium, 400X enlargement; B) COX-2 expression in circular myometrium + longitudinal myometrium(top lobe), 400X enlargement; C) COX-1 expression in endometrium(lower segment) + circular myometrium(upper segment), 200X enlargement; D) staining without primary antibody.

Table II

Uterine COX-2 Expression [a]

	day13-14	parturition
epithelial cells	++	–
stroma cells	–	++
circ. myometrium	++	+++
long. myometrium	++	+++

[a]Pregnant uterine tissue sections were stained using a polyclonal rabbit anti-cyclooxygenase 2(COX-2) antibody and viewed under a fluorescence microscope. Fluorescence intensity was graded visually:"++++":highest levels;"-":no observable immunostaining.

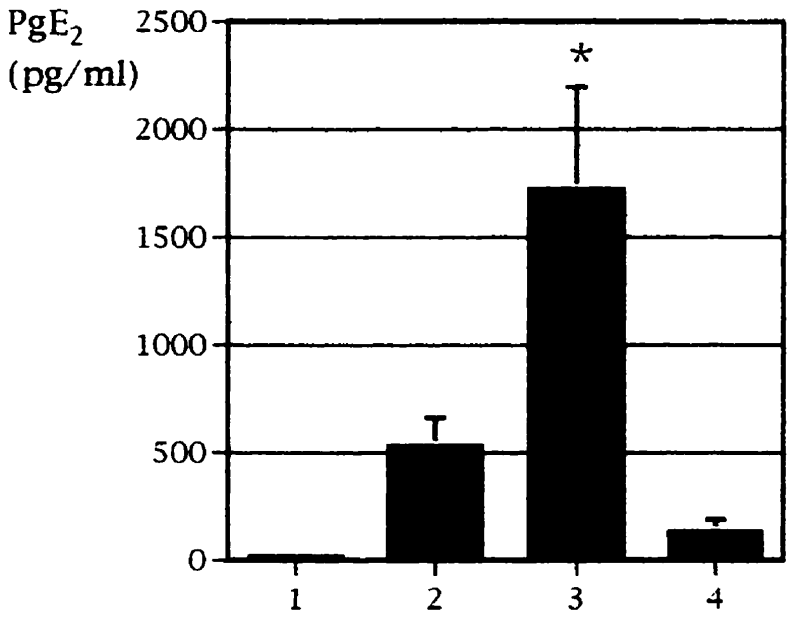

Fig.4 Prostaglandin E2 release from CHO-OTR cells. CHO-OTR cells were stimulated with oxytocin and/or oxytocin antagonist overnight. An Elisa Immunoassay was used for the quantitation. 1) control; 2)0.1uM oxytocin; 3)1.0uM oxytocin; 4)1.0uM oxytocin + 3.0uM oxytocin antagonist. Each bar represents the mean +/- S.E. of three independent experiments. All samples were measured twice. The value significantly different from control is denoted by an asterisks($P<0.01$).

COX-2 Western Blot

Since the OTR and COX-2 genes exhibit a similar expression profile in vivo, we decided to do a Western blot to observe if OTR stimulation altered the expression levels of the Cox-2 protein. Fig.5 shows that at concentrations of 0.1μM and 0.5μM of oxytocin, the COX-2 protein is readily induced. Without stimulation, the COX-2 protein seems to be undetectable. The upper bands coincide with the previously published doublet which have been shown to be the result of differential glycosylation(14,15). We approximate these bands to be 74 and 66kDa respectively.

Interestingly, we also observe bands with the approximate size of 28 and 17kDa as well. These could be either immunologically reactive fragments of Cox-2 or cross-reactive smaller proteins.

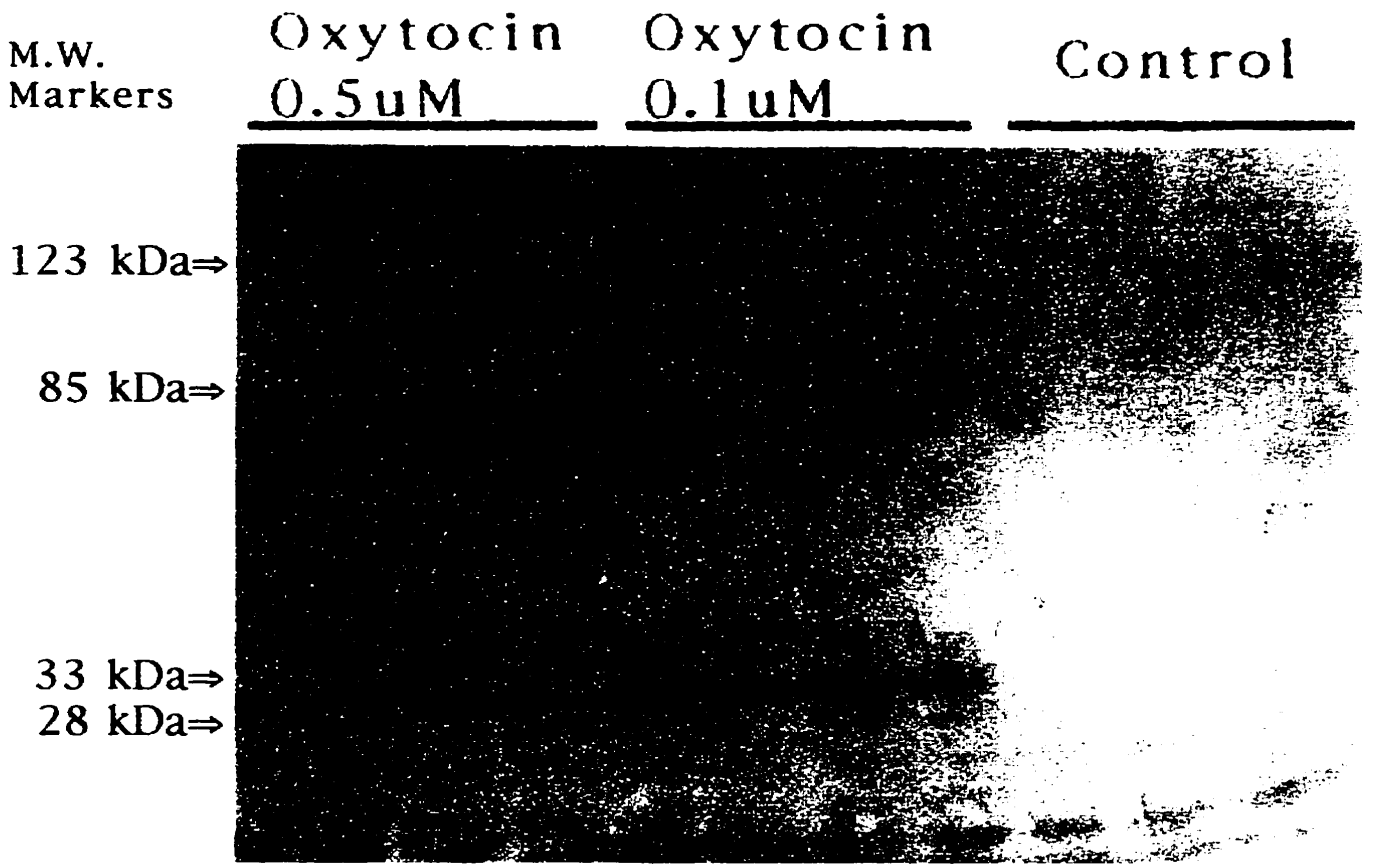

Fig.5 Western blot of COX-2 expression in CHO-OTR cells. CHO-OTR cells were stimulated with oxytocin overnight. The following day, the cells were lysed and the protein extract quantitated. 100ug was electrophoretically separated on a SDS-polyacrylamide gel with molecular weight(MW) markers. After transfer to a nitrocellulose membrane, they were immunoblotted with a polyclonal anti-COX-2 antibody and visualized with a secondary antibody conjugated to alkaline phosphatase. The concentration of OT used is as stated. Each experiment was done in quadruplicates. Four bands corresponding to sizes of 74 kDa, 69 kDa, 28kDa and 17 kDa were visualized.

DISCUSSION

The culminating stage in pregnancy is labour. It is the connection between prenatal and postnatal development. Many variables control its success but maternal cervical dilatation, softening of fetal membranes and more importantly the initiation of uterine contractions seem to be key factors(11,17). Given the diminished peripheral neuronal input in the uterus near term(18), endocrine hormones have been shown to be prominent in its regulation.

A classic example of such a hormone is fetal cortisol which has been shown to induce parturition in ruminants(19). Other important endocrine factors include prostaglandins and oxytocin. In both primates and our preferred animal model for parturition, the rat, they bring about contractions at term when administered(1,17). An understanding of their effects and molecular biology will uncover new avenues for the prevention of premature labor and dysmenorrhea. In the present study, we present data showing an interaction between both hormones that seem to be paracrine and autocrine in effect as assessed from this and other studies.

Chan et al. have shown that inhibition of uterine prostaglandin products in day 23 pregnant rats, leads to a decrease in the level of oxytocin receptor binding by approximately 70% without a change in affinity, therefore, prostaglandins seem to regulate oxytocin receptor expression(5). When Alexandrova et al. injected $PgF_{2\alpha}$ into day18 pregnant rats, they observed an approximately five fold increase in OT binding(20). Furthermore, in preterm women, Fuchs et al. have shown oxytocin injections induce an increase in plasma PgE_2 and 15-keto-13,14-dihydro-$PgF_{2\alpha}$ levels, a stable $PgF_{2\alpha}$ metabolite(21). An oxytocin antagonist was not used, therefore, although the study showed release of prostaglandins, demonstration of a direct relationship of the oxytocin addition and effects to the oxytocin receptor required further study. Additional studies include

the work done in ruminants. In non-pregnant ruminants, oxytocin released both from the posterior pituitary and granulosa cells of the corpus luteum induces $PgF_{2\alpha}$ release from endometrial epithelial cells(22,23). The

increased serum $PgF_{2\alpha}$, in turn, induces corpus luteum regression, luteolysis, and a drop in progesterone synthesis and eventually its serum levels. Therefore, collectively, the published data supports oxytocin inducing prostaglandin synthesis and release which in turn modulates oxytocin receptor expression and function.

Our present manuscript directly shows not only that this interaction occurs but more importantly which cyclooxgenase is involved. Prostaglandin synthesis has two rate limiting steps in its regulation. The first is the liberation of arachidonic acid from phospholipids by PLA_2 and the second is its conversion to PgH_2, the substrate for all prostaglandins and thromboxanes, by the constitutively expressed COX 1 and/or the immediately induced COX2(12,24). In the pregnant uterus, PLA_2 activity levels remain unchanged while cyclooxygenase activity increases(25). More importantly, in the rat, we have shown COX-2 expression increases more than COX-1. Therefore, a logical connection between oxytocin and COX-2 expression seemed understandable.

The initial experiments were done to determine if the same uterine cells express both OTR and COX-2 and whether the expression in these cells changed during the course of gestation. Immunohistochemical analysis of the pregnant uterus cryosections revealed marked changes. Within the myometrium, mostly myometrial cells expressed OTR and COX-2 while in the endometrium only epithelial cells expressed them. Cells that express one and not the other include the endometrial sub-epithelial stroma whose COX-2 expression rises dramatically at term. These cells don't express OTR as judged from our analysis, both from binding and protein analysis. The next logical step was to assess if the OTR is coupled to prostaglandin release, more specifically PgE_2. The addition of OT to CHO-OTR cells led to a 107 fold increase in the amount produced and released. When the OT antagonist was added we were able to substantially reduce this rise which indicates specificity to OT binding and its effects. The third and final step was to show that the OTR stimulates COX-2. The results from the western blot showed this indeed does occur. In conclusion, at least in the pregnant rat uterus, OT binding to OTR leads to COX-2 expression and this leads to increased prostaglandin synthesis and release. The released prostaglandins, in turn, would have a paracrine (endometrium to

myometrium) and autocrine (myometrium) effect leading to OTR expression. In addition, since $PgF_{2\alpha}$ is luteolytic(26), the decreased serum progesterone leads to increased gap junction formation(27,28) and therefore, increased pressure from the coordinated contractions.

Aside from their effects on the OTR, both prostaglandins mediate an OT-independent increase in uterine contractions, both frequency and basal tone, near term by binding to their respective receptors. From ligand binding studies, it has been shown that $PgF_{2\alpha}$ receptors are found mainly in the myometrial fractions and PgE_2 receptors are found in both compartments(29,30). Cloning and expression of the prostaglandin receptors has shown that the PgE_2 receptors EP1 and EP3D, and the $PgF_{2\alpha}$ receptor are all Gprotein-coupled seven transmembrane domain receptors which, when activated, increase cytosolic Ca^{2+} levels(7,8,9,31). Not all the receptors are coupled to Ca^{2+}. The PgE_2 receptor EP2 activates adenylate cyclase(32) and a rise in cAMP in myocytes which sustains the cells in a non-contractile state. Given the multitude of receptors for these prostaglandins and different secondary signals, additional studies on receptor localization and endocrine regulation during pregnancy would better define their function.

Both OTR and COX-2 are expressed in the endometrial epithelium at day13-14 of pregnancy. In these cells, we have also shown oxytocin expression(2). Exactly what the physiological effects of the produced prostaglandins might be in the mucosa remains undetermined. Endometrial vasodilation, and/or immune cell activation/suppression might be possible avenues(33).

The OTR has been shown to be coupled to IP_3 turnover and protein kinase C activation(PKC)(23). Previously, other studies have shown phorbol esters stimulate COX-2 expression(34), therefore, most probably PKC induces the COX-2 gene in the myometrium. Since cytokines are also coupled to PKC(35,36), these results would be consistant for the stroma as well.

In summary, the aforementioned data and collected studies support a better understanding of how oxytocin induces an increase in prostaglandin production in the pregnant rat uterus.

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PART III

DISCUSSION

From fertilization onwards, the zygote must bypass many obstacles just to implant and eventually form a maternal-fetal interface. The genetic factors responsible for the required steps are currently being discovered and investigated. Not all embryos succeed. It's estimated up to 33% fail the process. Of the embryos that do succeed past placentation, 3-16% are prematurely born, which varies depending on the region, and they account for approximately 80-85% of the newborns mortality and morbidity(Rush et al, 1976; Wilcox et al, 1988; Klebanoff et al, 1990). From a practical stand point, tocolytic therapy has many benefits. Prolongation of pregnancy would increase the probability of the fetus surviving not only birth but the required time for the major tissues such as the endocrine, immune, and respiratory system to mature. The question has become how and what factors should be targeted and inhibited to prevent contractions. This thesis adds to this knowledge and provides some new insight.

The contractile system is quite complex. The system has stimulants, inhibitors, and furthermore, integrates these factors to give a graded response. In respect to the wide array of stimulators, they all in some fashion or another raise cytosolic Ca^{2+} and MLCK activity(Huszar, 1986; Garfield et al, 1994). In the presence of inhibitors, their effects are dampened(Krall et al, 1984; Anwar et al, 1989) and depending on the hormonal milieu, their effects might be additive or synergistic(Hillhouse et al, 1993). In our laboratory, we have for the past decade focused our efforts on oxytocin, the strongest uterotonic agent known, and its receptor. In addition to oxytocin, personally, I added a new dimension of prostaglandin research. The data clearly indicates these two systems are not mutually exclusive but rather intertwined.

Exactly how oxytocin, cytokines and prostaglandins cooperated to induce labour remained poorly resolved at a molecular level. Our first aim was to establish endometrial cell lines which could be used to understand these relationships. From persistant efforts, we established four stromal and two epithelial cell lines. Careful analysis of differentiation markers and replication times allowed us to demarcate between epithelial and stromal cells.

In contrast to simple reasoning, the endometrium is much more complex than first perceived. Both stromal and epithelial cells require many factors to differentiate. Aside for the need of the basement membrane for epithelial cells, and extracellular matrix for stromal cells, an interactive autocrine and paracrine crosstalk between the cells, also, seems to be required. In light of this information, we found it imperative to recreate these conditions in vitro as closely as possible. The initial attempts to create an in vitro bi-layer as a simple model for the endometrium wasn't difficult but the experimental protocol was quite complex given co-culture of one cell atop another requires both cells to be immortalized and not transformed, and furthermore, the media conditions had to accommodate both cell lines. From a practical stand point, the cell lines provided us with a very good tool to study prostaglandin regulation.

Prior experiments in other laboratories had predetermined that cytokines and various hormones, oxytocin and steroids, were able to increase endometrial prostaglandin synthesis(Simon et al, 1993; Novy et al, 1977; Fortunato et al, 1994; Bry et al, 1993; Kauma et al, 1990; Mitchell et al, 1991; Matsuzaki et al, 1993; Gu et al, 1990; Wilson et al, 1982; Wilson, 1983; Wilson et al, 1987), and therefore, we decided to understand exactly how and what enzymes were involved and induced. As previously highlighted, infection of fetal membranes and the consequent immune response usually elicits increased prostaglandin production from the uterine decidua(Romero et al, 1994; Lamont et al, 1990) and 17-β estradiol increases their levels as well (Wilson et al, 1982; Wilson et al, 1983; Wilson et al, 1987); therefore, from our understanding, irrespective of the inducer whether endocrine or immune in origin, both systems are involved. Both act upstream of prostaglandin production.

One of the effects from the convergence of the immune and endocrine system and the rising uterine prostaglandin levels is initiation of labor. They are widely accepted as such for the following reasons: 1) administration to pregnant women at mid-trimester will lead to myometrial contractions, 2) administration of NSAID's in vivo and in vitro will inhibit myometrial contractions, 3) spontaneous parturition at term is associated with increased plasma concentrations, 4) intra-amniotic injection of arachidonic acid leads to the onset of labour, and 5) administered

prostaglandins lead to cervical dilatation and ripening(Gustavii et al, 1977; Turnbull et al, 1977; Flower, 1977; Fitzpatrick, 1977; Romero et al, 1991; Reddy et al, 1994).

The interesting phenomenon we and others have observed is that cytokines and factors such as oxytocin, lipopolysaccharide, and phorbol esters increase prostaglandin levels in Swiss 3T3, mast, macrophage and CUS-V4 cells by increasing the Cox-2 gene without a concomitant increase in Cox-1(Reddy et al, 1994; Murakami et al, 1994; Arslan et al, 1996). The result being selective inhibition of the Cox-2 gene has therapeutic value. This would be ideal since prostacyclin, whose concentrations are relatively high compared to the other prostaglandins found in the myometrium, inhibits and reduces contractions, therefore, complete inhibition of all the cyclooxygenase products would be counter productive(Satoh et al, 1981; Peplow et al, 1989).

The cloning, sequencing and de novo expression of the Cox-2 and OTR gene allowed us the means to make the link between the two in vivo and in vitro(Kujubu et al, 1991; Kimura et al, 1992). When we probed cryosections of pregnant uteruses, we observed both genes were expressed in the same cells at the same time, furthermore, they were both expressed at parturition. In vitro, when OT was added to CHO cells expressing OTR, we saw greater than 100 fold increase in the amount of PgE_2 released whose effect could be abrogated by a specific OT antagonist and, as well, we saw a dose dependent increase in Cox-2 expression. These results are in agreement with those published by Fuchs et al., Soloff et al., and Chan et al. who also measured prostaglandin release in vivo and from organ cultures after OT stimulation(Alexandrova et al, 1980; Husslein et al, 1981; Chan et al, 1992).

Since prostaglandins are also uterotonic, this would suggest an amplification of the contractile response. This may occur by two means. First, $PgF_{2\alpha}$ increases oxytocin receptor affinity for OT and itself couples to IP_3 turnover and, second PgE_2 has been shown to increase gap junction expression(Husslein et al, 1981; Chan et al, 1992; Garfield, 1980). By increasing OT binding, more prostaglandins are produced, this was evident from our results when increased amounts of OT addition led to larger

amounts of PgE_2 synthesized and released, which in turn amounts to increased cytosolic Ca^{2+}. Since prostaglandins depolarize the plasma membrane, this would decrease the threshold level for burst discharges elicited by OT and furthermore increase pacemaker cell activity. Therefore, both internal and external sources of calcium would be mobilized more effectively.

Fetal embryos which lack estrogen and progesterone receptors are born normally. Therefore, placental estrogen and progesterone receptors aren't required for parturition. Placental expression of luteotrophic factors therefore must be to some extent independent of these hormones. Furthermore, this would apply to other fetal membranes such as the amnion, chorion and yolk sac. In addition, as stated previously, MHCI, and MHCII knock out mice and lymphocyte depleted mice are born normally. Therefore, the initiation of parturition must not involve or can substitute for these factors. The decidua not only expresses cytokines but contains many bone marrow derived immune cells in addition to lymphocytes such as Dendritic and Langerhan cells. In conclusion, the data would indicate a decidual signal that could be open to and most likely is regulated by the placenta. We believe since the cytotrophoblast keeps dividing, this would increase the amount of luteotrophic factors released. These factors in turn would increase estrogen synthesis by promoting an increase in granulosa and thecal cell formation of cAMP. As parturition approaches, progesterone metabolism would increase or factors are released or synthesized by the placenta which would modify the progesterone receptor leading to its inhibition. For example dopamine in certain cells activates the progesterone receptor independent of progesterone(Power et al, 1991). We believe there are certain placental factors which would function in a similar manner starting as soon as the formation of the placenta(Ogle et al, 1981; Ogle et al, 1983; MacDonald et al, 1982). Synthetic progesterone antagonists such as RU486 would support this hypothesis(Haluska et al, 1994).

From the Cox-1 and Cox-2 gene knockout mice(Morham et al, 1995; Langenbach et al, 1995), the data seems to indicate fetal prostaglandins play a negligible role in the initiation of parturition. In the absence of Cox-1 and Cox-2 in the fetal membranes, parturition isn't compromised.

Collectively, from the animal studies, in vitro cell culture work, gene knock out experiments, and the pharmacological efforts, the data supports our hypothesis for the importance of decidual and myometrial eicosanoids.

PART IV

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PART V

CLAIMS FOR ORIGINAL RESEARCH

1. The establishment, immortalization, and characterization of novel temperature sensitive rat uterine epithelial and stromal cell lines.

2. The development of a protocol for forming a simple in vitro endometrial bi-layer using the cell lines.

3. The *Cox-2* gene is expressed in the rat uterus and the levels of the *Cox-2* gene transcripts increase during pregnancy and approaching term.

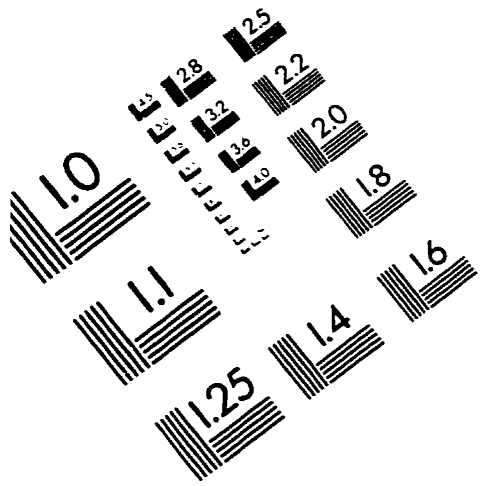
4. The demonstration that the *Cox-1* and *Cox-2* genes are expressed in both the endometrial epithelial and stromal cell lines and that from immunohistochemical staining both enzymes are expressed in the rat uterine epithelium, sub-epithelial stroma and myometrium.

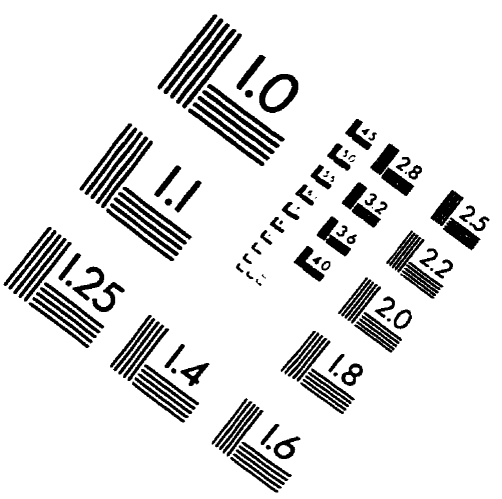
5. Determining that in stromal cells, the *Cox-2* gene is regulated by IL-1β and TNF-α.

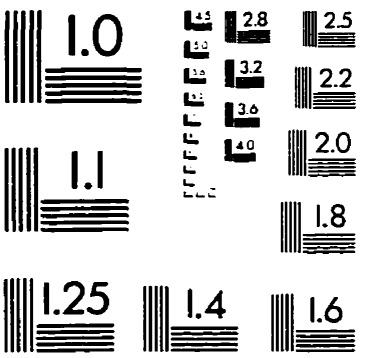
6. Showing that in Chinese Hamster Ovary cells overexpressing the oxytocin receptor stimulation of the oxytocin receptor leads to increased levels of the *Cox-2* enzyme with a concomitant rise in PgE_2 synthesis.

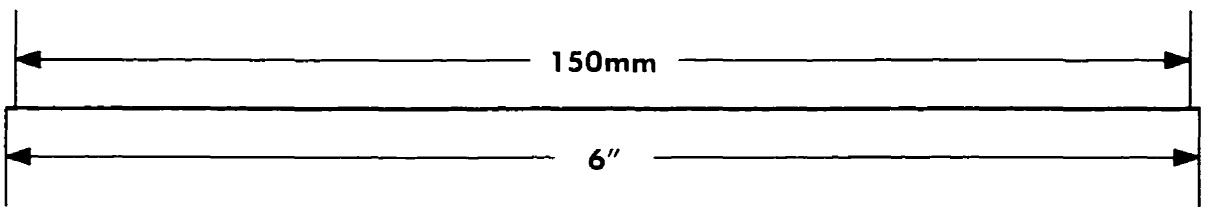
7. Demonstrating that 17β-estradiol increases the expression levels of the OTR gene in rat uterine sections cultured in vitro.

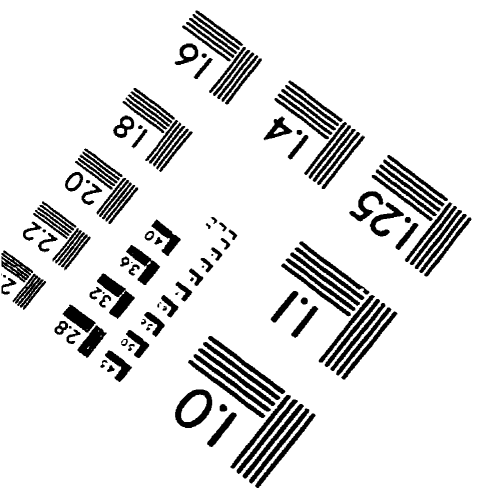
IMAGE EVALUATION
TEST TARGET (QA-3)

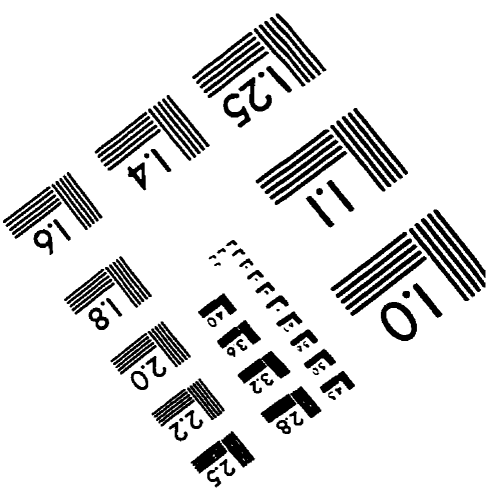












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