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Human relaxin, prolactin and placental lactogen in human intrauterine tissues

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HUMAN RELAXIN, PROLACTIN AND PLACENTAL LACTOGEN IN HUMAN INTRAUTERINE TISSUES

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

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ABSTRACT

The human placenta, fetal membranes and decidua can be classified as endocrine glands because of their abilities to produce hormones which maintain and ensure the success of a pregnancy. These hormones may enter the maternal and/or the fetal circulatory systems to act upon distant targets while others may be produced and act locally within the intrauterine compartment.

This study was designed to look at three circulatory hormones, relaxin, prolactin and placental lactogen, as bipolar hormones, local and distant. The sources of their production were studied in five different intrauterine tissues at two physiological time frames, before and after labour.

The corpus luteum is the source of circulating relaxin during pregnancy. To determine whether this hormone is produced locally in intrauterine tissues, two techniques have been used, immunocytochemistry and Northern analyses. An antiserum to a synthetic 14-amino acid sequence of the connecting peptide of human relaxin and two monoclonal antibodies to human relaxin were used to immunostain fetal membranes with adherent decidua and placental trophoblast. Poly(A)$^{+}$RNA prepared from five separate tissues, the amnion, chorionic membrane, decidua parietalis, basal plate and the placental trophoblast were hybridized to a 48-mer oligoprobe to human relaxin. Results from both techniques showed that the decidua parietalis and basalis, the chorion and the placental trophoblast synthesize and produce relaxin. The mRNA species in the placental trophoblast was shown to be 1.1kb, about 100 base pairs

smaller than the mRNA species in other tissues, suggesting different processing mechanisms for these two mRNA species for relaxin. Comparative quantitation of mRNA levels showed that the decidua parietalis expressed the gene for relaxin more than the other tissues. Also all tissues obtained after normal spontaneous delivery had a lower capability for relaxin synthesis than tissues obtained from term elective Cesarean section.

It is generally accepted that the decidua parietalis is the primary source of amniotic fluid prolactin. Whether this hormone is a product of other intrauterine tissues has not been thoroughly defined. Two polyclonal and four monoclonal antibodies were used to localize human prolactin (hPRL) in human intrauterine tissues. Northern analyses using a 48-mer oligoprobe and a 712 base pair cDNA probe for hPRL were performed to distinguish synthesized from sequestered hormone. Results showed that the decidua parietalis is indeed the major source of amniotic fluid prolactin and that the chorion laeve and the basal plate are additional sources. There was no significant difference in hPRL mRNA levels between Cesarean section and normal vaginal delivery tissues.

Human placental lactogen (hPL) is one of the major hormones secreted by the placental syncytiotrophoblast and is readily detected in the maternal circulation. In this study, a specific polyclonal antibody to hPL, a 48-mer oligoprobe and a 540 base pair cDNA probe to hPL were used to investigate and determine other possible intrauterine sources of this hormone. Results showed unequivocally that the syncytiotrophoblast is the classical source of hPL. In addition, some cells of the chorionic cytotrophoblast and the basal plate also synthesized hPL. Quantitative

analysis on Northern blots showed that the mRNA levels for hPL in these extra-sources were less than one percent of that in the classical source syncytiotrophoblast. It is not known whether these small amounts of hPL by these ectopic sources stay and function locally in the intrauterine tissues or whether they contribute to maternal circulation.

The differential production of the three hormones by intrauterine tissues presented in this dissertation provide further definition of a paracrine/autocrine system within these tissues.

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LIST OF ABBREVIATIONS

ABC	avidin-biotin immunoperoxidase complex
ATP	adenosine triphosphate
C	centigrade
cDNA	complementary deoxyribonucleic acid
cm	centimeter
C-peptide	connecting peptide
cpm	counts per minute
CRH	corticotropin releasing hormone
°	degrees
DAB	diaminobenzidine
dATP	deoxyadenosine triphosphate
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetate
ER	endoplasmic reticulum
Fig(s)	figure(s)
g	gram
GF	growth factor(s)
GnRH	gonadotropin releasing hormone
h	hour
hCC	human chorionic corticotropin
hCG	human chorionic gonadotropin
hCp14	a synthetic 14 amino acid sequence of the human relaxin connecting peptide
hCT	human chorionic thyrotropin
hGH-variant	human growth hormone-variant

LIST OF ABBREVIATIONS (CONT'D)

hPL	human placental lactogen
hPRL	human prolactin
IgG	immunoglobulin G
kb	kilobases
l	liter
M	moles
Mab	monoclonal antibody
MHC	major histocompatibility complex
min	minutes
ml	milliliter
mm	millimeter
mmol	millimoles
mRNA	messenger ribonucleic acid
ng	nanogram
NIH	National Institutes of Health
NIADDK	National Institute of Arthritis, Diabetes, Digestive and Kiney Diseases
NRS	normal rabbit serum
^{32}P	radioactive phosphorus
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
poly(A)$^{+}$	polyadenylic acid
POMC	pro-opiomelanocorticotropin
PRL-RF	prolactin releasing factor
RIA	radioimmunoassay

LIST OF ABBREVIATIONS (CONT'D)

^{35}S	radioactive sulfur
SDS	sodium dodecyl sulfate
SSC	sodium saline citrate
SSPE	sodium chloride, sodium phosphate and EDTA
TdT	terminal deoxynucleotidyl transferase
TNF	tissue necrosis factor
TRH	thyrotropin releasing hormone
tRNA	transfer ribonucleic acid
ug	microgram
ul	microliter
UV	ultra violet
VIP	vasoactive intestinal peptide
vol	volume
wt	weight
x	times (as in 6xSSPE)

CHAPTER I

INTRODUCTION

1. Gross examination of the normal term placenta and the fetal membranes

Shortly after the delivery of the fetus, the placenta with the torn fetal membranes adhering to its margins and the attached umbilical cord are expelled from the uterus. Parts of the decidua parietalis remain attached to the chorionic membrane to which it becomes closely associated in the last weeks of gestation.

The fetal membranes are composed of two major structures, the amnion and the chorion laeve. The amnion, the innermost of the membranes enveloping the fetus *in utero*, is a thin transparent layer of about 0.2 mm thick but contains tensile strength. It has no direct blood supply and can be readily peeled away from the adjacent chorion which is a layer of about 0.5 mm. Adposed to the chorion laeve are the decidua cells. The decidua at term is divided into two portions based on its location; the portion that adheres to the fetal membranes is called decidua parietalis and the portion that lies directly beneath the placenta is called decidua basalis (Fig. 1.1).

The human placenta (from the Greek plakous, meaning cake) at term is disk-shaped, about 4 cm in thickness and 18 cm in diameter and averages about 1/6 to 1/7 the weight of the fetus. Its fetal face is smooth being formed by the adherent amnion, with the umbilical cord attached, normally, near its center. The maternal face of a detached placenta usually has a film of loosely attached blood clots which when

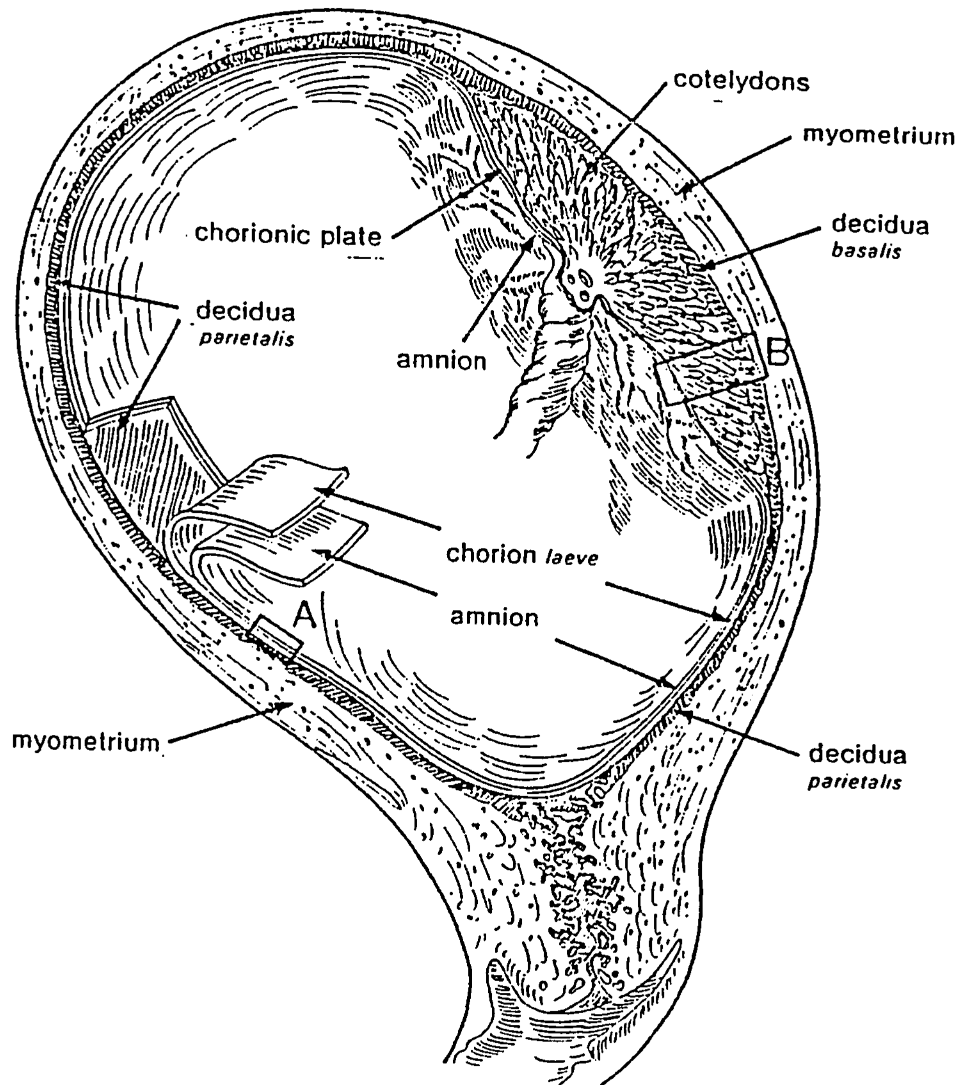

Figure 1.1. Full term placenta _in utero_.

A and B: sections for immunocytochemistry.

from: Williams Obstetrics, 16th edition, 1980.

removed, discloses a thin, grayish layer of decidua basalis. The basal plate is formed by the anchoring trophoblast and the decidua basalis, thus representing the interface of maternal and fetal tissues. The maternal surface of the placenta is divided into cotyledons or lobes by septa which are composed of projections of connective tissue elements which are in direct contact with the decidual cells and cellular trophoblast. On sectioning, the dark red villous tissue reflects the content of fetal blood and the loosely structured areas surrounding them represent intervillous space which contains trapped maternal blood.

2. Development

The ovum is fertilized in the Fallopian tube and reaches the uterine cavity as a morula, which rapidly converts into a blastocyst and loses its surrounding zona pellucida. A thin cellular layer called trophoblast develops to surround the blastocyst which normally attaches to the uterine mucosa about 6 days after fertilization and penetrates into the highly vascular endometrial connective tissue and becomes enclosed by it (Hamilton and Boyd, 1960). The embryo is completely implanted when it is 11 days of age. The endometrium goes through profound changes known as decidualization. The endometrial stromal cells enlarge and accumulate glycogen and lipid; then after this stromal edema occurs, polygonal or round decidual cells are formed within 3 or 4 days. Decidualization in the human, unlike that in the many animals studied, is a spontaneous phenomenon that occurs in the presence of a functional corpus luteum or in the presence of luteal

hormones, estrogen and progesterone (DeFeo, 1967). In human, in the absence of the embryo, the endometrium is sloughed during menstration, but if the implanted embryo is present, the decidual reaction persists. During this early stage of pregnancy the decidua is divided into three portions. The portion lying between blastocyst and myometrium is the decidua basalis; the portion covering the developing blastocyst and separating it from the rest of the uterine cavity is the decidua capsularis which eventually degenerates as pregnancy progresses; and the remainding endometrium is the decidua parietalis or decidua vera (Fig. 1.2).

The trophoblast rapidly differentiates into two layers. The inner layer is composed of mononuclear cells and is known as the cytotrophoblast whereas the outer layer consists of multinucleated cells and is known as the syncytiotrophoblast. This latter layer has been shown to be derived from the cytotrophoblast by different studies (Midgley et al., 1963; Enders, 1965; Kliman et al., 1986). The syncytium possesses spaces called lacunae that contain maternal blood. During the third week of pregnancy, the cytotrophoblast and the syncytiotrophoblast invade the surrounding decidua as solid cellular columns known as primary villi. Then the mesenchymal core is formed within these villi and converts them into secondary villi. By the end of the third week, the mesenchymal cells within the villi differentiate into blood capillaries and the resulting villi are termed tertiary villi.

The villi in association with the decidua basalis rapidly increase in number, branch profusely and form the chorion frondosum,

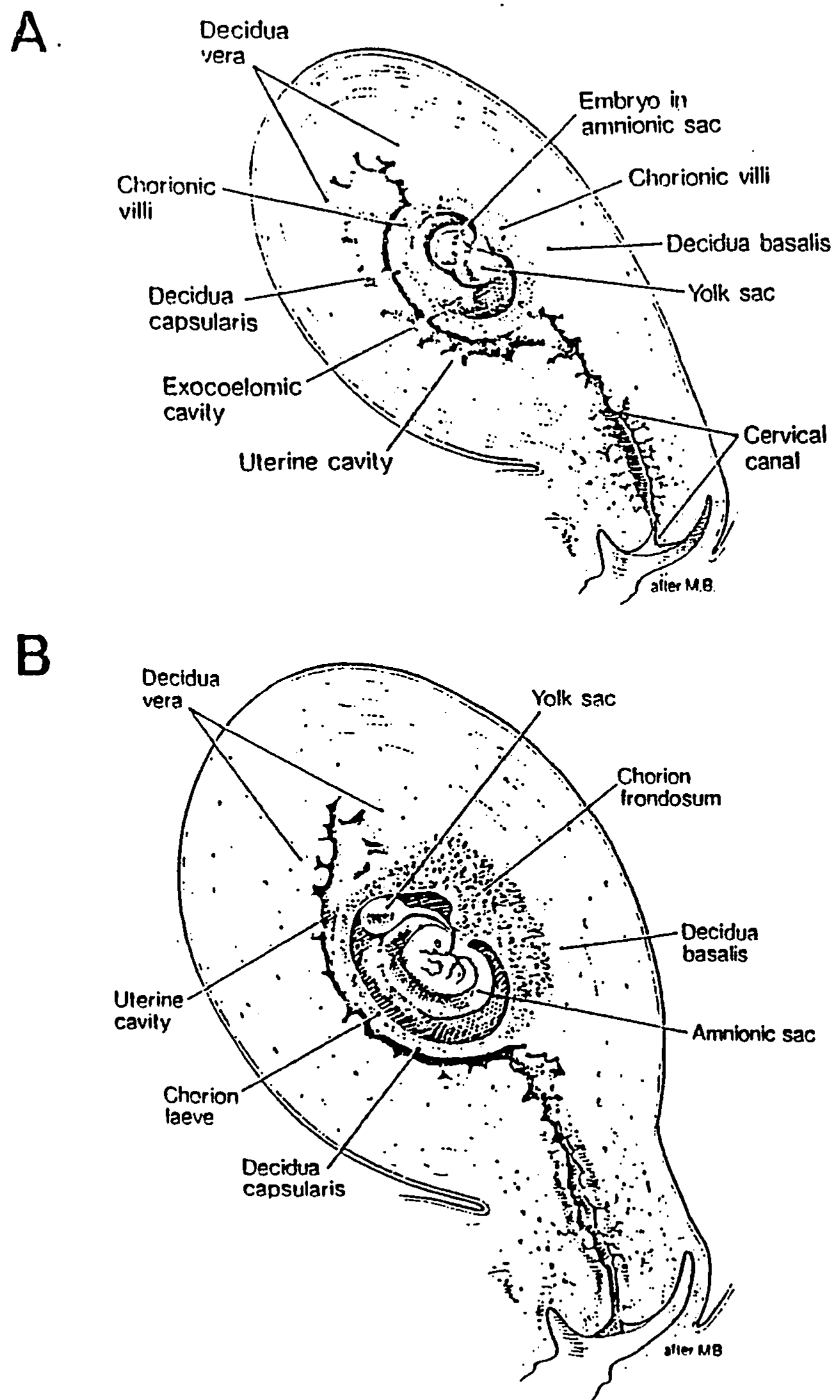

Figure 1.2. Formation of the placenta and fetal membranes.
A: Chorion frondosum and chorion laeve of early pregnancy
B: More advanced stage of pregnancy, showing atrophic chorion laeve and proliferated chorion frondosum. Three portions of the decidua are also illustrated. from: Williams Obstetrics, 16th edition, 1980.

the definitive fetal component of the placenta. Each of the main stem villi and its ramifications constitutes a placental cotyledon. The villi associated with the decidua capsularis become compressed and their blood supply is greatly reduced. These villi subsequently degenerate, producing a smooth relatively avascular area known as the chorion laeve. As the conceptus continues to enlarge, the decidua capsularis degenerates and disappears leaving the associated chorion laeve to fuse with the decidua parietalis, thus obliterating the uterine cavity (Fig. 1.2).

Two extraembryonic membranes not involved in the formation of the human placenta, the amnion and the yolk sac, also differentiate. The human yolk sac is the initial site of the fetal blood cell formation. The amnion develops into a small amniotic sac that covers the dorsal surface of the embryo. By the end of the first trimester, the amnion sac is fully expanded and this rapid growth brings the amnion into contact with the interior of both the chorion frondosum and laeve and results in obliteration of the chorionic sac (Fig. 1.2). The amnion never completely grows together with the chorion, however, so that in term placenta, it may be dislodged from the chorion rather easily.

3. Microscopic aspects of term placenta, fetal membranes and decidua

A. The amnion

At term, the human amnion is comprised of three layers: the epithelium, the basement membrane and the connective tissue. The amniotic epithelium consists of a single layer of nonciliated, cuboidal cells. On the basis of their ultrastuctural characteristics,

the human amniotic epithelial cells may be divided into two types (Thomas, 1965). One type was named the "Golgi type" since it exhibits a cytoplasm which shows membrane-bound vacuoles, a highly developed Golgi complex and distended cisternae of rough endoplasmic reticulum. This "Golgi type" cell has been suggested to have a role in secretion. The second type has a cytoplasm filled with coarse fibrils and few organelles and is named "fibrillar type". It has been suggested that this fibrillar cell type might be the "Golgi Type" cell at a resting physiological phase or it may have a role in absorption (Thomas, 1965). Wynn and French (1968) could not find such sharply defined types, instead they described the amniotic epithelium to consist of dark and light cells. The light cells differ from the dark cells principally in content of ribosomes, which is higher in the latter. The number of light cells increases as the amnion ages, these cells are probably more often inactive and are commonly degenerate. The amniotic epithelium and its basememt membrane are derived from the ectoderm. The connective tissue of the amnion has been described as a compact layer and a loose thin fibroblastic layer with some macrophages. The amount and structure of collagen of the compact layer, shown to be primarily types III and IV (Dieron and Bryant-Greenwood, 1989), probably determine the elasticity and strength of the amnion (Aplin et al., 1985). At any stage of development, the amnion is free of blood capillaries and nerve endings (Fig. 1.3).

B. The chorion laeve

The chorion laeve is separated from the amnion by a spongy intermediary zone of connective tissue where separation may take place.

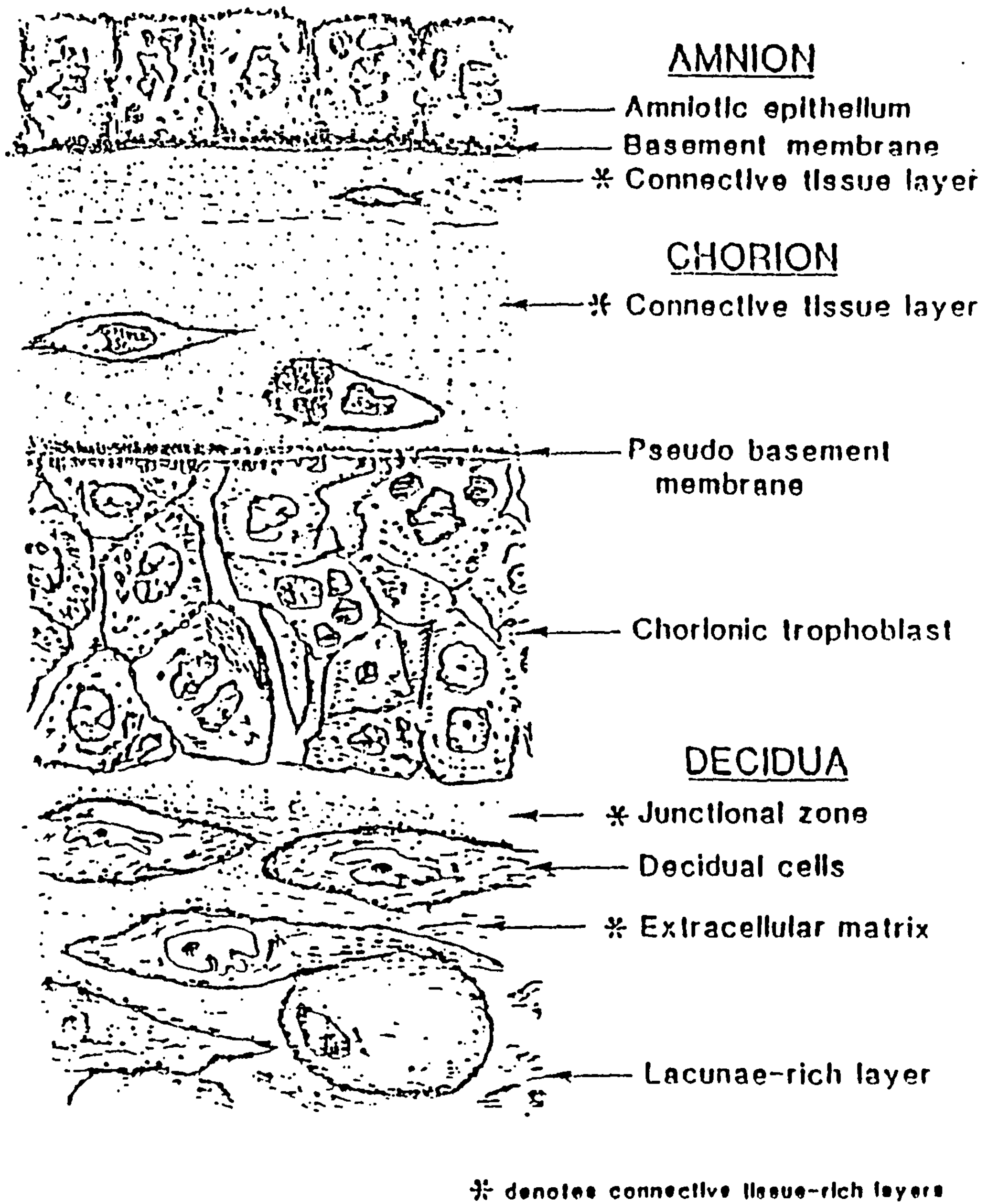

Figure 1.3. Morphological sketch of the fetal membranes and the decidua parietalis.

The chorion can be described as three layers: fibroblast layer, pseudo-basement membrane and chorionic cytotrophoblast (Fig. 1.3). The fibroblast layer under the amnion is a loose layer and contains fibroblasts and some macrophages. The collagen of this layer is types III (Aplin and Campbell, 1985; Dieron and Bryant-Greenwood, 1989). The pseudo-basement membrane is a layer of extracellular matrix and is named after its thin thickness and its location as a barrier between the fibroblast layer and the chorionic trophoblast (Bourne, 1960). The extracellular matrix fibrils extend from the pseudo-basement membrane up into the fibroblast layer and down to in between chorionic trophoblast cells in all layers (Ben-Resly et al., 1981). The external aspect of the chorion is the chorionic trophoblastic layer which is not homogenous in terms of its thickness and which is made of two to five irregular layers of cytotrophoblast cells. These cells possess abundant rough ER and Golgi membranes and are believed to synthesize and secrete extracellular matrix found in the cell layers (collagen types I and IV) and the pseudo-basement membrane (collagen type III and IV) (Aplin and Campbell, 1985; Dieron and Bryant-Greenwood, 1989). The chorion laeve is also free of blood vessels and nerve endings.

C. The placental syncytiotrophoblast

The chorionic villi form the major portion of the mature placenta. Their syncytiotrophoblastic covering layer is irregularly thinned and attenuated. The syncytial nuclei are dispersed and often appear aggregated to form multinucleated protusions from the villous surface known as syncytial knots (Fox, 1965). Cytotrophoblastic cells are still present in many villi though they are fewer in number and

less prominent than in the immature placenta. They tend to be flattened between the syncytiotrophoblast and the basement membrane and they do not form a continuous layer. Within the villi are numerous dilated fetal capillaries and some of them are situated towards the villous periphery in close approximation to the villous trophoblast. The villous stromal tissue is usually reduced to a thin compressed layer between the dilated villous capillaries and contains fibroblast, few Hofbauer cells and collagen (Fox, 1967).

D. The decidua

Fetal trophoblastic cells come into close association with maternal decidual cells at a number of points within the uterus. These cells can be distinguished from each other at the light-microscopic level by the stronger cytoplasmic basophilia of the cytotrophoblastic cells. After delivery, the decidua parietalis and basalis which are closely associated with the fetal membranes and the placenta respectively represent only a thin layer of these cells since the majority are left behind within the uterus.

D.1. Decidua parietalis

The decidua parietalis is composed of three layers: a surface layer or zona compacta that is in direct contact with the trophoblast of the chorionic laeve; a middle portion or zona spongiosa with glands and numerus small blood vessels; and a basal zone or zona basalis that lies along the myometrium. The zona compacta and spongiosa together form the functional zone and they are destined to be shed following parturition. The zona basalis, however, remains and gives rise to new endometrium.

The decidual cells are epithelioid and polygonal (Fig. 1.3). The lightly stained cytoplasm of these large cells contains numerous rough ER and Golgi membranes suggesting a role in protein secretion. They are separated from each other by extracellular matrix which consists of collagen types III, IV and V (Dieron and Bryant-Greenwood, 1989).

Within the decidua parietalis, many leucocytes are present. A high proportion are irregular in shape and found to be macrophages, while a few leucocytes are small and rounded and believed to be T and B lymphocytes (Bulmer and Johnson, 1984). Monoclonal antisera to cell surface antigens have been used extensively to identify numbers of maternal bone marrow derived cells within the decidua, however no definitive decidual cell marker has been reported yet. It has been shown that among the leucocyte cell populations in the first trimester decidua, there were 45% large granular lymphocytes, 19% macrophages, 8% T cells and that the other 28% did not stain to any cell marker (Starkey et al., 1988). Among the T lymphocytes, decidual suppressor cells were found to be present throughout normal pregnancy (Daya et al., 1985).

D.2. Decidua basalis

Unlike the decidua parietalis, the three layered structure of the decidua basalis is not well described, presumely due to trophoblastic invasion. However, histologically, the decidua basalis differs from the decidua parietalis in two respects. First, the decidua basalis has more arteries and dilated veins which have direct contact with the placental trophoblast by penetrating the basal plate, and second, it is invaded extensively by the trophoblast of the placenta.

Within the decidua basalis, there is a leucocyte population. The predominant maternal leucocytes at term are class II MHC-positive macrophages (Bulmer et al., 1988) which often extend processes around decidual cells and placental trophoblast cells.

4. Endocrinology of the placenta, fetal membranes and decidua

The placenta, the fetal membranes and the decidua are responsible for establishing effective communication between the mother and the developing fetus while maintaining the immune and genetic integrity of both individuals. They function as an alternative hypothalamic-pituitary-gonadal axis and form a network of endocrine, paracrine and autocrine systems within the fetal-placental-decidual unit. Ascheim and Zondek (1927) initially detected human chorionic gonadotropin (hCG) in urine of pregnant women and since then, many hormones have been shown to be produced by the human placenta, fetal membranes and decidua. Table 1.1 summarizes these. It should be noted that although the presence of these hormones in specific tissues is being rapidly established, an understanding of their physiology is still very limited. Three hormones have been specifically addressed in this thesis: relaxin, prolactin and placental lactogen.

5. Relaxin

A. General background

In 1926, Dr. Frederick Hisaw showed that a substance with the ability to relax the pubic ligament of the guinea pig was present in pregnant sow corpus luteum; and later, it was named "relaxin" (Fevold

Table 1.1

Hormones of the human placenta, fetal membranes and decidua

Hormone	Tissue*	Method**	Reference
1- Hypothalamic-like			
GnRH	P. cytotroph.	RIA	Siler-Khodr and Khodr, 1978a
			Siler-Khodr and Khodr, 1978b
	C. cytotroph.	BioA	Poisner and Poisner, 1985
CRH	Placenta	BioA	Shibasaki et al., 1979
	Dec., Amn.	ABC	Saijonmaa et al., 1988
TRH	Placenta	BioA	Gibbons et al., 1975
	C. cytotroph.	BioA	Mitnick et al., 1974
Somatostatin	P. cytotroph.	IP	Watkins and Yen, 1980
	Decidua	IF	Kumasaka et al., 1979
PRL-RF	Placenta	Purif.	Handwerger et al., 1987
2- Pituitary-like			
hCG	Syncytiotroph.	IP	Midgley and Pierce, 1962
	C. cytotroph.	BioA	Poisner et al., 1983
hPL	Syncytiotroph.	IA	Grumbach and Kaplan, 1964
hCC	Placenta	IA	Opsahl and Long, 1951
hCT	Placenta	BioA	Hershman and Starnes, 1969
ACTH	Placenta	RIA	Rees et al., 1975
hGH-variant	Placenta	RIA	Hennen et al., 1985
hPRL	Decidua	IF	Frame et al., 1979
	C. cytotroph.	IF	Frame et al., 1979

* P. cytotroph.: placental cytotrophoblast, C. cytotroph.: chorionic cytotrophoblast, Amn.: amnion, C.: chorion, Dec.: decidua.
**RIA: radioimmunoassay, BioA: bioassay, IF: immunofluorescence, ABC: immunolocalization by avidin-biotin complex, IP: immunoperoxidase, Purif.: purification, IA: immunoassay, EnzA: enzyme activity.

Table 1.1 (cont'd)

Hormones of the human placenta, fetal membranes and decidua

Hormone	Tissue*	Method**	Reference
3- Gonadal-like			
Estrogen	Placenta	BioA	Siiteri and MacDonald, 1966
	Amn., C., Dec.	EnzA	Gibb et al., 1988
Progesterone	Placenta	ISP	Bolte et al., 1964
	Amn., C., Dec.	EnzA	Gibb et all., 1988
Inhibin	P. cytotroph	IF	Petraglia et al, 1987
Relaxin	Decidua	BioA	Bigazzi et al, 1980
	Basal plate	Purif.	Yamamoto et al., 1981
	Placenta	Purif.	Fields et al., 1981
4- GI-like			
Gastrin, VIP	Placenta	IA	Attia et al., 1976
5- Growth factors			
Nerve GF	Placenta	BioA	Goldstein et al., 1981
TNF	Placenta, Dec.	RIA	Jaattela et al., 1988
PDGF	Placenta	NA	Taylor and Williams., 1988
Fibroblast GF	Placenta	Purif.	Gospodarowicz et al., 1985
Transforming GF	Placenta	BioA	Stromberg et al., 1982
	Placenta	Purif.	Frolik et al., 1983
Troph. derived GF	Placenta	BioA	Roy-Choudhury et al., 1982

* P. cytotroph.: placental cytotrophoblast, Amn.: amnion, C.: chorion, Dec.: decidua.
**RIA: radioimmunoassay, BioA: bioassay, IF: immunofluorescence, IP: immunoperoxidase, IA: immunoassay, EnzA: enzyme activity, ISP: in situ perfusion, Purif.: purification, NA: Northern analysis.

Table 1.1 (cont'd)

Hormones of the human placenta, fetal membranes and decidua

Hormone	Tissue*	Method**	Reference
6- Prostaglandins			
Prostaglandin E_2	Amn., C., Dec.	BioA	Okazaki et al., 1981
Prostaglandin F_2	Decidua	BioA	Okazaki et al., 1981
Thromboxane B_2	Placenta	EnzA	Mitchell et al., 1981 (b)
Leukotriene	Placenta, Amn., C., Dec.	BioA	Saeed and Mitchell, 1982
7- Opiate peptides			
POMC, Endorphin, encephalin	Placenta	IA	Liotta and Krieger, 1980
Neuropeptide gamma	P. cytotroph.	RIA,IF	Petraglia et al., 1989
8- Others			
Renin	Chorion	BioA	Skinner et al., 1968
Angiotensin	Placenta, Amn., C.	EnzA	Warren et al., 1984
1,25-diOH vit.D_3	Placenta	BioA	Whitsett et al, 1981
Atrial natriuretic peptide	Placenta	RIA	Craig and Beveniste, 1987

* P. cytotroph.: placental cytotrophoblast, Amn.: amnion, C.: chorion, Dec.: decidua.
**BioA: bioassay, EnzA: enzyme activity, IA: immunoassay, RIA: radioimmunoassay, IF: immunofluorescence.

et al., 1932). Research on relaxin was "at a low ebb" for the first 40 years after this initial discovery (Bryant-Greenwood, 1982) because of the general lack of technology for isolation and purification of peptides. Significant advances in the relaxin field were made after a sensitive bioassay was developed (Steinetz et al., 1960), however it was the publication of the procedure for the purification of porcine ovarian relaxin (Sherwood and O'Byrne, 1974) which allowed the field to fully develop. The primary amino acid sequence of porcine relaxin was then, for the first time, shown to consist of two peptide chains (A and B chains) of 22 and 31 amino acids respectively, covalently linked by two interchain disulfide bonds with an intradisulfide link in the A chain (Schwabe et al., 1976; James et al., 1977; Schwabe et al., 1977). The locations of these disulfide bonds are homologus with those of insulin and some insulin-like growth factors (James et al., 1977). A three dimensional model of relaxin based on computer graphics showed similarities between this hormone and insulin (Bedakar et al., 1977; Isaacs et al., 1978), especially their closely packed hydrophobic core and their overall pattern of folding (Bedarkar et al., 1982). It has been proposed that these two hormones have evolved by a process of divergent evolution involving an early gene duplication (Gowan et al., 1981; Kemp and Niall, 1984). However, it appears that, during the evolution, the structures of relaxins of different species have diverged more than have insulins. For example, while the primary sequences of pig and rat relaxins show low degree of homology (John et al., 1981), those of pig and rat insulins have high degree of homology (Blundell and Humbel, 1980). Isolation of relaxin from ovaries, corpora

lutea, uterus and placentas of different species is summarized chronologically in Table 1.2.

B. Biological activities of human relaxin

Since the observation by Hisaw on the relaxation of the pubic ligament of the guinea pig by relaxin, this hormone has been demonstrated to have other biological effects in a number of tissues in the reproductive tract: the uterus, the cervix, the pelvic ligament and the fetal membranes. Table 1.3 summarizes its biological roles.

It has been proposed that human relaxin functions as a paracrine hormone as well as a classical endocrine hormone (Bryant-Greenwood, 1982). The corpus luteum appears to be the source of systemic relaxin while the decidua and perhaps the placenta are the source(s) of relaxin used in a paracrine mode (Koay et al., 1985a).

C. Sources of human relaxin

The primary source of systemic relaxin in pregnant women is the corpus luteum and it has been shown by a porcine RIA that its concentration is 4 times higher in the ovarian vein, draining the ovary containing the corpus luteum of pregnancy, than in either the peripheral vein or the contralateral vein (Weiss et al., 1976). An homologous RIA for synthetic human relaxin derived from the H2 gene sequence has been recently used to show that low serum levels of relaxin (systemic) are not causatively related to the onset of labour before term (Eddie et al., 1986) in contrast to a previous report using heterologous RIA (Szlachter et al., 1982). However, the mean levels of relaxin in normal pregnancy measured by both assays showed similar pattern (Bell et al., 1987).

Table 1.2

Isolation of relaxin in different species

Species	Tissue source	Methods*	Reference
Pig	ovary	AAE, GF, IEC, IEF	Sherwood et al., 1974
Rat	ovary	AAE, GF, IEC, AAA	Sherwood, 1979
Cow	corpus luteum	AAE, GF, IEF	Fields et al., 1980
Shark	ovary	AAE, GF, HPLC, AAA	Reinig et al., 1981
Guinea pig	uterus	AE, GF, RIA	Nagao and Bryant-Greenwood, 1981
Human	placenta	AAE, GF, IEF	Fields and Larkin, 1981
	basal plate	AAE, GF, IEC	Yamamoto et al, 1981
Dogfish	ovary	AE, IEC, GF, HPLC, AAA, SA	Bullesbach et al., 1986
Horse	placenta	AAE, GF, IEC, AAA	Stewart and Papkoff, 1986
Cat	placenta	AE, RIA	Addiego et al., 1987
Skate	corpus luteum	AAE, GF, HPLC, AAA, SA	Bullesbach et al., 1987
Hamster	placenta	AE, BA	Steinetz et al., 1988
Sheep	placenta	AAE, GF, IEC, RIA	Wathes et al., 1988
Whale	corpus luteum	AAE, GF, IEC, HPLC, AAA, SA	Schwabe et al, 1989
Dog	placenta	TCA, GF, RIA, HPLC	Stewart, 1989

* AAE: acid acetone extraction, GF: gel filtration, IEC: ion-exchange chromatography, IEF: isoelectric focusing, AAA: amino acid analysis, HPLC: high performance liquid chromatography, AE: aqueous extraction, RIA: radio immunoassay, SA: sequence analysis, BA: bioassay, TCA: trifluoroacetic acid extraction.

Table 1.3

Biological roles of relaxin in human

Organ or tissue	Effects of relaxin	Reference
Uterus	Uterine quiescence	
	- alone	Wiqvist and Paul, 1958
	- with progesterone	Beck et al., 1982
	Uterine collagen content	Schwabe et al., 1978
Cervix	Cervical ripening in women at term by purified porcine relaxin	MacLennan et al., 1980 Evans et al., 1983 MacLennan et al., 1986a
Pelvic joint	Corpus luteal extracts from women possessed bioactivity on the guinea pig pelvic ligament	O'Byrne et al., 1978
	Inadequate increase of relaxin related to pelvic pain and pelvic laxity in late pregnant women	MacLennan et al., 1986b
Fetal membranes	Increase plasminogen and collagenolytic activities	Koay et al., 1986
	Influence prostaglandin E_2 production by the amnion	Lopez-Bernal et al., 1987

In recent years, evidence that relaxin is a product of non-corpus luteum tissues has accumulated. Bioactivity of relaxin was demonstrated in human decidual extracts (Bigazzi et al., 1980). Crude relaxin was extracted from human placenta and shown to be biologically active (Fields and Larkin, 1981). The basal plate of the placenta was used as a starting tissue for the demonstration of bio/immunoactive relaxin (Yamamoto et al., 1981; Schmidt et al., 1984).

A summary of the immunohistochemical localization of relaxin in human intrauterine tissues is presented in Table 1.4.

The presence of relaxin in the reproductive tissues of non-pregnant women has also been investigated. This hormone has been localized by immunoperoxidase staining in the non-pregnant corpus luteum, in the secretory phase endometrium (Yki-Jarvinen et al., 1983) and in the syncytiotrophoblast of a term placenta after removal of the corpus luteum (Yki-Jarvinen et al., 1984) using antiserum to porcine relaxin and by immunofluorescent staining in the granulosa cells of human follicles (Balboni et al., 1982).

Relaxin has been shown also to be a hormone of the male human. An homologus RIA for porcine relaxin identified high levels of relaxin in human seminal plasma (Loumaye et al., 1980), the source of which was shown to be the prostate gland (Essig et al., 1982). It has recently been shown that the human seminal relaxin is a product of the H2 gene, the same gene as human luteal relaxin (Shih et al., 1989)

D. The genes for relaxin

Relaxin belongs to a family of structurally related molecules representing the products of structurally related genes. The study of

Table 1.4

Immunolocalization of relaxin in human intrauterine tissues

Reference	Antiserum	Amnion	Chorion	Decidua	PT	BP
Fields and Larkin, 1981	rabbit anti-pRLX: R19.	NE	NE	NE	+ve con.	+ve occ.
Yki-Jarvinen et al., 1983	2 rabbit anti-pRLX: R6 and NIH RXN-P1.	NE	NE	+ve	+ve	NE
Koay et al., 1985	4 rabbit anti-pRLX: R6, R19, Gg and Fk.	+ve occ.	+ve con.	+ve con.	+ve weak.	+ve con.
Bryant-Greenwood et al., 1987	rabbit anti-pRLX, Gg.	-ve	+ve C: 4/4 S: 5/10	+ve C: 0/4 S: 2/10	NE	NE

Abbreviations:

PT: placental trophoblast, BP: basal plate, pRLX: porcine relaxin, NE: not examined, +ve: positive, -ve: negative, con.: consistently, occ.: occasionally, weak.: weakly, C: tissue from Cesarean section, S: tissue from normal spontaneous delivery.

human relaxin genes has been achieved by the two traditional techniques of molecular biology, cloning and sequencing, and based on prior elucidation of the studies of the rat and the pig relaxin genes. The study of the human relaxin genes required a different strategy from that of the rat and pig shown in Table 1.5, because the amino acid sequence of human relaxin was not available. Attempts at the isolation of human relaxin from human placenta and decidua (Yamamoto et al., 1981; Biggazi et al., 1980) demonstrated that its concentrations were too low to permit purification and sequencing of the peptide. The general approaches to the cloning of relaxin cDNA in the rat (Hudson et al., 1981) and the pig (Haley et al., 1982), based on amino acid sequence analysis of the peptide hormones, are summarized in Table 1.5. When comparing rat and pig relaxins, some regions of the connecting peptides (C peptide) were found to possess a higher degree of nucleotide sequence homology than the A and B chains (Haley et al., 1982).

Hudson and his colleagues had identified a region of the pig relaxin cDNA (corresponding to amino acids 45-94 of the C peptide) that showed a reasonably high degree of homology (71% at the nucleotide level) with rat relaxin cDNA. A 150 base pair probe was constructed from this fragment and was used to screen a human genomic library (Hudson et al., 1983). The isolated genomic clone lambda H7 was analyzed to provide the complete coding sequence of a human relaxin gene designated as H1, from which the preprorelaxin amino acid and mRNA sequences were deduced. Unlike the rat and porcine relaxin genes, the human gene H1 contains an extra 3.7 Kb intron within the C peptide

Table 1.5

General approaches used in the study of rat and pig relaxin genes

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Major steps*:

1. Amino acid sequence of the peptide hormones.
2. Predict mRNA sequences from amino acid sequences.
3. synthesize oligodeoxyribo-nucleotide primers to the predicted mRNAs sequences.
4. Isolate mRNA from rat ovaries and pig corpora lutea of pregnant animals.
5. Make single stranded cDNA from mRNA by reverse transcriptase then convert it to double stranded cDNA by polymerase I.
6. Insert double stranded cDNA into plasmid and clone the plasmid.
7. Detect cDNA clones containing relaxin gene sequence by radiolabelled oligonucleotide primers from step 3.
8. Clone the positive plaques and sequence them.

---

* Developed from Hudson et al., 1981; Haley et al., 1982.

coding region. Human relaxin mRNA codes for preprorelaxin which consists of a signal peptide of 25 amino acids, B chain of 32 amino acids, C peptide of 25 amino acids and an A chain of 24 amino acids (Fig. 1.4). The following year, Hudson and his colleagues used the same 150 basepair probe to screen a cDNA clone bank prepared from human pregnant ovarian tissue, and a second relaxin gene was reported (Hudson et al., 1984). This new gene was designated as human relaxin gene H2. Extensive comparison of the nucleotides within exon sequence of the two relaxin genes showed 90.5% homology (Fig. 1.5), however, the predicted amino acids sequences of these relaxin peptides contained considerably more variation (9 out of 56 amino acids are different). The two human relaxin genes were shown to be non allelic and to be present within the human genome on chromosome 9 (Crawford et al., 1984). In contrast, the human insulin gene has been located on the short arm of chromosome 11 (Owerbach et al., 1980).

The relaxin molecule synthesized from the predicted relaxin gene H1 amino acid sequence has been shown to possesss relaxin-like biological activity in the rat uterus (Tregear et al., 1982). This suggests that human relaxin gene H1 is a potentially active gene, but the expression of this gene has not yet been shown in any tissue. Human relaxin gene H2, however, is a functional gene since it was shown to be expressed in the human corpus luteum tissue of pregnancy (Hudson et al., 1984).

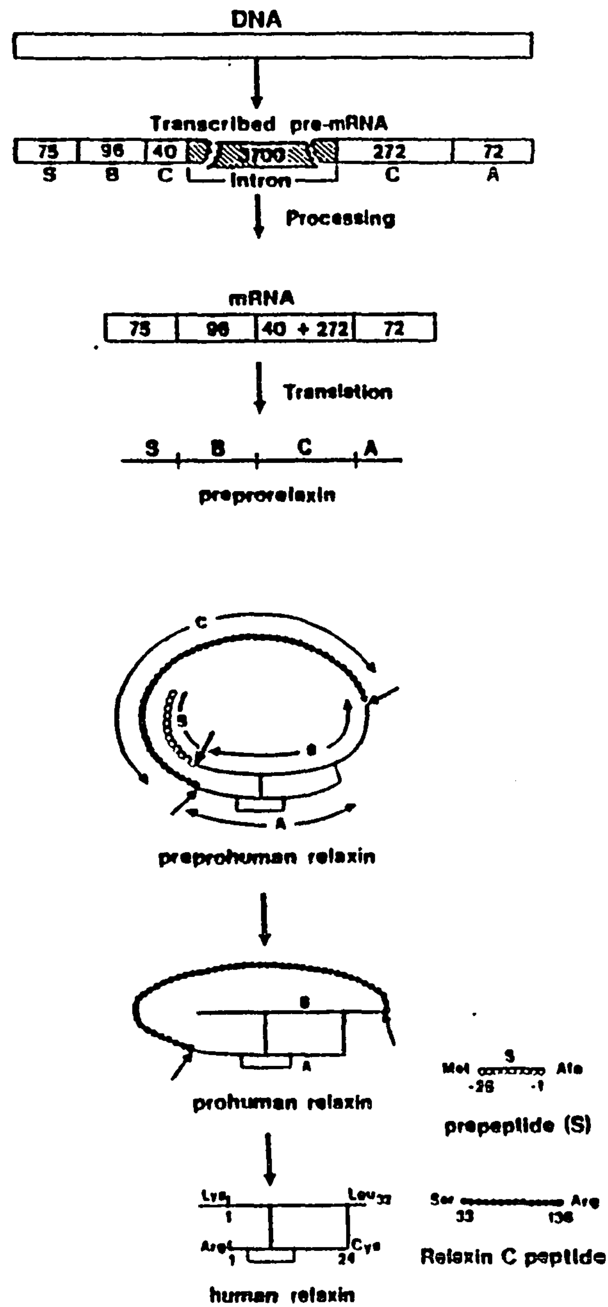

Figure 1.4. Schematic illustration from relaxin gene to the peptide hormone product.

from: Kemp and Niall, 1984.

Signal Peptide

B-chain

H2

H1

C-peptide

A-chain

Figure 1.5. Comparison of the amino acid and mRNA sequences of human relaxin H1 and H2.

from: Hudson et al., 1984.

6. Prolactin

A. General background

Human pituitary prolactin is a polypeptide hormone with a molecular weight of approximately 23,000 daltons. The molecule contains 199 amino acids with three disulfide bridges and has 26% homology with hGH and 27% homology with human placental lactogen (hPL) (Owerbach et al., 1981).

In 1961, it was shown that highly purified human growth hormone (hGH) possessed intrinsic lactogenic activity (Lyons et al, 1961.), and since many attempts to isolate a separate hPRL had failed, the existence of this hormone in primates was in doubt. All preparations from primate pituitary glands possessing lactogenic activity were shown to be contaminated by GH to varying degrees. This observation had led some investigators to suggest that perhaps in primate species, GH and PRL were one and the same hormone (Bewley and Li, 1970). However biological, immunological and clinical evidence steadily accumulated in favor of a search for a separate human pituitary PRL. In the isolation of primate PRL, the major problem was that GH was present in pituitary extracts at levels known to be 20-50 times those of PRL and it was impossible to use bioassays based upon lactogenic activity to monitor the fractionation procedures.

The first isolation of a primate PRL (monkey) was finally achieved through the use of an affinity chromatography technique (Guyda and Friesen, 1971). Shortly thereafter, hPRL was isolated by the same group by gel filtration and ion exchange chromatography (Hwang et al., 1972). Independently, the purification of hPRL by a procedure based

upon polyacrylamide gel electrophoresis was published (Lewis, 1971). Rapidly following these studies, specific RIAs were reported by Hwang et al. (1971) and by Bryant and Greenwood (1972) (who used hPRL isolated by Pasteels and his colleagues in Belgium).

B. Biological roles of pituitary prolactin

The functions of pituitary PRL vary in different species and will not be considered in detail here. PRL has wide-ranging actions either by itself or in synergism with steroid hormones. Some examples of the effects of PRL are related to reproduction (mammary development, lactation, luteotropic), growth (hair, gland, feather), osmoregulation (especially in lower vertebrates) and parental behavior (nest building in rabbits and birds) (Nicoll, 1974). In the human, the roles of maternal systemic PRL in breast development, synthesis and secretion of milk have been well studied.

C. Decidual prolactin

Following the development of the specific RIAs for hPRL, it was found to be present in large amounts in the amniotic fluid. This amniotic PRL was chemically, biologically and immunologically indistinguishable from pituitary PRL (Hwang et al., 1971; Tyson et al., 1972). The biological effects of amniotic fluid prolactin have not been well established although a variety of physiologic functions have been suggested as shown in Table 1.6. Recently, it has been shown that only amniotic fluid PRL could increased fetomaternal water flow, not exogenous pituitary PRL or ovine PRL (De Bakker-Teunissen et al., 1988). This is the first report suggesting that biological differences between pituitary and amniotic fluid PRL. The concentrations of

Table 1.6

Suggested biological roles of decidual prolactin

Observation	References
1. Regulations of electrolyte and water in amniotic fluid	
- Human or bovine pituitary PRL did not cross the chorion from the maternal side to the fetal side.	Riddick and Maslar, 1981
- Exogenous human or bovine pituitary PRL caused a decrease in fetomaternal water flow and in membrane permeability.	Tyson et al., 1972 Holt and Perks, 1975 Leontic and Tyson, 1977 Stray-Peterson, 1982 MaCoshen and Barc, 1985
- Exogenous human or bovine PRL did not have any effects on membrane permeability, but PRL extracted from amniotic fluid increased fetomaternal water flow.	De Bakker-Teunissen et al., 1988
- Low amniotic fluid prolactin in patients with chronic polyhydramnios.	Healy et al., 1983
- Defective receptor for PRL in the chorionic cytotrophoblast in patients with chronic polyhydramnios.	Healy, 1984
2. Fetal lung surfactant production	
- PRL stimulated phosphatidylcholine production in human fetal lung explants when added with cortisol and/or insulin.	Mendelson et al., 1981
- Correlation between tracheo-bronchial PRL and amniotic fluid PRL and lung surfactant in rhesus fetus.	Johnson et al., 1982
3. Parturition	
- PRL was hypothesized to have a role in the control of human labor.	Healy, 1984

amniotic fluid PRL were 100 to 200 times greater than those found in maternal or fetal serum (Hwang et al., 1971; Tyson et al., 1972). The major source of amniotic PRL was initially thought to be either the maternal pituitary (Josinovich et al., 1974) or the fetal pituitary (Fang and Kim, 1975). However, it was noticed that the changing concentrations of PRL in the amniotic fluid during human gestation did not occur in any recognizable pattern related to changes in maternal circulating PRL. During pregnancy, neither hypophysectomy nor the administration of bromocriptine in concentrations that suppressed maternal and fetal PRL production markedly cause a decrease in the amniotic fluid PRL concentrations (Riddick et al., 1979; Bigazzi et al., 1979). Since the maternal and fetal plasma PRLs are different from amniotic fluid PRL, the possibility that the latter might be produced locally in the uterus compartment was considered.

Prolactin was shown to be released into the incubating medium from flasks containing decidua but not from chorion, amnion, or placental tissue (Riddick and Kusmick, 1977). Data from two separate laboratories later confirmed the _de novo_ synthesis of hPRL from radiolabeled-amino acid precursors by decidua (Riddick et al., 1978) or by chorion-decidual tissue (Golander et al., 1978). The localization of PRL in the intrauterine tissues at different stages of pregnancy by immunofluorescence or immunohistochemistry has produced a variety of results as shown in Table 1.7.

Clements and colleagues used a molecular approach to show the presence of PRL mRNA in the pregnant intrauterine tissues by using a 616 base pair cDNA prolactin probe to hybridize to mRNA from those

Table 1.7

Immunolocalization of human prolactin in human intrauterine tissues

Reference	Antiserum	Amnion	Chorion	Decidua	PT
Healy et al., 1977	rabbit anti hPRL from NIH	+ve	-ve	NE	NE
Frame et al., 1979	rabbit anti hPRL from NIAMDD: AFP-1582-C	-ve	-ve	+ve A.S.	+ve weakly
Meuris et al., 1980	rabbit anti oPRL	NE	NE	+ve A.S.	NE
Kasai et al., 1981	rabbit anti hPRL	NE	NE	NE	+ve S.C.
Tomoda et al., 1983	rabbit anti hPRL from NIAMDD: AFP-C 11580	NE	-ve	-ve	+ve S.C.
Al-Timinii and Fox, 1986	rabbit anti hPRL from NIAMDD	-ve	-ve	+ve A.S.	+ve A.S.

Abbreviations:
PT: placental trophoblast, hPRL: human prolactin, oPRL: ovine prolactin, NIH: National Institute of Health, NIAMDD: National Institute of Arthritis, Metabolism and Digestive Disease, NE: not examined, -ve: negative, +ve: positive, A.S.: all cells, S.C.: some cells.

tissues. They found that mRNA from human decidua-chorion but not amnion or placental trophoblast gave a positive hybridization signal (Clements et al., 1983). The results of a cell free translation study (Taii et al, 1984) also confirmed de novo synthesis of PRL in the decidua but not in the placenta or amnion.

D. The human prolactin gene

Human prolactin, growth hormone and placental lactogen appear to have evolved from a common ancestral family gene by consecutive duplication and sequence divergence (Niall et al., 1971). Cooke et al. (1981) isolated mRNA from human pituitary prolactin-secreting adenomas and used it as template for synthesis of cDNA. The cDNA, inserted to pBR322 plasmid, was cloned, screened and the identified clone was then sequenced. The 914 base pair nucleotide sequence of the prolactin mRNA and the deduced amino acid sequence of the human preprolactin were reported (Fig. 1.6). The cloned human prolactin cDNA was later used as a probe to isolate and characterize human prolactin gene sequences in the human genome (Troung et al., 1984). The human prolactin has been shown to be present as a single copy per haploid genome and to be about 10 Kb long and containing 4 introns, three of which interrupt the coding sequence at the same locations as in the known GH and hPL genes (Fig. 1.7). Also, all the intron splice sites were unambiguously located following the GT-AG rule (Breathnach et al., 1978). The sequence for the 5 exons was identical to the previously reported human prolactin cDNA sequence (Cooke, et al., 1981).

The genes for hGH and hPL have been shown to be located on chromosome 17 (Owerbach and Rutter, 1980) whereas the gene for human

```
       -28                                    -20                                                -10
       met asn ile lys gly ser pro trp lys gly ser leu leu leu leu leu val ser asn leu leu leu cys gln ser val ala pro
AAAC   AUG AAC AUU AAA GGA UCG CCA UGG AAA GGG UCC CUC CUG CUG CUG CUG GUG UCA AAC CUG CUG CUG UGC CAG AGC GUG GCC CCC

1                                   10                                      20                                      30
leu pro ile cys pro gly gly ala ala arg cys gln val thr leu arg asp leu phe asp arg ala val val leu ser his tyr ile his
UUG CCC AUC UGU CCC GGC GGG GCU GCC CGA UGC CAG GUG ACC CUU CGA GAC CUG UUU GAC CGC GCC GUC GUC CUG UCC CAC UAC AUC CAU

                                    40                                      50                                      60
asn leu ser ser glu met phe ser glu phe asp lys arg tyr thr his gly arg gly phe ile thr lys ala ile asn ser cys his thr
AAC CUC UCC UCA GAA AUG UUC AGC GAA UUC GAU AAA CGG UAU ACC CAU GGC CGG GGG UUC AUU ACC AAG GCC AUC AAC AGC UGC CAC ACU

                                    70                                      80                                      90
ser ser leu ala thr pro glu asp lys glu gln ala gln gln met asn gln lys asp phe leu ser leu ile val ser ile leu arg ser
UCU UCC CUU GCC ACC CCC GAA GAC AAG GAG CAA GCC CAA CAG AUG AAU CAA AAA GAC UUU CUG AGC·CUG AUA GUC AGC AUA UUG CGA UCC

                                    100                                     110                                     120
trp asn glu pro leu tyr his leu val thr glu val arg gly met gln glu ala pro glu ala ile leu ser lys ala val glu ile glu
UGG AAU GAG CCU CUG UAU CAU CUG GUC ACG GAA GUA CGU GGU AUG CAA GAA GCC CCG GAG GCU AUC CUA UCC AAA GCU GUA GAG AUU GAG

                                    130                                     140                                     150
glu gln thr lys arg leu leu glu gly met glu leu ile val ser gln val·his pro glu thr lys glu asn glu ile tyr pro val trp
GAG CAA ACC AAA CGG CUU CUA GAG GGC AUG GAG CUG AUA GUC AGC CAG GUU CAU CCU GAA ACC AAA GAA AAU GAG AUC UAC CCU GUC UGG

                                    160                                     170                                     180
ser gly leu pro ser leu gln met ala asp glu glu ser arg leu ser ala tyr tyr asn leu leu his cys leu arg arg asp ser his
UCG GGA CUU CCA UCC CUG CAG AUG GCU GAU GAA GAG UCU CGC CUU UCU GCU UAU UAU AAC CUG CUC CAC UGC CUA CGC AGG GAU UCA CAU

                                    190                             199
lys ile asp asn tyr leu lys leu leu lys cys arg ile ile his asn asn asn cys OC
AAA AUC GAC AAU UAU CUC AAG CUC CUG AAG UGC CGA AUC AUC CAC AAC AAC AAC UGC UAA GCCCACAUCCAUUUCAUCUAUUUCUGAGAAGGUCCUUAAUGA

UCCGUUCCAUUGCAAGCUUCUUUUAGUUGUAUCUCUUUUGAAUCCAUGCUUGGGUGUAACAGGUCUCCUCUUAAAAAAUAAAAACUGACUCGUUAGAGACAUC
```

Figure 1.6. Prolactin mRNA sequence with the corresponding amino acid sequences.

from: Cooke et al., 1981.

Human Prolactin
Human Growth Hormone
Human Chorionic Somatomammotropin

Figure 1.7. Comparison of human prolactin, growth hormone and placental lactogen mRNA sequences.

from: Cooke et al., 1981.

prolactin was on chromosome 6 (Owerbach et al., 1981). The DNA sequence homologies by base pair of these hormones were 92% between hGH and hPL (Cooke et al., 1981), 42% between hPRL and hGH, and 41% between hPRL hPL (Owerbach et al., 1981). Comparison by codons, however, showed 78% homology between hGH and hPL, only 10% between hPRL and hGH and 9% between hPRL and hPL (Cooke et al., 1981).

As already described, there is much evidence to suggest that the decidua is the source of PRL found in the amniotic fluid. Takahashi et al. (1984) isolated mRNA from human decidua and used it as template to prepare single-stranded and then double stranded cDNA which was inserted in pBR322. The coding sequence for decidual PRL was found to be identical with that of human pituitary except for 4 silent nucleotide differences.

7. Human placental lactogen

A. General background

Human placental lactogen (hPL) is a single-chain polypeptide hormone with a molecular weight of 22,300. It is composed of 191 amino acids and contains two disulfide bridges but no carbohydrate moieties. hPL has 85% of its amino acid sequence identical to hGH and 27% to hPRL (Cooke et al., 1981).

In pregnant women, hPL is a major placental secretory product, accounting for 10% of all placental protein production at term (Suwa and Friesen, 1969). Its concentration in the maternal circulation gradually rises during the first trimester, dramatically rises in the second and third trimesters and reaches maximal levels near term. hPL

has been detected in the syncytiotrophoblast 5 to 12 days after implantation of the fertilized ovum (Beck, 1970). There is direct correlation between these concentrations and increasing placental mass (Sciarra et al., 1968). Immunohistochemical studies localized this hormone in placental trophoblast (De Ikonicoff and Cedard, 1973; Watkins, 1978; Kurman et al., 1984; Gosseye and Fox, 1984; Sasagawa et al., 1987) and in chorionic cytotrophoblast of fetal membranes from early pregnancy (Kurman et al., 1984; Sasagawa et al., 1987) and term (Kurman et al, 1984).

B. The hPL genes

Northern analysis using a cDNA probe to hPL showed that there was about a 4-5 fold greater population of hPL mRNA in total RNA from term placenta than in total first trimester RNA (McWilliams et al., 1977; Boime et al., 1982). *In situ* hybridization confirmed that hPL is synthesized in the syncytiotrophoblast of placental villi (McWilliams and Boime, 1980).

There are 5 hPL genes and all of them are clustered together with human growth hormone genes at band q22-q24 on chromosome 17 (Owerbach et al., 1980; George et al., 1981; Harper et al., 1982). Analysis of cDNAs of human placental mRNA demonstrated that two distinct hPL genes, hPL-1 and hPL-2, are transcribed giving rise to two distinct hPL mRNAS. The hPL-1 gene encodes 60% of placental hPL mRNA (Selby et al., 1984), while the hPL-2 gene encodes the remaining 40% (Barsh et al., 1983). The overall nucleotide sequence homologies for these two genes are 98% and they consist of five exons separated by 4 introns with correct splice site donors and acceptors, GT and AG for each of the 4 introns

(Miller and Eberdhardt, 1983). The hPL-3, hPL-4 and hPL-5 genes have not been characterized in detail.

C. Biological roles of hPL

When hPL was first isolated and purified, investigators anticipated a wide range of diagnostic and therapeutic uses for this growth hormone-like substance. However, there is no evidence for a unique biologic activity of this hormone. hPL has many of the somatotrophic and lactogenic properties of hGH and hPRL. It has been shown that hPL is approximately 50% as potent as hPRL in stimulating mucosal proliferation in pigeon crop sac assay (Josimovich and Maclaren, 1962) and that it is as potent as hPRL in stimulating the synthesis of casein, lactalbumin and lactoglobin in explants of mouse mammary glands (Turkington and Topper, 1966). Although hPL shares extensive structural homologies to hGH, it is only about 1% as potent as hGH in stimulating epiphyseal growth of the tibia (Kaplan and Grumbach, 1966) and weight gain in hypophysectomized rats (Arrezzuni et al., 1972). Another suggested role of hPL is as an insulin antagonist since it decreases glucose utilization and increases free fatty acid utilization (Turtle and Kipnis, 1967). Despite its many possible roles, hPL appears to be unessential to a successful pregnancy since there are two reports which demonstrate the delivery of normal offspring from mothers whose serum hPL was undetectable throughout pregnancy (Barbieri et al., 1986; Trapp et al., 1987).

8. Statement of the problems

The locus of control of parturition in women appears to be within the placental unit (Liggins, 1985) rather than fetal (like in the sheep and goat, Liggins, 1985) or maternal centered. This is an exclusion hypothesis in that studying the other approaches has not yielded a coherent credible account. Hence, the understanding of the events of human parturition should be based on local controls. Hormones appearing in the plasma from whatever source are classically regarded as systemic signals to a distant target. Prostaglandins are an exception and so might be all the growth factors.

The theme of this dissertation is to look at three endocrine hormones of the fetal/decidua/trophoblast as local hormones. At this stage of our techniques, without a handle on receptor number and distribution, we cannot identify them as truly paracrine or autocrine. However immunolocalization affords "a flash photogragh" of an *in vivo* instant as to where the hormones are in those particular tissues at that particular time. Furthermore, Northern analyses allow us to distinguish *in situ* hormone synthesis from accumulation.

Relaxin

The presence of relaxin in the human intrauterine tissues by immunohistochemistry has been discussed earlier. It should be noted that different polyclonal antibodies raised in rabbits against porcine relaxin were used in all of the reported studies. The specificity of these heterologus antisera must always be suspected of generating artifact and could not provide a definitive answer on whether the human amnion, chorion, decidua parietalis, basal plate or placental

trophoblast were capable of synthesizing relaxin. Thus, the first question addressed in this project was whether relaxin is locally produced by at least one type of intrauterine tissue during pregnancy.

An antiserum to a custom synthesized 14-amino acid segment of the C-peptide was used to localize relaxin C-peptide, preprorelaxin and/or prorelaxin in a number of intrauterine tissues. Monoclonal antibodies to human relaxin, gifts from Genentech Inc., have been most recently used to reexamine the problem. Finally in order to show relaxin synthesis, as opposed to sequestration, the presence of its mRNA was sought in these tissues. A 48 mer oligonucleotide probe was designed from the gene structure and used to hybridize with mRNA extracted from five separated tissues of the placenta and fetal membranes.

Prolactin

It is generally accepted that decidua parietalis is the primary source of human prolactin in amniotic fluid. Results from immunocytochemistry of this hormone in human intrauterine tissues were inconsistent (Table 3) while Northern analysis (Clements et al., 1983) and *in situ* hybridization (Coghlan et al., 1985) showed decidua partietalis as the only source of amniotic prolactin. Hence the second hypothesis is that decidua parietalis is not the only source of this hormone and that other types of pregnant intrauterine tissues are also capable of synthesizing prolactin.

Several polyclonal and monoclonal antibodies, a 48 mer oligonucleotide probe and a cDNA probe to hPRL were used to investigate other possible sources of amniotic fluid prolactin. Reproducable

positive results in the decidua parietalis served as a positive control and as standard for comparative quantitation of PRL mRNA isolated from other intrauterine tissues.

Human placental lactogen

Most studies have concentrated on the syncytiotrophoblast component of the placental villi as the classical site of hPL production. Immunohistochemistry of this hormone however showed some chorionic cytotrophoblast of first trimester and term fetal membranes to be positively stained (Kurman et al., 1984; Sasagawa et al., 1987). Hence, the third problem was to determine whether hPL was produced by other intrauterine tissues.

Immunohistochemistry using a polyclonal antibody and Northern analysis using a 48 mer oligonucleotide probe and a cDNA probe to hPL were performed. It is generally agreed that the syncytiotrophoblast is the major source of this hormone, so this tissue was used as a positive control in this study. A portion of human uterus was obtained through the Pathology Department. University of Hawaii, from a premenopausal patient undergoing hysterectomy for leiomyoma and was used for Northern analysis as a negative control.

9. Organization of the dissertation and allocation of contribution

The body of this dissertation is made up of 4 publications in refereed journals.

* An overview of the tissues and the hormones involved in this work, not fully described in these papers, is presented in Chapter 1. Since the 4 papers were published in different journals, the format for

each one of them is slightly different. Although there are references at the end of each chapter, the bibliography of the whole dissertation is listed in the last chapter.

* The title of Chapter II is "Immunocytochemical localization of prolactin and relaxin C-peptide in human decidua and placenta". This study was published in the Journal of Clinical Endocrinology and Metabolism, volume 65, number 2, 1987. My contribution as first author of this paper included conducting all the immunostaining, taking the photographs and approximately 50% of the preparation of the manucript.

* The PRL study is extended and presented in Chapter III with the title "The localization of prolactin and its mRNA in the human fetal membranes, decidua and placenta". The article was submitted to Trophoblast Research and is in press. I was responsible for all Northern analyses which included mRNA extraction, hybridization with the 48 mer oligonucleotide probe and quantitation, and the preparation of the first draft of this manuscript. Dr. S. Ali is first author because this work represented some parts originally used in his thesis which I repeated and extended.

* Chapter IV represents the work on hPL which was published in the American Journal of Obstetrics and Gynecology, volume 162, 1990 under the title "Immunocytochemical localization and mRNA concentrations for human placental lactogen in amnion, chorion, decidua and placenta". I was responsible for all Northern analyses which includes mRNA extraction, hybridization with the 48 mer oligoprobe and the cDNA probe, densitometric analysis and preparation of the first draft of this manuscript.

* "Human relaxin in the amnion, chorion, decidua and trophoblast by immunocytochemical localization and Northern analysis" is the fourth paper included in the dissertation as Chapter V. My contribution was the extraction of mRNA, hybridization, quantitation and preparation of the first draft of the manuscript. The article appeared in the Journal of Clinical Endocrinology and Metabolism, volume 70, 1990.

CHAPTER II

IMMUNOCYTOCHEMICAL LOCALIZATION OF PROLACTIN AND RELAXIN C-PEPTIDE IN HUMAN DECIDUA AND PLACENTA*

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Running Title: PROLACTIN AND RELAXIN C-PEPTIDE LOCALIZATION

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ABSTRACT

The production of prolactin by the human decidua is generally accepted, but the production of relaxin by this tissue is not. The two hormones were localized in decidual tissue using the avidin-biotin immunoperoxidase procedure with antisera to human prolactin and to a synthetic 14 amino-acid sequence of the connecting peptide of human relaxin (hCp14). The object of using the hCp14 antiserum was to verify relaxin production by the detection of the C-peptide and/or prorelaxin. Cells of the parietal decidua adherent to the fetal membranes stained with both antisera and immunostaining for both hormones in the same cell was seen. Also, the decidua-like cells of the placental basal plate stained with both antisera. The chorionic cytotrophoblast stained with the antiserum to hCp14 but not the antiserum to human prolactin, whereas the placental syncytiotrophoblast stained for prolactin and/or human placental lactogen (hPL) but not hCp14. The prolactin staining in all tissues was lost when anti-prolactin serum absorbed with human placental lactogen (hPL) was used. This finding suggests that the antiserum to prolactin could not distinguish between prolactin and hPL. It appears, therefore, that the parietal decidua cells and the decidua-like cells of the placental basal plate may be capable of producing both relaxin and prolactin, while the syncytiotrophoblast produces hPL and possibly prolactin.

The high concentrations of prolactin found in human amniotic fluid are produced locally within the uterus (1). The source of this hormone has been shown by indirect immunofluorescence to be the decidua and trophoblast cells (2). In addition, the syncytiotrophoblast has been suggested as a site of prolactin production by immunoperoxidase localization (3).

A polyclonal antiserum to porcine relaxin has been used with the avidin-biotin immunoperoxidase procedure to show that relaxin-like material can be localized in the cells of the human parietal decidua adherent to the fetal membranes as well as in the cells of the chorionic trophoblast and the placental basal plate (4). However, a heterologous antiserum is not optimal on the one hand and the technique of immunolocalization may not be able to distinguish between hormone production and sequestered hormone on the other. Therefore, the question of whether these cells produce relaxin is still open. An antiserum has been raised to a 14 amino-acid synthetic peptide representing a partial sequence of human relaxin connecting-peptide (hCp14) deduced from the gene sequence of human relaxin gene I (5). Some preliminary results of an RIA for this synthetic peptide (6) suggested that the antiserum can detect prorelaxin, its C-peptide and metabolic fragments derived therefrom in human serum taken throughout pregnancy. The aim of this study was two-fold: 1) to demonstrate the presence of relaxin C-peptide material in uterine and placental cells; 2) to study simultaneously the prolactin producing cells in order to determine whether they are the same or different from those producing relaxin.

MATERIALS AND METHODS

Materials

A sequence of 14 amino-acids of the connecting peptide of human relaxin (79-92) of human relaxin gene I (5) was custom synthesized by Peninsula Labs., CA. Amino-acid analysis revealed it to be 97% pure and it migrated as a single spot on thin layer chromatography and electrophoresis. This sequence was searched against all other known protein sequences by the Protein Identification Resource, National Biomedical Research Foundation, Georgetown University Medical Center. It had a low score of 30, with segments #28-30 having some similarity to two kinds of AIDS-virus proteins. Human prolactin was kindly provided by Dr. U. J. Lewis, La Jolla, CA and its antiserum by the National Hormone and Pituitary Program (anti hPRL-IC-2, AFP-C12081). A sample of highly purified human placental lactogen (hPL) was kindly provided by Dr. A. Parlow, Torrance, CA. Other materials used and their sources were: Vectastain ABC Kit, Vector Laboratories, Inc., Burlingame, CA; 3-amino-9- ethyl-carbazole N, N'-dimethylformamide, 30% hydrogen peroxide, and glyceryl- glycerine, Sigma Chemical Co., St. Louis, MO. The buffers used in the immunostaining procedure were 0.075 M phosphate, 0.15 M NaCl, pH 7.2 (PBS), further diluted 1:5 just before use, and 0.1 M acetate buffer, pH 5.2.

Antiserum Production (hCp14)

An antiserum to hCp14 was raised in a New Zealand white rabbit using hCp 14-thyroglobulin as immunogen (7). The injection schedule of

Vaitukaitus, et al was followed (8). Titration of the antiserum was carried out prior to its use with ^{125}I-labelled hCp14 and precipitation of the bound fraction with anti-rabbit gamma globulin. Maximum binding was 85%, and the antiserum did not bind ^{125}I-labelled porcine relaxin. In a RIA for hCp14, unlabelled porcine relaxin produced no inhibition. Human plasma reacted in the RIA, but the dose-response was not parallel to that of hCp14. This suggests that the immunoactivity is related to the C-peptide and to its metabolic fragments (6).

The optimum dilution for each antiserum employed to obtain maximal immunocytochemical staining in the syncytiotrophoblast and decidua was determined experimentally by using a range of antiserum concentrations on serial sections of tissue.

Tissue Preparation

Placentas with attached fetal membranes were collected from women after normal spontaneous term delivery (n=4) and from one woman after an elective Cesarean section with no labor at Kapiolani Children's Medical Center, Honolulu, HI. The tissues were immediately taken to the laboratory on ice and processed within one hour after expulsion or removal. Representative slices, approximately 10 mm thick, were cut across the full thickness of the placenta from different areas; likewise, 1-cm pieces of fetal membranes with adherent decidua were dissected out. The sampled tissues were immediately fixed in Bouin's solution (16-24 h at 25 C) and then processed for paraffin embedding. Five-micron sections were cut and mounted on glass slides.

Immunocytochemical Staining

Deparaffinized and rehydrated sections were immunostained for relaxin connecting peptide using the avidin-biotin immunoperoxidase method (9). The sections were pretreated with 0.3% hydrogen peroxide for 7 min to remove endogenous peroxidase activity and with a 1:50 dilution of normal goat serum in PBS for 20 min to saturate nonspecific binding sites for immunoglobulin G (IgG). For the immunostaining, the sections were sequentially exposed to the following solutions at 25 C: appropriately diluted antiserum (or nonimmune control serum) for 60 min, 1:250 diluted biotinylated goat antirabbit IgG for 30 min, and avidin-biotinylated horseradish peroxidase complex (Vectastain ABC reagent) for 45 min. After each step, the sections were thoroughly rinsed in PBS for 9 min (three changes, 3 min each). Peroxidase activity was demonstrated cytochemically by incubation with a peroxidase substrate con- taining 0.02% (wt/vol) 3 amino-9-ethyl-carbazole and 0.03% (vol/vol) hydrogen peroxide in 0.1 M acetate buffer, pH 5.2 for 15 min. The sections then were rinsed in water, coverslips were mounted with glyceryl-glycerine, and the slides were examined by bright field microscopy. The reddish-brown chromogenic product was readily differentiated from background staining without need for further counter-staining. Sections of each sample were stained with hematoxylin and eosin. Examination of these sections verified that adequate preservation of cell morphology was obtained with the procedures employed.

Method specificity was tested by substitution of the primary antiserum with normal rabbit serum (NRS). Antiserum specificity was demonstrated by preabsorption of the prolactin antiserum with prolactin or hPL and the antiserum to hCp14 with hCp14 antigen for 24h at 4°C, before application to the sections. In addition, the antiserum to hCp14 was preabsorbed with thyroglobulin (50-2000 ng/ml) for 24h at 4°C, since this antiserum was raised by immunization with hCp14 conjugated to this large protein.

RESULTS

The antigens prolactin and hCp14 were localized in several sections of parietal decidua adjacent to the fetal membranes in all five tissues studied. Both hormones were localized in a large number of the decidual cells in a pattern similar to that previously shown for relaxin using antisera to porcine relaxin (4). Fig 1A shows these cells stained with antihuman prolactin serum diluted 1:500. A group of distinctly shaped cells is shown in Fig 1a at higher magnification, which clearly shows the stain located in the cytoplasm of the cells. Fig 1B shows the staining using antiserum to hCp14 diluted 1:500 in a section close to, but not directly adjacent to, that shown in Fig 1A. The same group of cells is also shown in inset Fig 1b at a higher magnification. The control studies carried out simultaneously included replacement of the antisera to prolactin and hCp14 with NRS or antisera preabsorbed with either human prolactin, hCp14 or thyroglobulin. The non-specific

background staining with these controls was low, thus enhancing the specific differential stained results shown in Figs 1A and 1B.

Immunostaining with the antiserum to hCp14 was seen in the chorionic cytotrophoblast of the fetal membranes (Fig 1C) in a manner identical to that observed with the antisera to porcine relaxin (4). In addition, the antiserum to hCp14 stained the amniotic epithelial cells (Fig 1C), although some of this staining was non-specific as shown by some staining of these cells after preabsorption of the antiserum with hCp14 (200 ng/ml) (Fig 1D). On the other hand, amniotic epithelial cells showed almost no staining with NRS (Fig 1E). Neither chorionic cytotrophoblast nor amniotic epithelium stained with the antiserum to human prolactin (not shown).

The distribution of cells stained in the placenta with antiserum to human prolactin (1:750 dilution) is shown in Fig 2A. The syncytiotrophoblast at the periphery of the placental basal plate and, to a lesser extent, the decidua-like cells of the placental basal plate stained. Staining with anti-prolactin serum also was evident in the syncytiotrophoblast throughout the chorionic villi. Preabsorption of the anti-prolactin serum with 150 ng/ml human prolactin resulted in no immunostaining (not shown). Moreover, when this antiserum was preabsorbed with a wide range of concentrations of highly purified hPL (5-1000 ng/ml), the staining was completely abolished (Fig 2B) in a manner similar to when the antiserum was preabsorbed with prolactin itself (not shown).

Localization in an adjacent tissue section with the antiserum to hCp14 (1:500 dilution) is shown in Fig 2C. There was a different distribution of immunostain in comparison to that using antiserum to prolactin. The decidua-like cells of the placental basal plate showed specific staining, but there was no staining in the syncytiotrophoblast, either around the basalplate or throughout the chorionic villi (not shown). Preabsorption of the antiserum with hCp14 (200 ng/ml) resulted in reduced staining of the decidual-like cells in the placental basal plate (Fig 2D).

The results using the different antisera were consistent in all 5 specimens studied, although the intensity of staining varied between specimens. There was no difference in the tissues obtained from the single specimen obtained at elective Cesarean section or those obtained after normal spontaneous labor and delivery.

DISCUSSION

The detection of human prorelaxin-like material or the C-peptide of relaxin with an antiserum to a synthetic 14 amino-acid sequence of the connecting peptide of human relaxin (anti-hCp14) has been used to provide further evidence for relaxin production by the cells of the human uterus and placenta. The demonstration that the cells of the parietal decidua and chorionic cytotrophoblast stained with the anti-hCp14 serum, as well as with antiserum to porcine relaxin (4), suggests that these cells produce relaxin. We are unable to explain the staining with antiserum to hCp14 in the amniotic epithelium.

While these cells immunostain with anti-porcine relaxin serum (4), the recent demonstration of relaxin receptors in these cells suggests that the relaxin on them may be receptor bound (10), in which case immunostaining with hCp14 antiserum would not be expected.

The simultaneous localization of prolactin and the C-peptide of relaxin in the decidua-like cells of the basal plate and the parietal decidua cells provides evidence that these cells may be the site of production of both protein hormones. The cells in Fig 1a and 1b are not completely identical because these sections were not directly adjacent to one another and the thickness of the sections led to the loss of some cells in Fig 1b. However, both antigens were localized clearly within the cytoplasm of the same cells. Unfortunately, double labelling techniques using the avidin-biotin system are not suitable for the detection of two antigens produced by the same cell (11).

Our results show that the antiserum to human prolactin used for this study cannot distinguish between prolactin and hPL. Work done with a different technique, the detection of the mRNA for the hormone, in which the cross-reactivity of structurally related hormones presents less of a problem, suggests that prolactin is produced by the decidua and not the trophoblast (12), whereas hPL is produced by the trophoblast (13). However, these hybridization techniques have their own limitations in sensitivity, so that there is still the possibility that there is some hPL in the decidua and placental basal plate and some prolactin in the trophoblast. We have recently localized these hormones with a polyclonal antiserum to hPL and a monoclonal antiserum to prolactin, selected on

the basis of RIA and supplied by Dr. J. Friesen, University of Manitoba. The polyclonal antiserum to hPL, which in RIA does not detect human prolactin, stains the trophoblast but not the parietal decidua or the decidua-like cells of the basal plate. The monoclonal antiserum to human prolactin stains the parietal decidua and placental basal plate cells but also stains the syncytiotrophoblast very lightly (to be published).

With this enhanced immunospecificity and the hybridization results showing no mRNA for prolactin detected in the trophoblast (12), the results suggest hormone binding to receptor rather than hormone production. What is needed to resolve these important relationships is a combination of high immuno-sensitivity with higher levels of sensitivity in the hybridization technique.

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Figure 2.1. Immunolocalization of prolactin in human parietal decidua and relaxin C-peptide (hCp14) in human parietal decidua and human fetal membranes.

A. Section of the parietal decidua stained with anteserum to human prolactin (1:500 dilution) and the avidin-biotin procedure (x160).

a. Inset: Higher magnification of area contained within the sqare in A (x375).

B. Section of specimen shown in A treated with antiserum to hCp14 (1:500 dilution) (x160). Note the same cells which stained positively for prolactin in A, and a, also stained positively for hCp14 B, b.

b. Inset: Higher magnification of area contained within the square in B (x375).

Sections through the fetal menbranes C-E, showing the amniotic epithelium (e), chorionic cytotrophoblast (ch) and decidua (d) (x160).

C. Stained with antiserum to hCp14 (1:500). Note staining in the amniotic epithelium, chorionic cytotrophoblast and decidua cells.

D. Serial section stained with antiserum to hCp14 (1:500) preabsorbed with hCp14 (22 ng/ml). Note absence of immunostaining, except in the amniotic epithelium.

E. Serial section stained with NRS at 1:500 dilution.

A
a
B
b

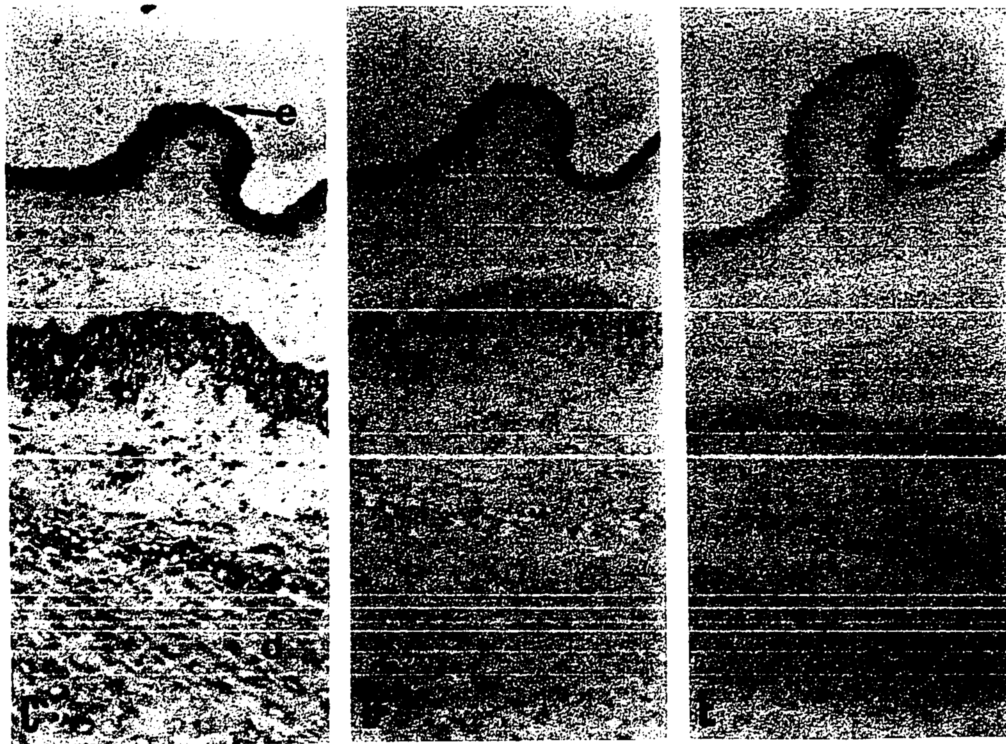

e

Figure 2.2. Immunolocalization of human prolactin in placental syncytiotrophoblast and relaxin C-peptide in the placental basal plate.

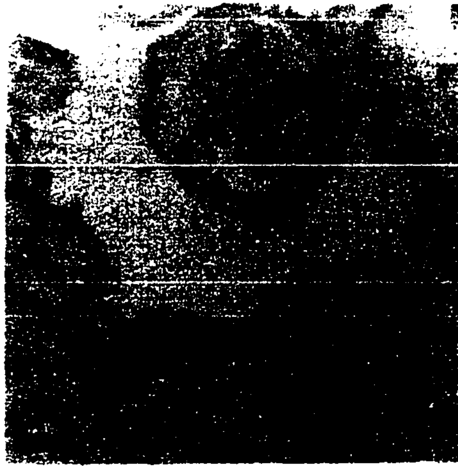
A. Section through the placental villi treated with anteserum to prolactin (1:750 dilution). Note intense staining of the syncytiotrophoblast (s) (x550).

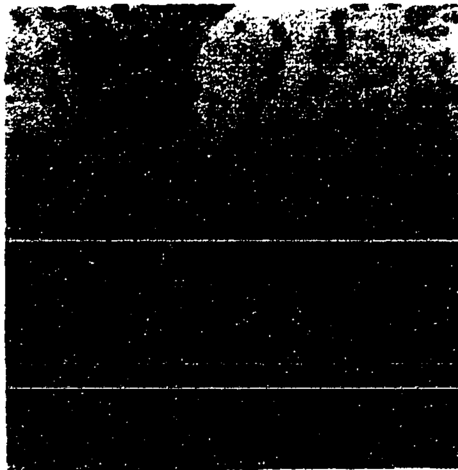
B. Section of specimen shown in A treated with antiserum to human prolactin preabsorbed with hPL (50 ng/ml). Note loss of staining in the syncytiotrophoblast (x550).

C. Section of the basal plate stained with antiserum to hCp14 (1:500 dilution) (x175).

D. Section of specimen shown in C treated with antiserum to hCp14 preabsorbed with hCp14 (200 ng/ml). Note also some non-specific staining in the cells and connective tissue outside the basal plate area. This was also present in the NRS controls (not shown).

A





CHAPTER III

THE LOCALIZATION OF PROLACTIN AND ITS mRNA IN THE HUMAN FETAL MEMBRANES, DECIDUA AND PLACENTA

submitted to Trophoblast Research
in press

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Running Title: INTRAUTERINE PROLACTIN

INTRODUCTION

The maternal parietal decidua has been proposed and generally accepted as a primary source of the human prolactin (hPRL) in amniotic fluid (Riddick & Kusmik, 1977). Whether hPRL is also a product of other intrauterine tissues, the syncytiotrophoblast of the placenta and/or the cytotrophoblast which is incorporated into the fetal chorion early in development is still controversial.

Immunocytochemistry with fluorescent antibody demonstrated unequivocally the presence of hPRL in the cytoplasm of decidual cells obtained after labor and delivery at term (Frame et al., 1979) but fluorescent cells were also found in the chorionic cytotrophoblast of the fetal membranes. Frozen sections stained with the avidin-biotin immunoperoxidase method confirmed the presence of hPRL in the decidual cells but not in the cytotrophoblast of the fetal membranes (Bryant-Greenwood et al., 1987).

The detection of hPRL in the placental trophoblast by immunocytochemistry is difficult because of the presence of very high tissue levels of human placental lactogen (hPL). This problem was addressed by Tomoda et al. (1983), using an antiserum to hPRL preabsorbed with hPL; specific staining for hPRL in the placental trophoblast was obtained suggesting that the placental trophoblast was indeed a source.

Confirmatory evidence for hPRL production by the decidua and chorion laeve was obtained by the demonstration of specific hPRL mRNA in these tissues. Hybridization was not detected with mRNA from the

placenta, suggesting that this tissue does not express the hPRL gene at a detectable level (Clements et al., 1983). More recently hPRL mRNA was identified in the decidual cells by in situ hybridization histochemistry, but results on the placental trophoblast were not reported (Coghlan et al., 1985).

The availability of more sensitive methods for immunocytochemistry and Northern analysis have allowed a reexamination of this problem using the human fetal membranes with attached parietal decidua as well as the placental villous trophoblast and basal plate. An attempt to define more precisely the cells of origin of hPRL within the pregnant human uterus was made using in situ hybridization histochemistry.

MATERIALS AND METHODS

Immunocytochemistry: antisera and antigens

A polyclonal rabbit antiserum to hPRL was a gifts from the National Hormone and Pituitary Agency NIDDK (hPRL-3, AFP-C11580). Dr. H. Friesen, University of Winnipeg, Manitoba, generously provided 4 different monoclonal antibodies made and selected for their lack of cross-reactivity with hPL in RIA (Laboratory numbers 12B1, GC3, 8A5 and 1B5). The monoclonal antibodies gave identical results in immunocytochemistry and 12B1 was therefore used for most of these studies at a 1:32 dilution.

Preabsorption studies were carried out using hPRL from NIDDK of iodination grade hPRL-1-7 (AFP-9900), a sample of Dr. H. Friesen's hPRL (85-8-8) and highly purified hPL kindly supplied by Dr. A. Parlow.

Placentas with attached fetal membranes were collected from women after elective Cesarean section at term who had not undergone labor (n=5) at Kapiolani Women's & Children's Medical Center (Honolulu, HI). The tissues were immediately taken to the laboratory on ice and processed within 1h after removal. Representative slices (10mm thick) were cut across the placenta and basal plate and 1cm pieces of the fetal membranes with adherent decidua were dissected. The sampled tissues were immediately fixed in Bouin's solution (16-24h at 25C) and then processed for paraplast embedding. Seven-micron sections were cut and mounted on glass slides.

Immunocytochemical Staining

Deparaffinized and rehydrated sections were immunostained for PRL using the avidin-biotin immunoperoxidase method (Hsu et al., 1981). The sections were pretreated with 0.3% hydrogen peroxide for 5min to remove endogenous peroxidase activity and with 0.5% normal rabbit serum in PBS (for the polyclonal antiserum) and normal horse serum (for the monoclonal antibodies) for 20min to saturate nonspecific binding sites for IgG. For the immunostaining, the sections were sequentially exposed to the following solutions at 25C. The reagents used for the monoclonal antibodies are given in parenthesis. Appropriately diluted primary antiserum to hPRL, 1:500 (1:32) or control non-immune serum 1:500 (1:32 mouse ascites fluid, ICN Immunobiologicals Inc. Ill.) for 60min, 1:250 diluted biotinylated goat anti-rabbit immunoglobulin (1:250 diluted biotinylated horse anti-mouse immunoglobulin) for 30min, and avidin-biotin horseradish peroxidase complex, Vectastain ABC reagent, for 45min. After each step, the sections were thoroughly

rinsed in PBS for 9min (three changes, 3min each). Peroxidase activity was demonstrated cytochemically by incubation with a peroxidase substrate containing diaminobenzidine (DAB) 0.5mg/ml with 0.01% hydrogen peroxide in Tris buffer 0.1M, pH 7.2. The sections were rinsed in water and counterstained with hematoxylin, coverslips were mounted with Permount (Allied Fisher Scientific, CA) and the slides examined by bright field microscopy.

Preabsorption studies were carried out by incubation of the primary antibody at the concentration used for immunocytochemistry in a range of concentrations of antigen (1-2000ng/ml), for 48h at 4C.

Isolation of Poly(A)$^+$RNA

Placentas with attached membranes were obtained from women after elective term Cesarean section, without labor (n=20) and after normal spontaneous delivery (n=10), at Kapiolani Women's & Children's Medical Center (Honolulu, HI) and were brought to the laboratory on ice within 1h of delivery. The tissue samples (8g) were obtained by separating the amnion from chorio-decidua and then scraping the decidual cells from the chorion laeve. The basal plate was sliced carefully from the placental maternal surface and villous trophoblast collected from the central region of the placenta thereby avoiding the basal and chorionic plates. It was necessary to pool tissues from several placentas in order to obtain 8g, for amnion or chorion 2 placentas were used, and for decidua or basal plate 4 placentas were used. Histological examination of the harvested chorion laeve and decidua showed the decidua to be homogeneous whereas the chorion even after quite vigorous scraping was always contaminated by some decidual cells. Control

tissue was obtained from the uterus (myometrium and endometrium) of a premenopausal patient who underwent hysterectomy for leiomyoma. The tissues were homogenized in guanidinium thiocyanate-phenol-chloroform and total RNA was prepared by the method of Choczynsky and Sacchi (1987). Poly(A)$^+$RNA was isolated by affinity chromatography on oligo(dT) cellulose using the method of Aviv and Leder (1972). The total RNA was passed through the oligo(dT) column twice and poly(A)$^+$RNA was then eluted and precipitated with ethanol. Poly(A)$^+$RNA fractions were stored at -80C in 70% ethanol until use.

Northern Analysis

Poly(A)$^+$RNA (11-20ug) from different tissues was denatured in 0.66M formaldehyde, 50% v/v formamide, and electrophoresed on 1.2% agarose gel containing 0.66M formaldehyde. An RNA ladder (Bethesda Research Laboratories, MD) was used as a molecular size marker. The gel was stained with 0.5ug/ml ethidium bromide for 30min and photographed under UV light. RNA was transferred from agarose gels to Schleicher & Schuell Nytran nylon membranes (0.45um) in 10xSSPE (1x is NaCl 8.7g/l, NaH_2PO_4. H_2O 1.4g/l, EDTA 0.37g/l, NaOH to pH 7.5) by capillary transfer overnight. The membranes were then rinsed in 5xSSPE and baked at 80C for 1h and the blots stored dessicated at 4C until use.

A 48-mer oligonucleotide with perfect complementarity to the mRNA sequence that encodes amino acids 60-75 (Table 3.1) of hPRL was kindly made by Drs. W. Scott Young III and Michael J. Brownstein, National Institute of Mental Health, Bethesda, MD. The oligonucleotide was made by solid phase synthesis on a DNA synthesizer (Applied Biosystems,

Foster City, CA) and purified on 8% polyacrylamide and 8M urea preparative sequencing gel. The homologies of the 48-mer probe for hPRL mRNA to the related mRNA sequences, human growth hormone (hGH) and hPL show a 25% similarity at the codon level (within the same region), and 58% similarity at the nucleotide level respectively. It is unlikely that the hPRL probe would hybridize to hGH or hPL mRNAs because the homologous bases are spread through the entire length of the probe.

This probe was labeled with (^{32}P)dATP (>3000 Ci/mmol, New England Nuclear, MA) and terminal deoxynucleotidyl transferase (Bethesda, Research Laboratories, MD). Hybridization was carried out using the methods described by Conner et al. (1983) and Schleicher & Schuell (1987) or by Wood (1987). The blots were sealed in plastic bags while still wet and placed in X-ray cassettes. Exposure to Fuji RX film was done with intensifying screens at -70C for the times indicated in the figure legends.

The hPRL cDNA was a generous gift from Dr. Nancy Cooke, University of Pennsylvania (Cooke et al., 1981).

The 712 base pair cDNA for hPRL was labelled using [alpha ^{32}P] dATP (>3000 Ci/mmol, New England Nuclear, MA) and Klenow fragment. Prehybridization and hybridization of the nylon membranes were carried out at 37C in a shaking water bath. The hybridization solution contained 5% Dextran sulfate, 5xSSPE, 5xDenhardt's reagent, 50% formamide, 0.1% SDS, 100ng/ml denatured SS DNA and the probe at $5x10^6$cpm/ml. After hybridization the filters were washed four times,

two washes in 6xSSPE, 0.1% SDS at room temperature for 15min each, once in 3xSSPE, 0.1% SDS at 50C for 30min and finally in 1xSSPE for 15min at room temperature. The filters were exposed to X-ray film as described above.

Quantitation of Northern blots

Results were quantified by scanning densitometry using a Video Densitometer (BioRad, Inc., Model 620) with a 1-D Analyst Software for IBM-PC. In order to assess the amount of poly(A)$^+$RNA spotted onto the filters each filter was additionally hybridized with a 27-mer human beta-actin probe (Clonetech Labs, Inc.). The amount of hybridization obtained with this probe varied from tissue to tissue: amnion (6.0 $\pm$ 1.1, n=4), chorion (9.9 $\pm$ 1.1, n=4), decidua (7.3 $\pm$ 1.4, n=6), placental basal plate (3.1 $\pm$ 0.9, n=8), placental trophoblast (4.8 $\pm$ 0.8, n=7). Therefore it was not possible to use the beta-actin probe as a standard between tissues. The hybridization signal was used as a standard (100%); the signal obtained with the 48-mer hPRL oligoprobe and poly(A)$^+$RNA from the decidua.

In situ Hybridization

Small pieces (1cm) of placental trophoblast at the junction with the fetal membranes were selected. The membranes with adhering decidua were rolled around the placental trophoblast tissue and frozen over liquid nitrogen in a small plastic weighing boat. The frozen tissue was then stored at -80C until use. Human pituitaries as control tissue were generously provided by Dr. W.W. Tourtellote, National Neurological Research Bank, CA. The frozen tissues were sent on dry ice. 10 micron sections were cut on a cryostat and thaw mounted on gelatin/chrome alum

(1g/0.1g) coated slides. The slides were placed on a slightly warm surface for approximately 1min and placed at -80C until use.

The 48-mer hPRL probe and control of the same length, rat vasopressin exon C (Table 3.1), were labelled with (alpha-^{35}S) dATP rather than ^{32}P to obtain better resolution at the cellular level after liquid emulsion (NTB-2) autoradiography. Hybridization solution (45 ul) containing 3-10x10^{5}cpm of the labelled probe in 4xSSC (pH 7.2) (1xSSC=150mM NaCl, 15mM Na citrate), 50% formamide, 1xDenhardt's, 10%(wt/v) dextran sulfate, 500ug/ml sheared single-stranded DNA, 250ug/ml yeast tRNA, and 100mM dithiothreitol was applied to each section and covered with small squares of parafilm. The slides were incubated in a humid chamber for 20-24h at 37C. The slides were dipped in NTB-2 (Nuclear Track Beta, Kodak) liquid emulsion for different exposure times. After development, the tissue sections were stained with a 0.5% solution of Toluidine blue diluted 1:1 with absolute ethanol. They were then dipped in 95%, 100% ethanol and xylene (1min each), and mounted with coverslips and a drop of immersion oil using nail paint.

RESULTS

Immunocytochemistry

The parietal decidua cells adhering to the fetal membranes stained with all antibodies to hPRL, Fig. 1A (polyclonal), Fig. 1C (monoclonal). The polyclonal antiserum also stained the amniotic epithelial cells, but not the cells of the chorionic cytotrophoblast.

In contrast, the monoclonal antibodies showed little staining in the amniotic epithelium but distinct stain in the chorionic cytotrophoblast cells (Fig. 1C), not well shown in the black and white prints (Fig. 1A, 1C). The respective controls, normal rabbit serum (Fig. 1B) and mouse ascites fluid (Fig. 1D) showed low background staining.

Preabsorption of the polyclonal antiserum with hPRL (NIDDK) gave reduction, but not complete loss of stain in the decidua cells at 100 and 1000ng/ml. We were unable to show reduction of staining by preabsorption of the monoclonal antibodies with concentrations of hPRL up to 2000ng/ml with samples of hPRL from NIDDK or Dr. H. Friesen.

The syncytiotrophoblast of the placental villi are shown stained with polyclonal antiserum (Fig. 1E) and with monoclonal antibodies (Fig. 1F). It can be seen that the polyclonal antibody gave an uneven stain in the syncytiotrophoblast whereas the monoclonal antiserum produced a light but uniform stain throughout the syncytiotrophoblast. Both antisera stained the decidua-like cells of the placental basal plate (not shown).

Preabsorption of the polyclonal antiserum with 10 and 100ng/ml hPRL (NIDDK) resulted in much reduced staining in the placental basal plate cells but only a slight reduction in staining of the syncytiotrophoblast. In contrast, when this polyclonal antiserum was preabsorbed with hPL, staining was reduced at 100ng/ml and completely abolished at 2000ng/ml. The monoclonal antibody when preabsorbed with the hPRL (NIDDK) showed no reduction in immunostain in the syncytiotrophoblast or basal plate cells, but it did show a reduction

at 1500ng/ml hPRL from Dr. Friesen (to which these antibodies were raised). However, there was also a reduction of staining with the monoclonal antibodies when it was preabsorbed with hPL (100ng/ml) in both the syncytiotrophoblast and the decidua-like cells of the basal plate.

Northern Analysis

Filters with poly(A)$^+$RNA from different tissues: amnion, chorion, decidua pareitalis, placental villous trophoblast and placental basal plate were prepared and hybridized with either the 3' end-labelled 48-mer oligoprobe or the random primed labelled hPRL cDNA probe. Poly(A)$^+$RNA from human uterus showed no hybridization with either probe.

The 48-mer oligoprobe hybridized strongly to poly(A)$^+$RNA from the decidua and the enriched chorion (Fig. 2A, lanes c and b respectively). The signal obtained at 1.2kb was identical to that obtained with mRNA from the decidua hybridized with the cDNA probe (Fig. 2B, lane b). The oligoprobe gave a weak but distinct hybridization with mRNA from the placental villous trophoblast (Fig. 2A, lane d); this was smaller (0.9kb) than the signal from decidua (1.2kb, Fig. 2A, lane c). Poly(A)$^+$RNA from the placental basal plate hybridized as two bands, one equivalent to the decidua (1.2kb) and one to trophoblast (0.9kb) (Fig. 2A, lane e). With the cDNA probe there were no signals with mRNA from villous trophoblast (Fig. 2B, lane a); the enriched chorion however gave a signal somewhat weaker than but similar to that obtained with mRNA from the decidua (not shown).

In order to study the specificity of the hybridization at 0.9kb with mRNA from trophoblast and basal plate and the 48-mer oligoprobe, the hybridization was repeated and washes were performed in tetra methyl-ammonium chloride, under conditions of high stringency (Wood et al., 1985). Under these conditions there was no hybridization obtained in the 0.9kb region and mRNA from the villous trophoblast (Fig. 2C, lane a), although the hybridization obtained with poly(A)$^+$RNA from decidua (Fig. 2C, lane b) and chorion (not shown) at 1.2kb was still present.

The results with tissues obtained after Cesarean section and the oligoprobe in the 1.2kb region are shown after densitometric analysis (Table 2). The hybridization signal with poly(A)$^+$RNA and decidua (1.2kb) was used as a standard (100%) and each tissue calculated as a percentage of this, the results were 36 ± 12 (n=4) from the chorion laeve, 17 ± 10 (n=7) from placental basal plate. There was no positive hybridization signal obtained from amnion, placental trophoblast or uterus (negative control).

Initially there appeared to be less poly(A)$^+$RNA in placental tissues obtained after spontaneous labor and delivery. This was found to be due to the difference in time between expulsion/removal and collection at the hospital and work-up in the laboratory with these two types of tissues. When this time was kept constant the results obtained were identical between the two sets of tissues.

In situ Hybridization

A more precise localization fo hPRL mRNA in amnion, chorion and decidua and the syncytiotrophoblast was attempted using in situ

hybridization. A human pituitary gland was used as a positive control. An autoradiograph of cells of the anterior pituitary at high power magnification showing abundant PRL mRNA-oligomer probe hybrid exposed to NTB-2 emulsion for 2 weeks is shown in Fig. 3A. Human decidua cells gave a similar positive hybridization signal (Fig. 3B), but a doubling of the exposure time (4 weeks) was required to obtain this signal. The pattern of grains lay only within the decidual cell boundary with fewer or no grains in the extracellular matrix or the chorionic cytotrophoblast. Thus Fig. 3C shows the chorionic cytotrophoblast cells at the junction of the decidua with no grains over the chorionic cytotrophoblast cells. No grains were seen over the amniotic epithelial cells (not shown) thus hPRL could be detected only in the decidua with this technique.

Rat vasopression mRNA-sense oligonucleotide (48-mer) used as a negative control with the same hybridization conditions gave very little background in the decidual cells after a four week exposure to liquid emulsion (NTB-2) (Fig. 3D).

A hPRL mRNA:probe hybrid signal was not detected in the trophoblast after a 6 weeks exposure, suggesting that the levels of mRNA were below the limits of the _in situ_ hybridization methodology employed or it was absent. It should be noted that the exposure times for hPRL mRNA by Northern analyses for the decidua:trophoblast were in the ratio of 1:10. Decidual mRNA for hPRL by _in situ_ hybridization required 4 weeks exposure with this probe and suggests the need for greater sensitivity.

DISCUSSION

The results using three techniques on five intrauterine tissues from human pregnancy are summarized in Table 3. There is general agreement between the 3 methods although the in situ hybridization histochemistry performed with a 48-mer oligoprobe proved less sensitive than either Northern analysis or immunocytochemistry.

The three techniques confirmed the parietal decidua as the major intrauterine source of hPRL. The in situ hybridization showed a high density of grains over selected decidua cells, whereas all the antisera stained the decidua cells with uniform intensity. This observation suggests that hPRL is synthesized discontinuously and that there is storage of the hormone.

The amniotic epithelium was stained by the polyclonal antiserum to hPRL whereas the monoclonal antibodies did not stain these cells. The absence of mRNA for hPRL by Northern analysis with both probes suggests that there is no synthesis of hPRL by these cells and that the polyclonal antiserum detects receptor bound hormone; a suggestion previously made by Healy et al. (1977) and by McCoshen et al. (1982).

The monoclonal antibodies stained the cytotrophoblast cell layer of the chorion laeve whereas the polyclonal antiserum did not. Northern analysis with the two probes showed hybridization with mRNA from chorion laeve albeit less than with mRNA from the decidua. Although we carefully scraped the decidual cells from the chorion, there could still be some contamination by decidual cells, but it appears unlikely to account for the degree of hybridization obtained. It seems more probable therefore that the cytotrophoblast of the

chorion laeve does indeed produce prolactin. In situ hybridization histochemistry was unable to add to any further resolution.

The immunolocalization and preabsorption studies for hPRL in the placenta demonstrate the difficulty, even with highly selected monoclonal antibodies, of detecting hPRL in the presence of high tissue levels of hPL. Northern analysis with the 48-mer hPRL probe under moderate stringency conditions gave hybridization with mRNA from placental trophoblast and seemingly afforded an elegant solution. However the signal was smaller in size than that obtained with mRNA from the decidua. Two separate hybridization signals were obtained with this probe and mRNA from the placental basal plate. The two sizes corresponded to the bands obtained from decidua (1.2kb) and villous trophoblast (0.9kb) and thus reflected the dual origin (fetal and maternal) of this tissue. However, the cDNA probe for hPRL showed no hybridization with mRNA from the trophoblast and the equivalent signal was absent with the mRNA from placental basal plate. When the filters after hybridization with the 48-mer probe were washed in tetra methylammonium chloride under conditions of high stringency the signal obtained with the trophoblast and its equivalent from placental basal plate were then absent. Thus the results with the oligoprobe became consistent with those obtained with the cDNA probe, and allow a conclusion that the placenta is not a site of hPRL synthesis. It is possible that the placenta may express an incomplete mRNA for hPRL or that the 48-mer probe may detect a hPRL-like message not hybridizing with the cDNA probe. It appears unlikely that the signal is due to cross-reactivity with mRNA for hPL since the signal obtained with a

cDNA probe for hPL is larger (1.1kb) (to be published). Finally, the decidua-like cells of the placental basal plate, are a site of authentic hPRL synthesis as assessed by Northern analysis and immunolocalization.

SUMMARY

Three techniques, immunocytochemistry, Northern analysis and *in situ* hybridization histochemistry have been used to reassess the sources of intrauterine hPRL in pregnancy. There is little doubt that the parietal decidua is the major intrauterine source of hPRL whilst the cytotrophoblast of the chorion laeve and the placental basal plate are additional sources which may contribute to the levels of hPRL in amniotic fluid.

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Figure 3.1.

Immunolocalization of hPRL in the fetal membranes, decidua (A-D) and placental trophoblast (E,F). x200

(A) Stained with polyclonal antiserum (1:500). Note staining in the amniotic epithelium (e), and decidua (d), no staining of the amnion (a) or chorionic cytotrophoblast (ch).

(B) Serial section stained with normal rabbit serum (1:500).

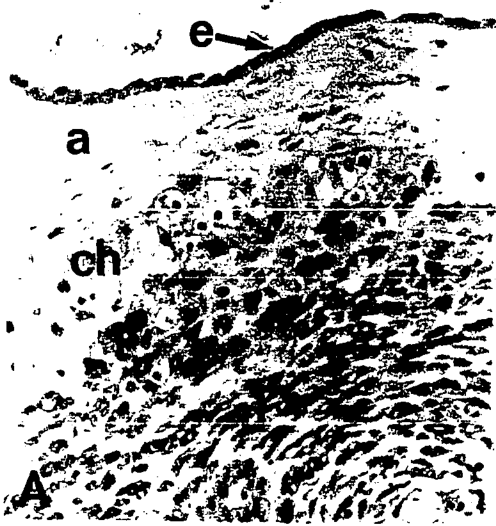
(C) Stained with monoclonal antibody 12B1 (1:32). Note there is staining in the chorionic cytotrophoblast but this is difficult to see in black and white. Staining in the cytoplasm of the decidual cells is clear.

(D) Serial section stained with mouse ascites fluid (1:32).

(E) Stained with polyclonal antiserum (1:500). Note uneven stain in placental syncytiotrophoblast(s).

(F) Stained with monoclonal antibody (1:32). Note lighter but uniform stain in the placental syncytiotrophoblast(s).

All sections were counterstained with hematoxylin.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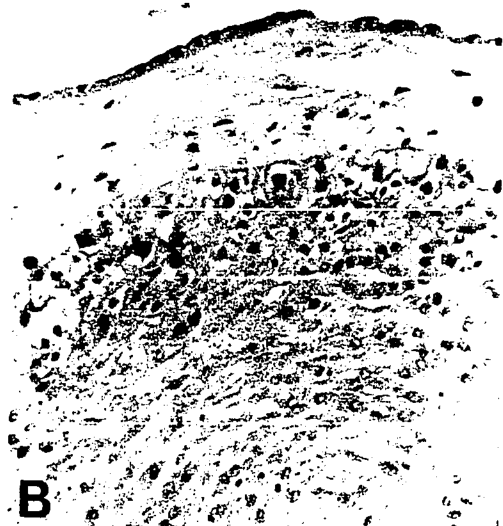
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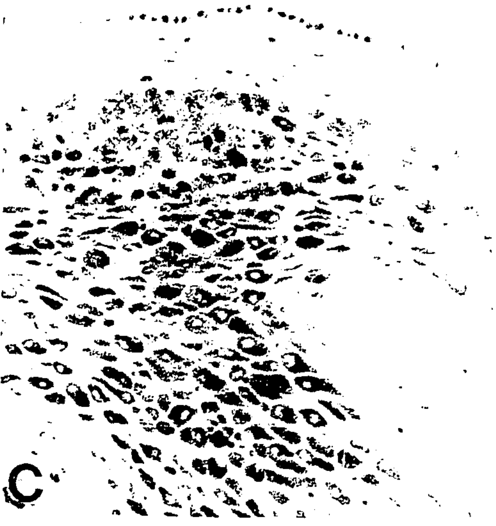
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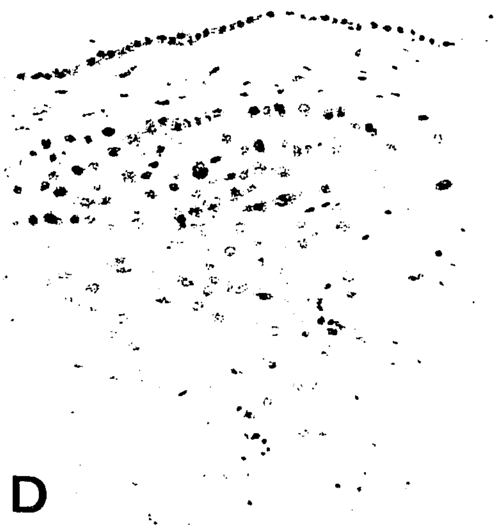
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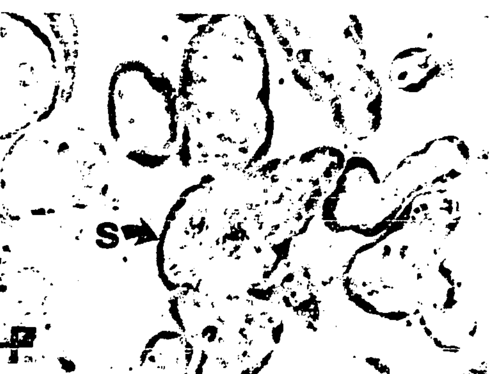
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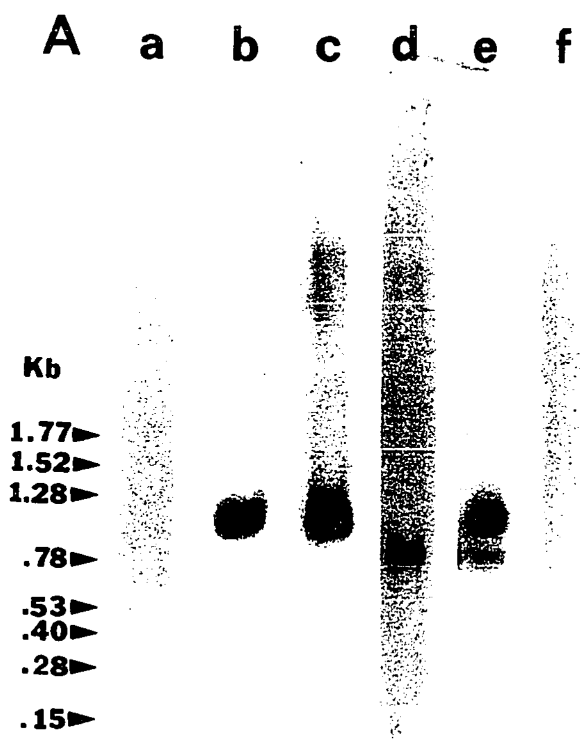
Figure 3.2.

Northern analyses of poly(A)$^+$RNA from human amnion, enriched chorion, decidua parietalis, villous trophoblast and placental basal plate.

(A) Poly(A)$^+$RNA (20ug) from human amnion (pooled) (lane a), enriched chorion (lane b), decidua parietalis (lane c), villous trophoblast (lane d), placental basal plate (lane e) and uterus (lane f), hybridized with a hPRL 48-mer oligoprobe 3'-end labelled with alpha ^{32}P. The filter was hybridized under moderate stringency conditions and exposed to Fuji RX film for two days at -20C.

(B) Poly(A)$^+$RNA from villous trophoblast (11ug) (lane a) and decidua parietalis (16ug) (lane b) hybridized with a random prime ^{32}P-labelled cDNA probe to hPRL. The filter was hybridized under moderate stringency conditions, washed and exposed to film for 24h at -20C.

(C) Poly(A)$^+$RNA (20ug) from villous trophoblast (lane a) and decidua parietalis (lane b) hybridized with the 48-mer oligoprobe as in Figure 2A except that washes were carried out with the addition of tetra methylammonium chloride under conditions of high stringency and exposed for 7 days at -20C.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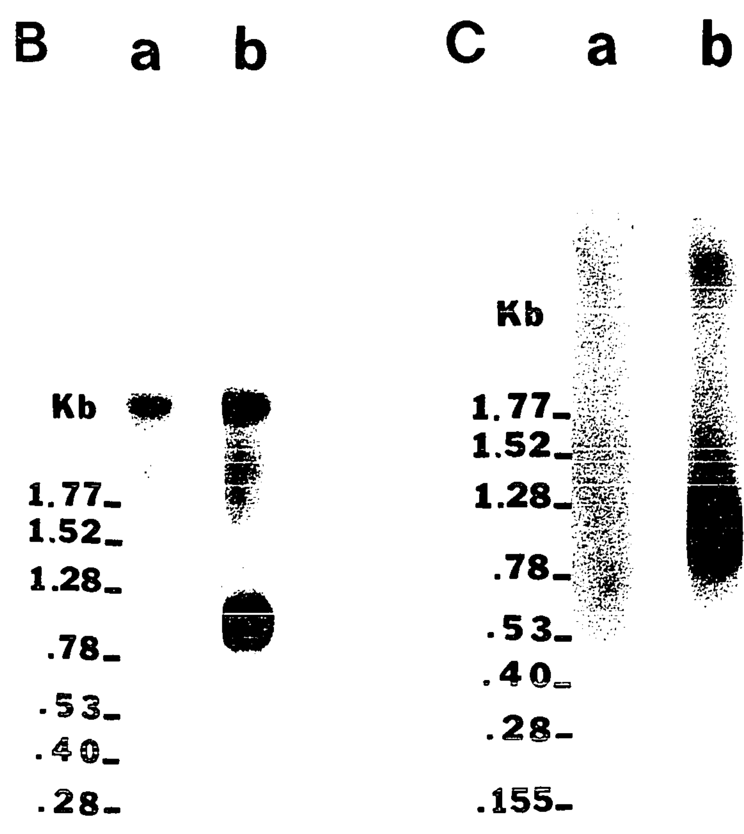
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Figure 3.3.

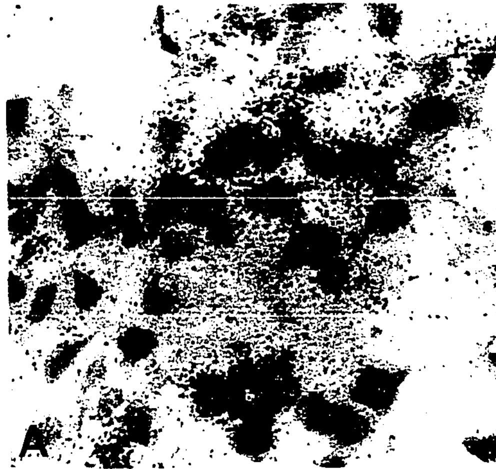
PRL mRNA localization in situ in the human anterior pituitary and decidua using an ^{35}S-labelled 48-mer synthetic oligonucleotide. The slides were exposed to Kodak NTB-2 liquid emulsion for autoradiography.

(A) Autoradiograph showing PRL mRNA localization in the anterior pituitary gland control.
Exposure time: 2 weeks. (x300).

(B) PRL mRNA in the parietal decidua cells.
Exposure time: 4 weeks. (x850).

(C) Low power of section showing both decidua cells (d) and the adjacent cytotrophoblast cells (cy) with PRL mRNA hybridization signals only over the decidua cells.
Exposure time: 4 weeks. (x300).

(D) Negative control. Decidua cells treated with rat vasopressin mRNA-sense probe.
Exposure time: 4 weeks. (x850).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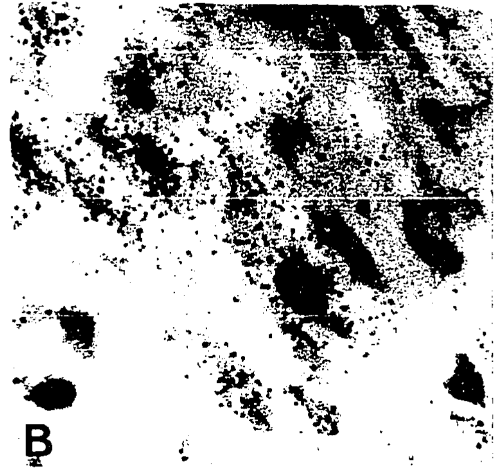
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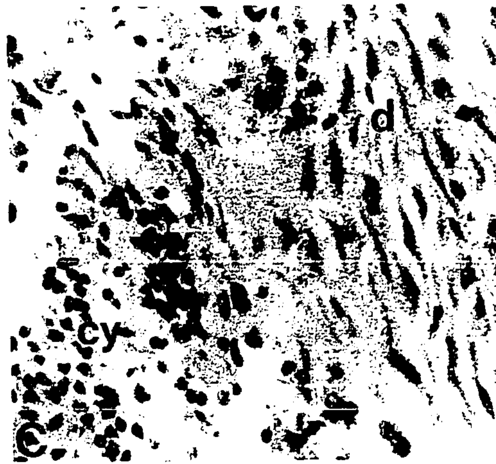
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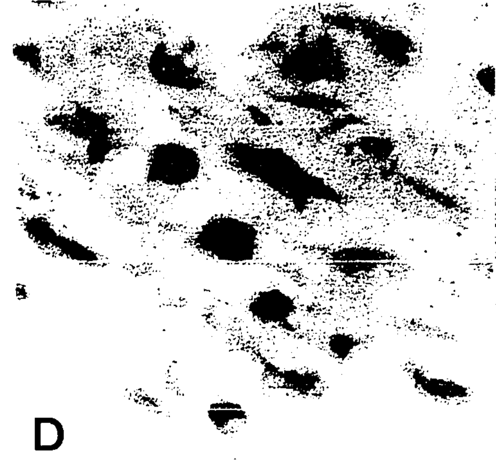
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Table 3.1

Sequences of 48-mer probes for hPRL and mVP

Gene	Amino acids	DNA probe sequence (48N)
Human Prolactin	60-75	5'- CAT-CTG-TTG-GGC-TTG-CTC-CTT-GTC TTC-GGG-GGT-GGC-AAG-GGA-AGA-AGT - 3'
Rat Vasopressin	Exon C 131 - 146	5'- GCT-GGG-ACA-CAA-GAG-TCC-GTG-GAT TCT-GCC-AAG-CCC-CGG-GTC-TAC-TGA - 3'

Table3.2

Densitometric analysis of Northern blots on poly(A)$^+$RNA (20ug) from intrauterine tissues (8g) and a 48-mer oligoprobe for hPRL. Conditions of high stringency were used throughout for hybridization. Only hybridization at the 1.2kb region was used for the calculation.

Tissues	Percentage of decidua hybridization signal	
Amnion	0	(6)*
Chorion laeve	36 ± 12	(4)
Decidua parietalis	100	(9)
Villous trophoblast	0	(8)
Placental basal plate	17 ± 10	(7)

*numbers in parenthesis are numbers of poly(A)$^+$RNA preparations.

Table3.3

Summary of results for the localization of hPRL and its mRNA in intrauterine tissues.

Tissue	Immuno-localization	Northern analysis	*In situ* hybridization
Amnion	±[1]	-	-
Chorion laeve	±[2]	+	-
Decidua parietalis	+++	++++	++
Placental basal plate	++	++	not examined
Placental trophoblast	+	-	-

[1]Polyclonal antiserum positive
[2]Monoclonal antibody positive

CHAPTER IV

IMMUNOCYTOCHEMICAL LOCALIZATION AND mRNA CONCENTRATIONS FOR HUMAN PLACENTAL LACTOGEN IN AMNION, CHORION, DECIDUA AND PLACENTA

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Running title: INTRAUTERINE HUMAN PLACENTAL LACTOGEN

Immunohistochemistry and Northern analyses showed the extravillous chorionic cytotrophoblast and the placental basal plate to be minor, additional sources of human placental lactogen; the major classical source being the syncytiotrophoblast.

Human placental lactogen (hPL) is one of the major hormones secreted by the placental syncytiotrophoblast and detected in the maternal circulation. Other sources of this hormone in intrauterine tissues at term, have been sought using immunohistochemistry and Northern analysis. Avidin-biotin immunoperoxidase staining with a specific polyclonal antibody to hPL showed this hormone to be present in groups of cells at the interface between chorionic cytotrophoblast and decidua parietalis and in some cells of the basal plate in addition to the classical source, the syncytiotrophoblast. Hybridization of poly(A)$^+$RNA extracted from amnion, chorion, decidua parietalis, basal plate and placental trophoblast with a radiolabelled 48 mer oligonucleotide and a 540 basepair cDNA probe to hPL showed the placental trophoblast to be the major source of hPL and the extravillous chorion and basal plate to be additional minor sources.

Key words: Human placental lactogen, placental trophoblast, chorionic cytotrophoblast, decidua, basal plate.

The syncytiotrophoblast surrounding the placental villi is the classical site of hPL production, as shown by immunohistochemical localization (1,2), mRNA isolation and Northern analysis (3) and *in situ* hybridization histochemistry (4). Although many studies have concentrated appropriately on the placental trophoblastic tissue as the source of systemic hPL, very few studies have investigated other intrauterine sources and routes of secretion.

A distinctive form of trophoblast in the fetal membranes designated "intermediate trophoblast" by Kurman and colleagues (5) was demonstrated in different intrauterine locations and shown by immunolocalization to contain a number of pregnancy specific proteins including hPL. This study used tissues from 12 days post-conception to term. More recently the extravillous trophoblast of the chorion laeve was shown to immunostain with antiserum to hPL between 6-22 weeks of gestation (6). Neither study demonstrated the synthesis of hPL by these tissues. In addition, little attention has been paid to hPL receptors in non-fetal intrauterine tissues. An interest in our laboratory is the decidua and fetal membranes as sources and targets of peptide hormones, nominally classified as systemic or endocrine hormones.

We have repeated the immunolocalization of hPL and sought evidence of synthesis by isolation of mRNA and Northern analysis in amnion and chorion of the fetal membranes, decidua parietalis, basal plate as well as in the classical site of production of the systemic hormone the placental trophoblast.

MATERIALS AND METHODS

Immunocytochemistry

Placentas with attached fetal membranes were collected from women after elective Cesarean section at term with no labor (n=5) at Kapiolani Medical Center for Women and Children (Honolulu, HI). The tissues were immediately taken on ice to the laboratory and representative slices (10mm thick) were cut across the placenta and basal plate and 1cm pieces of the fetal membranes with adherent decidua were rolled. The samples of tissue were immediately fixed in Bouin's solution (16-24h at 25C) and then processed for paraplast embedding. Seven-micron sections were cut and mounted on glass slides.

Deparaffinized and rehydrated sections were immunostained for hPL using the avidin-biotin immunoperoxidase method (7). The sections were pretreated with 0.3% hydrogen peroxide for 8min to remove endogenous peroxidase activity and with 0.5% normal goat serum in PBS for 20min to saturate nonspecific biding sites for immunoglobulin G. For the immunostaining, the sections were sequentially exposed to the following solutions at 25C: rabbit anti-hPL (1:5000) Lot # D-R-2-10, kindly provided by Dr. H. Friesen, University of Manitoba, Winnipeg, Canada, or normal rabbit serum (1:5000) (Sigma Chem. Co., MO) for 60min, 1:250 diluted biotinylated goat anti-rabbit immunoglobulin for 30min, and avidin-biotin horseradish peroxidase complex (Vectastain ABC reagent) for 45min. After each step, the sections were thoroughly rinsed in PBS for 9min (three changes, 3min each). Peroxidase activity was demonstrated cytochemically by incubation with peroxidase substrate

containing diaminobenzidine (DAB) 0.5mg/ml with 0.02% hydrogen peroxide in Tris buffer 0.01M pH 7.2. The sections were rinsed in water and counterstained with hematoxylin, coverslips were mounted with Permount (Allied Fisher Scientific, CA) and the slides examined by bright field microscopy. Method specificity was tested by substitution of the primary antiserum with normal rabbit serum. Antiserum specificity was demonstrated by preabsorption of the antiserum with highly purified hPL (1-2000ng/ml) kindly provided by Dr. A.F. Parlow (AFP-6561B) or highly purified human prolactin (1-2000ng/ml) a gift from the National Hormone and Pituitary Program NIDDK (NIDDK-hPRL-1-7). The antigen and antiserum were incubated for 48h at 4C before application to the sections.

Other materials used and their sources were: Vectastain ABC kit, Vector Laboratories, Inc. CA; 30% hydrogen peroxide, Allied Fisher Scientific, MO. The buffer PBS was 0.075M phosphate, 0.75M NaCl, pH 7.2, further diluted 1:5 just before use.

Northern analysis

Probes: A 48 mer probe was custom synthesized by Peninsula Labs., Inc. (Belmont, CA). The region corresponding to the amino-acid sequence 97-112 was chosen with a DNA probe sequence (48N) 5' - GTG ATA GTC ATC GCT GTC CGA GGT GTC ATA CAC CAG GTT GTT GGC GAA - 3'. This sequence minimized the homology with human prolactin (22.9%) but still shared 81% homology with human growth hormone (hGH). In addition, a cDNA probe of 540 base pairs corresponding to the codons for amino acid 60 to the end of the peptide and the contiguous 3' noncoding region of hPL was

kindly provided by Dr. I. Boime, University of Washington, St. Louis, Missouri.

Tissues: Human placentas with attached membranes were collected from women after elective term Cesarean section, without labor (n=15) and after normal spontaneous delivery (n=10) at Kapiolani Medical Center for Women and Children (Honolulu, HI). Tissues were processed for RNA extraction within 1h of delivery. The tissue samples (8g) were obtained from combined amnion-chorion-decidua or from each type of tissue by separating the amnion from chorio-decidua then scraping the decidual cells from the chorion laeve. In addition, fetal placental tissue was cut from the central region of the placenta by avoiding the basal and chorionic plates, basal plate tissue was also collected by careful slicing from the maternal surface of the placenta. It was necessary to pool tissues from several placentas in order to obtain 8g, for amnion and chorion 2 placentas were used, and for decidua or basal plate 4 placentas were used. Histological examination of the harvested chorion laeve and scraped decidua showed the latter to be homogeneous whereas the chorion laeve was always contaminated by some decidual cells even after vigorous scraping. Control tissue was obtained via the Pathology department, University of Hawaii, from the uterus (myometrium and endometrium) of a premenopausal patient undergoing hysterectomy for leiomyoma. Total RNA was isolated by extraction with guanidine thiocyanate-phenol-chloroform (8). Poly(A)$^+$RNA was isolated by affinity chromatography on oligo(dT) cellulose (9), denatured in 0.66M formaldehyde, 50% v/v formamide, and electrophoresed on 1.2% agarose gel containing 0.66M formaldehyde, RNA

ladder (Bethesda Research Laboratories, MD) and 18S, 28S rRNAs were used as molecular size markers.

^{32}P-labelling of probes and hybridization: The 48 mer oligoprobe was labelled with [alpha-^{32}P] dATP (3000 Ci/mmol; NEN) and terminal deoxynucleotidyl transferase, TDT, (Bethesda Res. Labs. MD). The reaction mixture (25 ul) with the addition of 30 units of TDT, was incubated at 37C for 5-10min, the reaction was stopped by the addition of 400 ul TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 7.6). The mixture was applied to a Sep-pak C_{18} cartridge and the labelled probe was eluted with methanol, and dried under a stream of nitrogen gas. Hybridization was carried out using the basic method of Conner et al (10) and Berent et al (11) with post-hybridization washes as described by Scheicher & Schuell (12). The nylon membranes were prehybridized for 6h at 55C in 6xSSPE (1xSSPE is NaCl 8.7g/l, $NaH_2PO_4.H_2O$ 1.4g/l, EDTA 0.37g/l, NaOH to pH 7.5), 10xDenhardts and 0.1% SDS, 250 ug/ml tRNA and salmon sperm DNA. The prehybridization buffer was removed and the hybridization solution plus the labelled probe were added. Hybridization was performed at 55C for 20h. The blots were washed in 6xSSPE/0.1% SDS three times at room temperature for 15min each, then in 6xSSPE/0.1% SDS at 55C for 15min. The final wash was 6 x SSPE without SDS at room temperature for 15min. The blots were exposed to Fuji XR film in a cassette with intensifying screens at -70C for 2h to one day as described in the figure. After autoradiography, the filters were washed by boiling in distilled water for 5min and stored for two months before reprobing with the hPL cDNA probe or other probes. No signs

of diminishing quality were noticed after reprobing the filters to 4 times.

The plasmid pBR322 containing 540 base pair cDNA probe to hPL was cut with restriction endonuclease Pst 1. The probe was random prime labelled using [alpha-^{32}P] dATP (>3000 Ci/mmol; NEN) and the random primer labelling system (Bethesda Research Laboratories, MD). Prehybridization and hybridization were performed at 42C. The hybridization solution contained 5% Dextran sulfate, 5xSSPE, 5xDenhardt's reagent, 50% formamide, 0.1% SDS, 100 ng/ml denatured ss DNA and the probe at $5x10^6$ counts/ml. After hybridization the filters were washed four times, twice in 6xSSPE, 0.1% SDS at room temperature for 15min each. once in 3xSSPE, 0.1% SDS at 50C for 30 min and finally in 1xSSPE for 15min at room temperature. The filters were exposed to X-ray film as described above.

A 27 mer human B-actin probe (Clonetech Labs, Inc.) was used as a control to assess the amount of poly(A)$^+$RNA spotted onto the filters. However, the B-actin probe could not be used as a standard between tissues since the amount of hybridization obtained with this probe varied from tissue to tissue: amnion (6.0 ± 1.1, n=4), chorion (9.9 ± 1.1, n=4), decidua (7.3 ± 1.4, n=6). placental basal plate (3.1 ± 0.9, n=8) and placental trophoblast (4.8 ± 0.8, n=7).

Densitometric analysis: Results were quantified by scanning densitometry using a Video Densitometer (BioRad, Inc., Model 620) with a 1-D Analyst Software for IBM-PC. Hybridization signal intensity was expressed as area under peak of intensity for the individual poly(A)$^+$RNA band. The hybridization signal obtained with the 48 mer

hPL probe and poly(A)$^+$RNA from the placental trophoblast was assigned a relative number of 100 and the signals obtained from hybridization of poly(A)$^+$RNA from the other tissues were expressed as a percentage of this.

RESULTS

Immunolocalization

Immunostaining was often present in groups of cells at the interface between the chorionic cytotrophoblast of the fetal membranes and the adhering decidua. These positively stained cells were present in 3 out of the 5 elective Cesarean section tissues examined (Fig. 4.1). It was difficult to tell from their position if these were true chorionic cytotrophoblast cells or decidua cells, the latter are often found embedded in the cytotrophoblast cell layer. The interface cells appeared identical to those described as "intermediate trophoblast" which was shown to have characteristic biochemical features and a diverse morphological expression (5). They did not stain when control rabbit serum at the same concentration as primary antiserum was used (Fig. 4.2).

The area where the placental basal plate joins the placental trophoblast is shown in Figs. 4.3-6. The syncytiotrophoblast surrounding the placental villi stained darkly, the classical site of hPL production. However, some trophoblast cells interspersed with the decidua-like cells of the placental basal plate also stained (Fig. 4.3). These cells were present in only 2 of the 5 tissues examined and

one of these specimens was from the same patient which showed positive staining of the chorionic cytotrophoblast cells.

When the antiserum to hPL was preabsorbed with highly purified hPL over a range 1-2000ng/ml, staining was appropriately decreased at 1000ng/ml in both the placental syncytiotrophoblast and the basal plate (Fig. 4.4). Staining was also decreased in the cells of the chorionic cytotrophoblast of the fetal membranes (not shown). When the antiserum to hPL was preabsorbed with highly purified human prolactin over the same concentration range, staining was not diminished at any site demonstrating the specificity of this antiserum. A section treated with antiserum preabsorbed with 1000ng/ml human prolactin is shown in Fig. 4.5. There was no staining in the placenta or basal plate when normal rabbit serum was used in place of the immune serum (Fig. 4.6).

Northern Analysis

Filters with total RNA, poly(A)$^+$RNA and poly(A)$^-$RNA (composed of rRNA and tRNA) from different tissues: amnion+chorion+decidua, amnion, chorion, decidua parietalis, placental villous trophoblast and placental basal plate were hybridized with either the 3′ end-labelled 48-mer oligoprobe or the random primed labelled hPL 540 bp cDNA probe.

The 48-mer oligoprobe hybridized strongly to poly(A)$^+$RNA from placental villous trophoblast (Fig. 4.7, lane 5). Total RNA from this tissue also gave a positive signal (Fig. 4.7, lane 7) which was stronger than that obtained from poly(A)$^+$RNA from amnion+chorion+ decidua (Fig. 4.7, lane 2) indicating the relative abundance of mRNA for hPL in placental trophoblast by comparison to the fetal membranes

and decidua. There was no signal with total RNA from amnion+chorion+decidua (Fig. 4.7, lane 4) on this filter because exposure to X-ray film was carried out for only 2h, a longer exposure may have resulted in a positive signal. Poly(A)$^{-}$RNA from the placental trophoblast and the amnion+chorion+decidua gave no positive signal (Fig. 4.7, lanes 6 and 3 respectively). When the same filter was rehybridized with the cDNA probe, an identical pattern of positive signals was observed with both poly(A)$^{+}$RNA and total RNA from placental trophoblast (Fig. 4.8, lanes 5 and 7) and with poly(A)$^{+}$RNA from amnion+chorion+decidua (Fig. 4.8, lane 2). Thus, the results from these two probes were the same and the positive signals obtained were at the 1.1 kilobase region. Results from Cesarean section tissues and normal vaginal delivery were similar in pattern and only that of Cesarean section tissues are shown in Fig. 4.9. The hybridization obtained with the 48 mer probe and the poly(A)$^{+}$RNA from the placental trophoblast gave the strongest signal (Fig. 4.9, lane 1), followed by that from the placental basal plate (Fig. 4.9, lane 2). Weaker signals were observed from the poly(A)$^{+}$RNA from the decidua parietalis and the chorion (Fig. 4.9, lanes 3 and 4 respectively). There was no positive hybridization signal obtained from the amnion or the uterus which served as a negative control (Fig. 4.9, lanes 5 and 6 respectively).

Comparative quantitation of the hPL mRNA levels in these intrauterine tissues by densitometric analysis using the signal from poly(A)$^{+}$RNA from the placental trophoblast of the Cesarean section tissues as a standard (100%) are shown in Table I. Comparisons were made, using Student's t test, between the levels of mRNA for hPL in

the placental tissues (trophoblast and basal plate) obtained after term elective Cesarean section and spontaneous vaginal delivery, there were no significant differences. However, there was significantly less mRNA for hPL in the decidua parietalis ($p<0.005$) and the chorion laeve ($p<0.005$) after normal vaginal delivery than in these tissues obtained prior to labor at elective Cesarean section. There were no significant differences in amounts of mRNA for hPL in the decidua parietalis versus the chorion laeve when these were compared in tissues collected under the same conditions.

COMMENT

Immunolocalization and Northern analysis show the abundant presence of hPL in the classical tissue of origin for the systemic hormone, the placental trophoblast. This is the first report however to identify unequivocally other sources of intrauterine synthesis of hPL and poses the questions of whether these sources are merely a minor contribution to the maternal plasma pool or whether the hPL synthesised is released and acts locally. The demonstration and distribution of hPL receptors in these tissues would help to clarify its function.

The avidin-biotin immunoperoxidase technique has been used in this study with an antiserum to hPL on tissue with high endogenous concentrations of human prolactin. We have demonstrated the specificity of the hPL immunolocalization by preabsorption of the antiserum to hPL and loss of immunostain by hPL but not by prolactin. In addition, the localization of hPL-positively stained cells in the fetal membranes and within the placental basal plate is different from that obtained with

highly specific monoclonal antibodies to human prolactin (Ali et al., in press).

The immunostaining results have been carried out in parallel with Northern analyses, first with a 48 mer oligoprobe to hPL and thence with a cDNA probe by using the same filters for hybridization. Since the hybridization and post-hybridization washes of the two probes were performed at moderate stringency, the possibility of cross-hybridization with mRNAs either for human prolactin or for placental growth hormone-variant (13) was eliminated.

The results using both techniques agree that the major intrauterine source of hPL is the villous trophoblast. In addition both techniques showed that the extravillous trophoblast of fetal origin and containing "intermediate trophoblast" contains populations of cells which express the hPL gene, confirming previous immunolocalization studies (5,6). However maternal decidua (14), which does not immunostain to hPL, when scraped from the chorion gave amounts of hPL mRNA by Northern analysis approximately equal to that shown for extravillous chorionic cytotrophoblast. It seems likely that, in the absence of the use of specific cell markers for the trophoblast cells in our decidual samples, the decidual mRNA for hPL was due to a contamination from "intermediate trophoblast". The problem is compounded by our inability to distinguish "intermediate trophoblast" and decidual cells in the scraped decidual samples. In previous work the presence of hPL in these cells demonstrated by immunocytochemistry has been used as a marker per se and justification for the term "intermediate trophoblast" for these cells (5). There was no evidence

of hPL production by the amnion by either Northern analysis or immunocytochemistry.

There were significantly lower levels of hPL mRNA in both the chorion and scraped decidua but not in the trophoblast after spontaneous vaginal delivery than in similar tissues collected after elective term Cesarean section. This suggests either a loss of cellular integrity during the course of labor and delivery in the chorion and decidua but not in the placental tissues, where no such difference was found, or that the relatively larger amounts of mRNA for hPL in the placental tissues obscured any small quantitative differences.

In summary, this study shows the placental syncytiotrophoblast is not the sole intrauterine source of hPL, although it is still unquestionably the major one. It is not yet known whether the extra-trophoblast sources express the gene for hPL throughout gestation, but from immunocytochemical studies carried out throughout gestation, it appears likely that they do (5,6). Technically it seems very difficult if not impossible to obtain decidual tissue free from extravillous trophoblast (intermediate trophoblast) cells. It is not known whether the hPL from the fetal membranes and basal plate merely contribute to the large maternal pool provided by the syncytiotrophoblast or whether they may have independent, local or paracrine functions.

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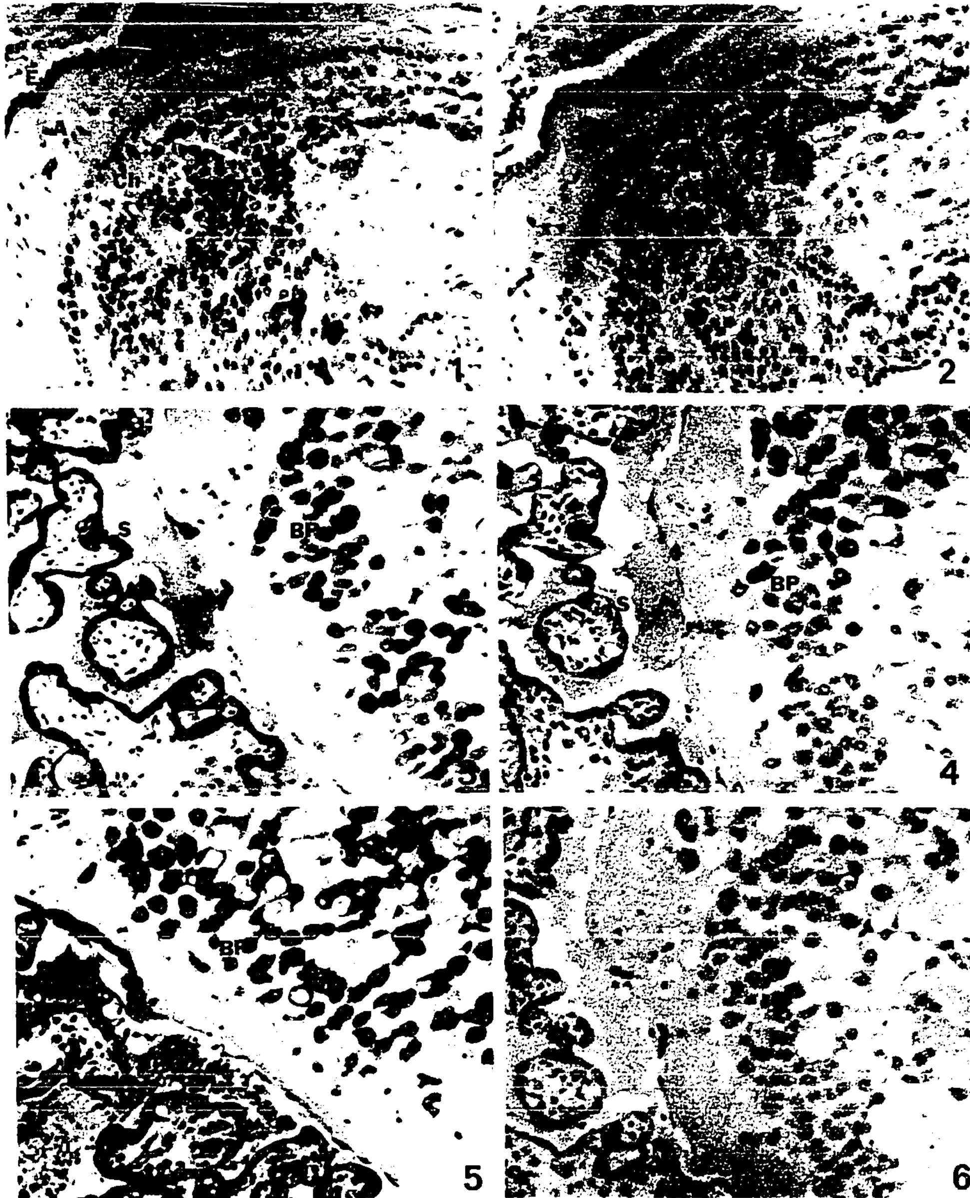
Figures 4.1-6.

Immunolocalization of hPL in the fetal membranes (4.1-2) and at the junction of the placental trophoblast and basal plate (4.3-6).

1. Immunostaining in cells (arrow) found at the interface between the chorionic cytotrophoblast (ch) of the chorion laeve and the decidua. There was no staining in the amniotic epithelium (e) or amnion (a).
2. Serial section stained with normal rabbit serum (control) at 1:500, the same concentration as primary antibody.
3. Dark staining for hPL in the syncytiotrophoblast (s) surrounding the placental villi. Some cells of the placental basal plate (bp) also stained positively for hPL.
4. Serial section stained with antiserum to hPL (1:5000) preabsorbed with 1000ng/ml hPL. Staining in the syncytiotrophoblast (s) and the basal plate cells (bp) was much reduced.
5. Serial section stained with antiserum to hPL (1:5000) preabsorbed with 1000ng/ml human prolactin. Note that staining was not diminished in either the syncytiotrophoblast (s) or the basal plate cells (bp) showing the specificity of the reaction with hPL.
6. Serial section stained with normal rabbit serum (control) at 1:5000, the same concentration as primary antibody.


E
A
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2
S
BP
S
BP
4
BP
5
6

Figure 4.7.

Northern analysis of RNAs from human amnion+chorion+ decidua and placental trophoblast from Cesarean section term placenta with a 48 mer oligoprobe to hPL.

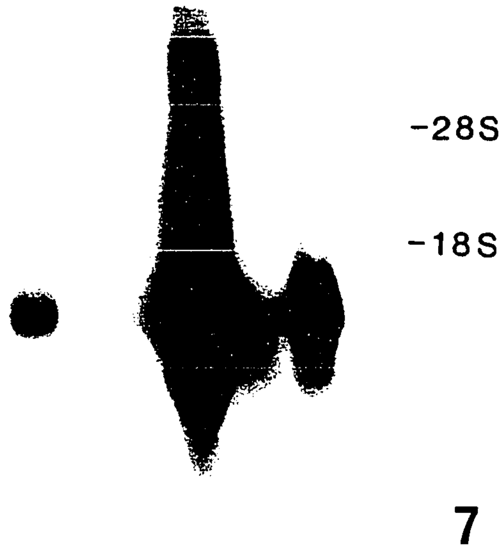
Amnion+chorion+decidua: poly(A)$^{+}$RNA (20ug, lane 2), poly(A)$^{-}$RNA (20ug, lane 3), total RNA (20ug, lane 4). Placental trophoblast: poly(A)$^{+}$RNA (20ug, lane 5), poly(A)$^{-}$RNA (20ug, lane 6), total RNA (20ug, lane 7). Hybridized with a hPL 48-mer oligoprobe 3'-end labelled with alpha ^{32}P. The filter was hybridized under moderate stringency conditions as described and exposed to Fuji RX film for 2h. Lane 1 and lane 8 were ribosomal markers.

Figure 4.8.

Northern analysis of RNAs from amnion+chorion+decidua and placental trophoblast with a cDNA probe to hPL.

The same filter as in figure 4.7 was hybridized with a random prime ^{32}P-labelled cDNA probe to hPL. The moderate stringency hybridization conditions were described. The film was exposed for 24h.

1 2 3 4 5 6 7 8



1 2 3 4 5 6 7 8

Figure 4.9.

Northern analysis of poly(A)$^+$RNA from separated amnion, chorion, decidua, basal plate and placental trophoblast from Cesarean section term tissues with a 48-mer oligoprobe to hPL.

Poly(A)$^+$RNA from placental trophoblast (4ug, lane 1), basal plate (4ug, lane 2), decidua (20ug, lane 3), chorion (20ug, lane 4), amnion (20ug, lane 5) and uterus (20ug, lane 6) hybridized with a 48-mer oligoprobe under moderate stringency conditions as described. The film was exposed for 6h.

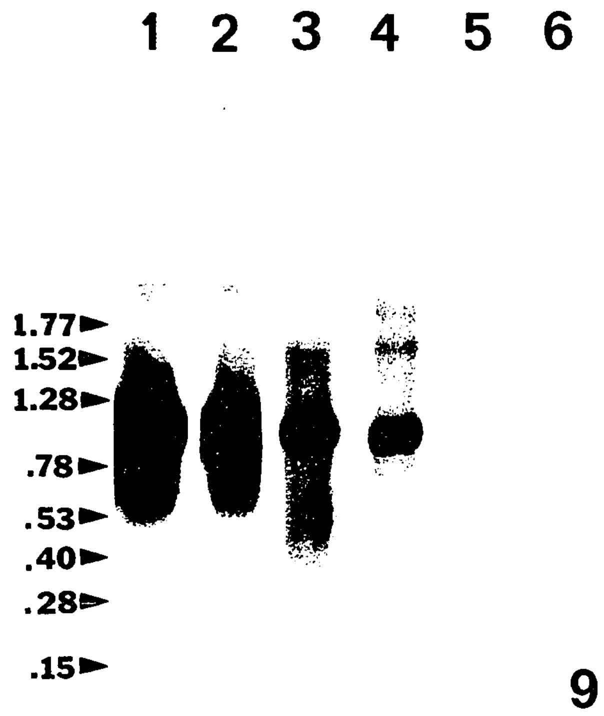

1
2
3
4
5
6
1.77
1.52
1.28
.78
.53
.40
.28
.15
9

Table 4.1

Comparative quantitation of human placental lactogen mRNA levels in intrauterine tissues obtained after Cesarean section and normal vaginal delivery. The signal from placental trophoblast was used as standard (100%).

Tissues	Cesarean section		Normal vaginal delivery	
Placental trophoblast	100.00	(n=8)	95.44 ± 21.89	(n=5)
Placental basal plate	58.80 ± 25.95	(n=5)	76.02 ± 12.01	(n=3)
Decidua parietalis	0.68 ± 0.08*	(n=3)	0.36 ± 0.04*	(n=4)
Chorion laeve	0.57 ± 0.05*	(n=3)	0.28 ± 0.04*	(n=4)
Amnion	0.00	(n=3)	0.00	(n=3)

Cesarean section versus normal vaginal delivery *$p<0.005$

CHAPTER V

HUMAN RELAXIN IN THE AMNION, CHORION, DECIDUA PARIETALIS, BASAL PLATE AND PLACENTAL TROPHOBLAST BY IMMUNOCYTOCHEMISTRY AND NORTHERN ANALYSIS

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Short title: Intrauterine Human Relaxin.

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ABSTRACT

Immunocytochemistry and Northern analysis were used to show that relaxin is a product of intrauterine human tissues of pregnancy. In addition, tissues from a patient without ovaries had similar results on both immunocytochemitry and Northern analysis as tissues from intact patients. The parietal decidua was clearly the major source of relaxin within the uterus and the relaxin mRNA (1.2 kilobases) from this tissue was detected with a 48-mer oligonucleotide probe designed to hybridize with both H1 and H2 relaxin gene transcripts. The mRNA isolated from the placental trophoblast was slightly smaller (1.1 kilobases), and the placental basal plate which has both maternal and fetal cells contained relaxin mRNAs of both sizes. Two monoclonal antibodies (Mabs) raised to synthetic human relaxin (H2) gave different patterns of localization in the fetal membranes, decidua and placenta. One Mab (RLX8) stained the chorionic cytotrophoblast in the fetal membranes and all of the cells in the placental basal plate. The other Mab (RLX6) stained the chorionic cytotrophoblast in some instances and selectively stained the decidua-like cells of the placental syncytiotrophoblast whereas Mab RLX8 failed to detect this relaxin. Tissues obtained after spontanious labor and delivery contained significantly less relaxin mRNA than tissues obtained at elective cesarean section without labor, but their hormone contents, as judged by immunocytochemistry, were not different.

We conclude that the relaxin gene (H2) is expressed in intrauterine tissues, but that expression and hormone synthesis are not ubiquitous. Whether the relaxin gene H1 is expressed has not been determined.

INTRODUCTION

The human genome has two genes for relaxin (H1 and H2), both situated on chromosome 9 (1). The available data suggest that these genes code for polypeptide that are significantly different in amino acid sequence and that transcription appears to be limited to the H2 gene in the corpus luteum of pregnancy (2) and in human seminal fluid (3). In addition to these studies, the isolation and detection by bioassay and RIA in human decidua and placenta suggest that these tissues are additional sources of relaxin (4-6). The coexistence of systemic and local relaxins in the pregnant woman from the corpus luteum and within the intrauterine compartments, respectively, has implications for the control of pregnancy and parturition.

Immunocytochemical localization with antisera to porcine relaxin showed positive staining in the cells of the parietal decidua and the chorionic cytotrophoblast of the fetal membranes as well as in the cells of the placental basal plate (7). An antiserum to a synthetic 14 amino acid segment of the connecting peptide of human relaxin was used to distinguish production from sequestered hormone (8), a similar immunolocalization pattern was found within the fetal membranes, decidua parietalis and basal plate. Despite this evidence, the concept of decidual relaxins is not generally accepted (9).

The consistency of immunolocalization of relaxin in the placenta is poor. Yki-Jarvinen and colleagues (10) used an anti-porcine relaxin (R6) and showed the syncytiotrophoblast to be consistently immunostained. We used a number of different anti-porcine relaxin sera including the R6, and found immunostaining in the syncytiotrophoblast

of only 2 out of 20 specimens (7). The availability of homologous monoclonal antibodies Mabs) to epitopes of human relaxin (H2) allowed us to reexamine the localization patterns of relaxin in these maternal and fetal tissues. We have isolated poly$(A)^+$RNA from amnion, chorion, decidua parietalis, basal plate and placental trophoblast and used these for Northern analyses with a specific 48-mer oligonucleotide probe to human relaxins.

MATERIALS AND METHODS

Immunocytochemistry: antiserum, antigen and tissues

Eight Mabs prepared against synthetic human relaxin H2 were gifts from Genentech Inc (San Francisco, CA). These antibodies are of the immunoglobulin G_1 (IgG_1) isotype and were purified on protein A-Sepharose and stored in sterile phosphate-buffered salin (PBS). Each was tested over a range of dilutions on amnion, chorion, decidual rolls and on placenta. The two (RLX6 #6451-60 and RLX8 #24-6956) selected for further study showed the greatest differences in immunolocalization patterns in these tissues. A Mab to human immunodeficiency virus (HIV; 10F6 #8974-44) prepared in the identical manner as the relaxin Mabs was used at the appropriate concentrations as a control to both relaxin Mabs.

Placentas with attached fetal membranes were collected from women after elective Cesarean section at term who had not undergone labor (n=5) and from women after normal vaginal delivery at term (n=3) from Kapiolani Medical Center for Women and Children (Honolulu, HI). Another

full term normal vaginal delivered placenta was kindly obtained by Dr. G. Letterie, Tripler Army Medical Center (Honolulu, HI), from a patient who underwent oophorectomy at 11 weeks of pregnancy. The tissues were immediately taken to the laboratory on ice and processed within 1h after removal. Representative slices (1 cm thick) were cut across the placenta and basal plate, and 1-cm pieces of the fetal membranes with adherent decidua were rolled. The sampled tissues were immediately fixed in Bouin's solution (16-24h at 25C) and then processed for paraplast embedding. Seven-micron sections were cut and mounted on glass slides.

Immunocytochemical Staining

Deparaffinized and rehydrated sections were immunostained for human relaxin using the avidin-biotin immunoperoxidase method (11). The sections were pretreated with 0.3% hydrogen peroxide for 10min to remove endogenous peroxidase activity and with 0.5% normal horse serum in PBS for 20min to saturate nonspecific binding sites for IgG. For the immunostaining, the sections were sequentially exposed to the following solutions at room temperature except where specified: Mab against human relaxin (100ug/ml for RLX6 or 40ug/ml for RLX8) or the control Mab at the same concentration as relaxin Mab for 18h at 4C, 1:250 diluted biotinylated horse anti-mouse immunoglobulin (1:250) for 30min, and avidin-biotin horseradish peroxidase complex (Vectastain ABC reagent) for 45min. After each step, the sections were thoroughly rinsed in PBS for 9min (three changes, 3min each). Peroxidase activity was demonstrated cytochemically by incubation with a peroxidase substrate containing diaminobenzidine (DAB) 0.5mg/ml with 0.01% hydrogen peroxide

in Tris buffer 0.1M, pH 7.2. The sections were rinsed in water and counterstained with hematoxylin, coverslips were mounted with Pro-Texx (Baxter Scientific, Honolulu,HI) and the slides were examined by bright field microscopy. Method specificity was tested by substitution of the Mab to human relaxin with Mab to HIV.

Isolation of mRNA

Human placentas with attached membranes were obtained from patients after elective term Cesarean section, without labor (n=20) and after normal spontaneous delivery (n=14), at Kapiolani Medical Center (Honolulu, HI) and were brought to the laboratory on ice. Tissues were processed for the extraction of RNA within 1h of delivery. Each tissue type (8g) was obtained by separating the amnion from chorio-decidua and then scraping the parietal decidual cells from the chorion laeve. The basal plate was sliced carefully from the placental maternal surface and the placental trophoblast was cut from the central region of the placentas, thereby avoiding the basal and chorionic plates. It was necessary to pool tissues from several placentas in order to obtain enough tissue, for amnion or chorion 2 placentas were used, and for decidua or basal plate 4 placentas were pooled. Histological examination of the harvested chorion laeve and decidua showed the latter to be less contaminated by chorionic cytotrophoblast cells than the chorion was contaminated by decidual cells. In addition mRNA was extracted from combined amnion+chorion+decidua and from placental trophoblast obtained from the patient who had undergone ovariectomy at 11 weeks of gestation and delivered spontaneously at full term. Control tissue was kindly provided by the Pathology Department, University of

Hawaii, from the uterus (myometrium and endometrium) of a premenopausal patient who had a hysterectomy for leiomyoma. This tissue was used as a control because of its availability at the time of this study. The tissue was homogenized in guanidinium thiocyanate-phenol-chloroform and total RNA was prepared by the method of Chomczynsky and Sacchi (12). Poly(A)$^+$RNA was isolated by affinity chromatography on oligo(dT) cellulose (13). Poly(A)$^+$RNA fractions were stored at -80C in 75% ethanol until use.

Northern analysis

The poly(A)$^+$RNA (20ug) from different tissues was denatured in 0.66M formaldehyde, 50% (vol/vol) formamide, and electrophoresed on 1.2% agarose gel containing 0.66M formaldehyde. An RNA ladder (Bethesda Research Laboratories, Gaithesburg, MD) was used as a molecular size marker. The gel was stained (in the dark) with 0.5ug/ml ethidium bromide for 30min and photographed under UV light to insure the quality and quantity of poly(A)$^+$RNA loaded in each lane. Poly(A)$^+$RNA was transferred from agarose gels to Schleicher & Schuell's Nytran nylon membranes (0.45um) in 10xSSPE (1x is NaCl 8.7g/l, $NaH_2PO_4.H_2O$ 1.4g/l, EDTA 0.37g/l, NaOH to pH 7.5) by capillary transfer overnight. The membranes were then rinsed in 5xSSPE and baked at 80C for 1h in a vacuum oven and the blots were then stored dessicated at 4C until use.

A 48-mer oligonucleotide with perfect homology to human relaxin genes H1 and H2 (2, 14) was kindly made by Drs. W. Scott Young III and Michael J. Brownstein, National Institute of Mental Health, (Bethesda, MD). The probe was made by solid phase synthesis on a DNA synthesizer (Applied Biosystems, Foster City, CA) and purified on 8% polyacrylamide

and 8M urea preparative sequencing gel. The sequence of the oligoprobe was from a region of the B-chain (amino acids 11 through 26) where both human relaxin genes H1 and H2 are identical: 5' - GGT GCT CAT GCC GCA AAT GGC AAT CTG CGC GCG AAC TAA TTC GCG GCC - 3'. This was labelled with [alpha-^{32}P]dATP (>3000 Ci/mmol, New England Nuclear, Boston, MA) and terminal deoxynucleotidyl transferase (Bethesda Research Laboratories). Hybridization was carried out using a modification that paralleled the methods described by Conner et al. (15) and Berent et al. (16) with post-hybridization washes as described by Schleicher & Schuell (17). The Nytran membranes were prehybridized for 4-6 h at 55C in 6xSSPE, 10xDenhardt's (1xDenhardt's is 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, and 0.02% bovine serum albumin), 0.1% SDS, 250ug/ml tRNA and salmon sperm DNA. After 4-6h, the prehybridization solution was replaced by the hybridization solution which contained 6xSSPE, 5xDenhardts, 0.1% SDS and 250ug/ml yeast tRNA and salmon sperm DNA. The ^{32}P-labeled oligonucleotide probe was used for hybridization which was performed at 55C for 20h. The blots were washed in 6xSSPE/0.1% SDS three times at room temperature for 15 min, and then in 6xSSPE/0.1% SDS at 60C for 15min. The final wash was in 6xSSPE without SDS at room temperature for 15min. The blots were sealed in plastic bags while still wet and exposed to Fuji XR film in cassettes with intensifying screens at -70C for the times indicated in the figures. The filters were washed by boiling in distilled water for 5min and stored before reprobing with other probes. No signs of diminishing quality were noticed after reprobing the filters 3 to 4 times.

A 27 mer human B-actin probe (Clonetech Labs,Inc., Palo Alto, CA) was used as a control to assess the amount of poly(A)$^+$RNA spotted onto the filters. However, this could not be used as a standard between tissues, since the amount of hybridization obtained varied from tissue to tissue: amnion (6.0 ± 1.1, n=4), chorion (9.9 ± 1.1, n=4), decidua (7.3 ± 1.4, n=6), basal plate (3.1 ± 0.9, n=8) and placental trophoblast (4.8 ± 0.8, n=7).

Densitometric analysis

Results were quantified by scanning densitometry using a Video Densitometer (model 620, BioRad, Inc., Richmond, CA) with a 1-D Analyst Software for IBM-PC. Hybridization signal intensity was expressed as area under peak of intensity for individual poly(A)$^+$RNA band. The hybridization signal obtained with the 48 mer human relaxin probe and poly(A)$^+$RNA of decidua parietalis from Cesarean section specimens was assigned a relative number of 100 and the signals obtained from hybridization of poly(A)$^+$RNA from other tissues were expressed as a percentage of this.

RESULTS

Immunocytochemistry

There was considerable variation in the degree of immunostaining with the two Mab RLX6 and RLX8 in the tissues studied, not unlike the degree shown by different polyclonal antisera (7, 10). The RLX8 (Fig. 5.1A) tended to stain the extravillous cytotrophoblast in the fetal membranes with greater intensity than the RLX6 Mab (Fig. 5.1B). The parietal decidua cells adhering to the chorion showed about the same

degree of cytoplasmic stain (Fig. 5.1A,B). The control Mab showed no staining in any cell type of the fetal membranes or decidua (Fig. 5.1C). The low level of staining in the cytotrophoblast in the fetal membranes with Mab RLX6 can be seen more clearly at higher magnification (Fig. 5.1D) and shows the infiltration into this cell layer of decidual cells which stained positively.

The tissues from the patient with no ovaries are shown in Fig 5.1E and 5.1F stained with Mab RLX6. In this specimen, obtained after spontaneous labour and delivery at term, the amniotic epithelium and chorionic macrophages in the extracellular matrix stained. In addition, the cytotrophoblast of the chorion stained more heavily than that shown in Fig. 5.1B, stained with the same Mab. Patches of syncytiotrophoblast of placenta stained in this patient with Mab RLX6 (Fig. 5.1F) but failed to stain with Mab RLX8 (not shown).

The results on the placental tissues with the two Mab showed more clearcut differences than in the cytotrophoblast of the fetal membranes. RLX6 localized to certain areas of the syncytiotrophoblast whereas RLX8 failed to stain these areas. The same region of a placenta obtained after spontaneous labor and delivery is shown in serial sections with the two Mabs in Fig. 5.1G and 5.1H respectively. The RLX8 Mab failed to stain any region of the placental syncytiotrophoblast from tissues obtained after cesarean section. whereas RLX6 detected relaxin in patches of syncytiotrophoblast in three of these tissues. The RLX6 also detected relaxin positively in all four of the placentas obtained after spontaneous labor and delivery whereas RLX8 stained only marginally in two of the four.

The Mab RLX8 which localized relaxin more than RLX6 Mab in the extravillous trophoblast of the fetal membranes, also localized to more of the cells in the placental basal plate (not shown). RLX6 however was more selective in the cells it stained in the basal plate as shown in Fig. 5.1I.

Northern analysis

Northern blots of the Cesarean section tissues are shown in figure 5.2. The 48-mer oligoprobe hybridized to poly(A)$^+$RNA from the decidua parietalis (lane C), the placental trophoblast (lane D), the basal plate (lane E) and the chorion laeve (lane B). There was no positive hybridization signal obtained from the poly(A)$^+$RNA from the amnion (lane A) or from the uterus which served as negative control (lane F). The positive signals were located at the 1.2 kilobase (kb) region for the decidua, chorion laeve and basal plate and at the 1.1kb region for the placental trophoblast. The poly(A)$^+$RNA from the pooled amnion, chorion and decidua and from the placental trophoblast of the patient without ovaries gave hybridization signals with intensities comparable to those from normal spontaneous delivery tissue (not shown).

Densitometric scanning of the data obtained from Northern analyses of poly(A)$^+$RNA from human intrauterine tissues (cesarean section and normal spontaneous delivery) with the 48-mer oligoprobe is shown in Figure 5.3. The signals were measured at the 1.2kb region of all tissues except the placental trophoblast, which was measured at 1.1kb. By using Student's t-test, the results showed that there was significantly less mRNA for human relaxin in tissues obtained after

normal vaginal delivery than in tissues obtained from cesarean section delivery before labor: the decidua parietalis ($n=4$: $P<0.005$), the chorion laeve ($n=4$: $P<0.01$), the basal plate ($n=3$: $P<0.005$) and the placental trophoblast ($n=3$: $P<0.01$). In both cesarean section tissues and those obtained after spontaneous labor and delivery, the amount of relaxin mRNA decreased in the order decidua > placental trophoblast > placental basal plate > chorion > amnion. A summary of the immunostaining results with the two Mabs and Northern analyses is given in Table 5.1.

To check the specificity of the 48-mer oligoprobe, filters were rehybridized and washes were performed in tetra-methylammonium chloride, under conditions of high stringency at 75C (18). Positive hybridization signals were still observed, although they were less intense than when moderate stringent conditions was used. Northern blots under condition of high stringency are shown with poly(A)$^+$RNA of the decidua parietalis (Fig. 5.3, lane A) and the placental trophoblast (Fig. 5.3, lane B); they are of lower intensity but of the same size (1.2 and 1.1 kb, respectively).

DISCUSSION

The results demonstrate the value of concurrent Northern analyses of separated intrauterine tissues in interpreting immunocytochemical staining. For relaxin, the latter has employed antibodies that are heterologous, and polyclonal or homologous, and monoclonal and selected by criteria other than the detection of endogenous cellular or membrane-bound relaxins. The results here with Mabs to synthetic human

relaxin H2 are clearly similar to those previously obtained with polyclonal antisera to porcine relaxin (7, 10) and with an antiserum to a synthetic portion of human relaxin C-peptide (8). All antibodies stain the cells of the decidua parietalis. This maternal tissue yielded the highest concentrations of relaxin mRNA from intrauterine tissues of pregnant women at term.

Fetal tissues gave anomalous results; the chorion, including the extra villous trophoblast of the fetal membranes, gave Northern blots consistent with low levels of relxin message. This may reflect the thechnical difficulty of obtaining this tissue entirely free of contamination by mRNA-rich decidual cells. Hence, the immunostaining with one or sometimes the other Mab suggests that receptor-bound relaxins, rather than synthesized relaxins, are being detected by what may be configurational antibodies. We have evidence that this tissue is indeed contain receptors for relaxin (19). Similarly, staining of the amniotic epithelium occured occasionally, and the Northern analyses showed no relaxin mRNA, suggesting that these Mabs may be detecting receptor-bound relaxin from a decidual source.

Staining and Northern analysis of the fetal villous trophoblast show relaxin mRNA and the translated hormone by one Mab, similar to the staining in the syncytiotrophoblast shown by some polyclonal antisera to procine relaxin (10). Interestingly, the mRNA was approximately 100 basepairs smaller than that isolated from maternal decidua. This suggests either a difference in the length of poly(A) tail or an alternative splicing mechanism for relaxin mRNA in the placenta (20).

The placental basalplate contains maternal decidual-like cells juxtaposed to fetalcells. It was not surprising, therefore, to find that the mRNA from this region hybridized at both the 1.1 and 1.2 kb regions, consistent with the notion of a mixed maternal/fetal tissue. Immunostaining showed the decidua-like cells to always be positive, whereas other cell types additionally stained with one Mab, indicating recptor-bound relaxin. The Mab produce to the same synthetic antigen and selected by enzyme-linked immunosorbent assay based on this standard in vitro evidentlydiffer in epitope specificity. If these are predominantly configurational, it might be expected that different Mabs may detect, or not detect, relaxin bound to its receptor or detect intracellular relaxin, or not, at different stages of its route from synthesis to secretion. Recent studies of Mabs to human GH showed the majority to be sensitive to the folded structure of human GH (21).

The results from the immunolocalization of relaxin in conjunction with the detection of its mRNA showed unequivocally that both maternal (decidua) and fetal (trophoblast) tissues express a human relaxin gene(s). This is the first observation that relaxin is made/produced by intrauterine tissues (Ali, S. M., unpublished observations). The use of tissues from a patient without ovaries provided further evidence of production in the intrauterine compartments, rather than sequestration from an ovarian source. The loss of relaxin mRNA in tissues obtained after spontanious labor and delivery versus those obtained at term elective cesarean section suggests either a parturition-related specific decline of transcription, nonspecific cell damage, or death after labor. In this study using fixed tissues we were unable to detect

any differences in the intensity of staining; such differences were apparent when frozen sections were used in a previous study (22).

We specifically designed our oligonucleotide probe to detect transcripts from both human genes H1 and H2 (2), and the Mabs were raised to the H2 sequence. These Mabs might cross-react with an H1 gene product, but H1 gene expression has yet to be demonstrated.

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Figures 5.1.

Immunolocalization of human relaxin in the fetal membranes, decidua and placenta with Mabs to human relaxin. Sections through the fetal membranes A-C and E show the amniotic epithelium (e), chorionic cytotrophoblast (cy) and decidua (d). Sections of the placenta F-H show the syncytiotrophoblast.

A. Stained with Mab RLX8 (40ug/ml). Note the staining in the extravillous cytotrophoblast cells and the decidua. X200

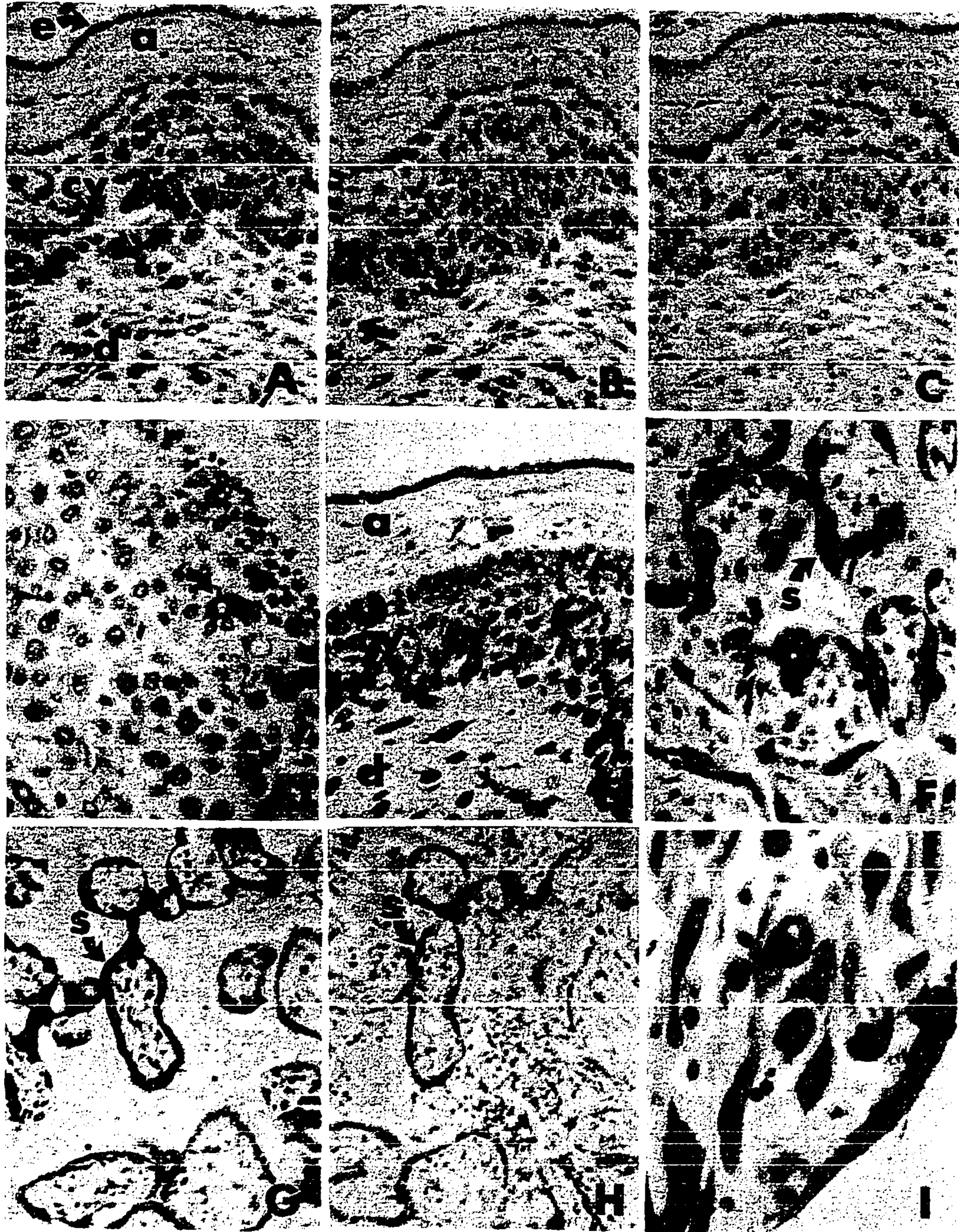
B. Serial section stained with Mab RLX6 (100ug/ml). Note less staining with this antibody in the cytotrophoblast cells. The staining with this antibody in the decidual cells is the same as with Mab RLX8. X200

C. Serial section stained with Mab to HIV (100ug/ml) as a control. X200

D. The chorionic cytotrophoblast stained with Mab RLX6 clearly shows only light staining of these cells which are infiltrated by the decidua cells (arrow). X336

E. Fetal membranes and decidua of a patient after spontaneous labour and delivery at term without ovaries. The amniotic epithelium and macrophages in the chorionic extracellular matrix stained more darkly in this specimen. Although this was stained more with Mab RLX6 the cytotrophoblast cells stained more than in 1B. X200

F. The placenta of the same patient without ovaries showing patchy syncytiotrophoblast staining with Mab RLX6. X200


e
a
sy
d
A
B
C
a
d
s
F
s
G
H
I

G. Stained with Mab RLX6, this placenta from a spontaneous labour and delivery shows dark syncytiotrophoblast stainig in patches. X200

H. Serial section stained with Mab RLX8 shows almost complete absence of staining in the same areas of syncytiotrophoblast stained in 1G. X200

I. The basal plate region of one placenta stained with Mab RLX6 shows selectively of stain to different cells in this region. Mab RLX8 (not shown) stained equally all cells in the basal plate. X400

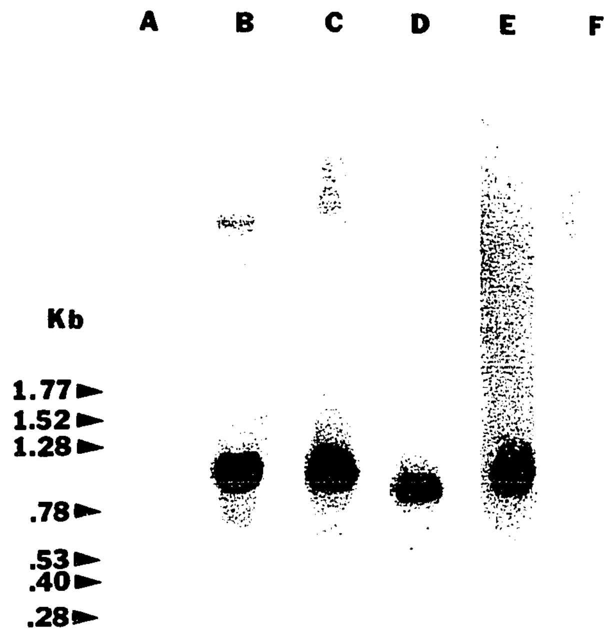
All sections were counterstained with hematoxylin.

Figure 5.2.

Northern analyses of poly(A)$^+$RNA from human amnion, chorion, decidua parietalis, placental trophoblast and basal plate obtained at term elective cesarean section without labor. Poly(A)$^+$RNA (20ug) from human amnion (lane A), chorion (lane B), decidua parietalis (lane C), placental trophoblast (lane D), basal plate (lane E) and uterus (lane F) hybridized with a human relaxin 48-mer oligonucleotide probe 3'-end labbelled with alpha ^{32}P. The filter was hybridized under moderate stringincy conditions as described in Materials and Methods and was exposed to Fuji RX film for 5 days at -20C.

Figure 5.3.

Poly(A)+RNA (20ug) from decidua parietalis (lane A) and placental trophoblast (lane B) hybridized with the human relaxin 48-mer oligonucleotide as in Figure 5.2A except that washes were carried out with addition of tetramethylammonium chloride under conditions of high stringincy and exposed for 7 days at -20C.


A
B
C
D
E
F
Kb
1.77
1.52
1.28
.78
.53
.40
.28
2

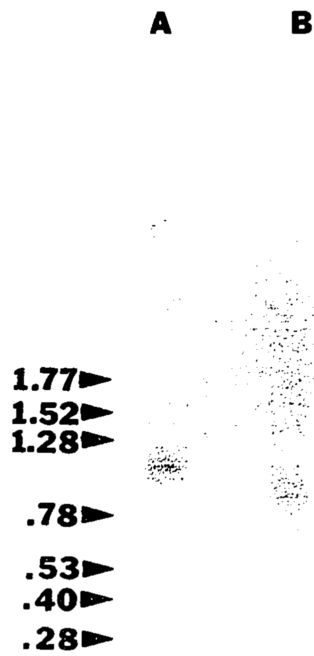

A
B
1.77
1.52
1.28
.78
.53
.40
.28
3

Figure 5.4.

Comparative quantitation of relaxin mRNA levels in human intrauterine tissues obtained from term elective Cesarean section ( ) and normal vaginal delivery ( ). After autoradiography, positive signals were evaluated by densitometry using a BioRad Model 620 with software for IBM-PC. The bars represent the mean ± SD obtained from three or four separate Northern analyses. Bar graph data are expressed in relation to the strongest signal obtained from Cesarean decidua parietalis which was assigned as 100. A: amnion, C: chorion, D: decidua parietalis, PT: placental trophoblast and BP: basal plate.

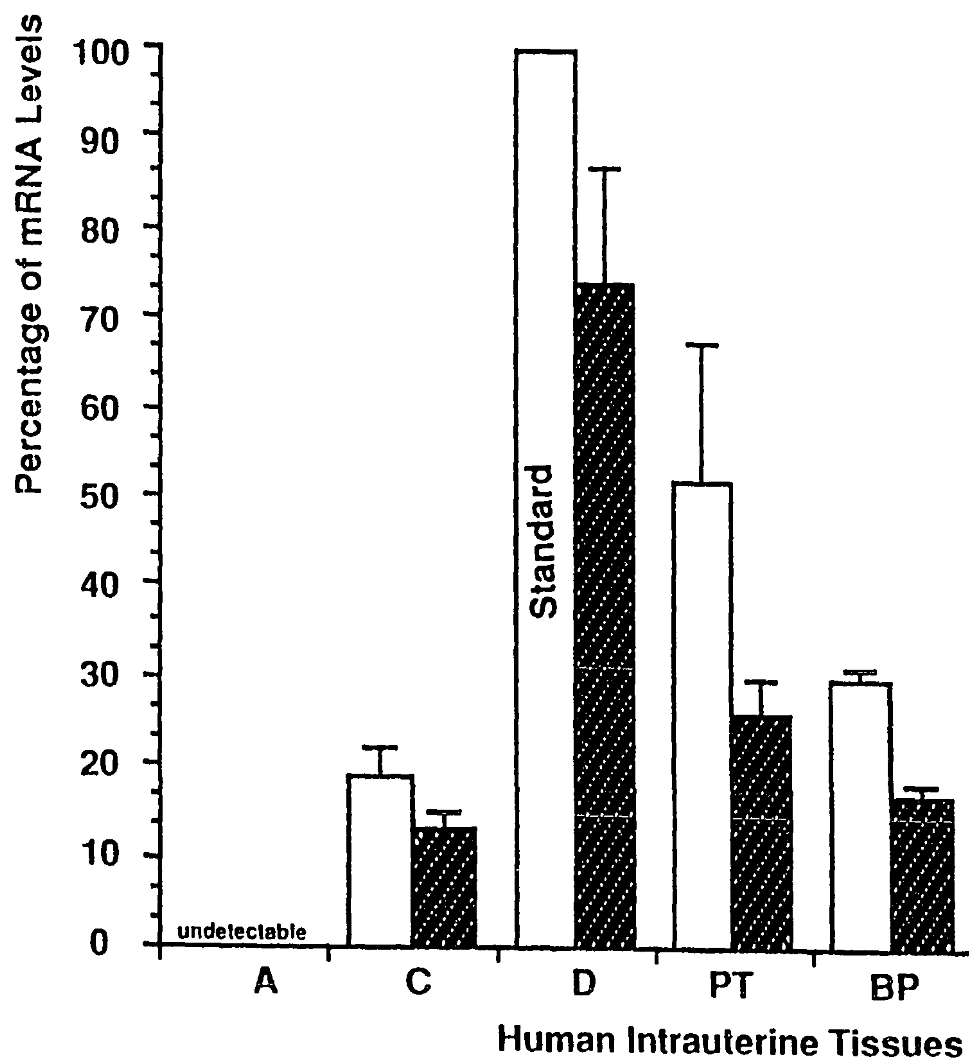

Percentage of mRNA Levels
100
90
80
70
60
50
40
30
20
10
0
undetectable
Standard
A
C
D
PT
BP
Human Intrauterine Tissues

Table 5.1

Summary of relaxin immunostaining and Northern analyses in human intrauterine tissues

TISSUE	NORTHERN ANALYSIS	IMMUNOCYTOCHEMISTRY Mab. RLX6	Mab. RLX8
Amnion	-	±	±
Chorion (cytotrophoblast)	+[1]	+	++
Decidua parietalis	++++[1]	+++	+++
Placental basal plate	++[1,2]	+	++
Placental trophoblast	+++[2]	++	-

[1] 1.2kb
[2] 1.1kb

CHAPTER VI

POSTSCRIPT

1. Summary

The production of three hormones, human relaxin, prolactin and placental lactogen, were sought in five intrauterine tissue types by immunocytochemistry and Northern analysis. The results are summarized in a summary table and the significant findings of this dissertation identified:

* For almost a decade, the presence of relaxin in the placenta and the basal plate has been shown by RIA (Bigazzi et al., 1980; Fields and Larkin, 1981; Yamamoto et al., 1981 and Schmidt et al., 1984). Immunocytochemistry using porcine relaxin as primary antibody localized relaxin in cells of the decidua parietalis and basalis (Yki-Jarvinen et al., 1983; Koay et al., 1985). Our data here show for the first time the presence of mRNA for relaxin in the decidua parietalis, chorion, basal plate and placental trophoblast, a valuable correlate of the immunocytochemical results.

* There are two species of mRNA for relaxin. The 1.2kb species is expressed in the decidua parietalis, chorion and basal plate whereas the 1.1 species is expressed only in the placental trophoblast.

* Relaxin mRNA concentrations are diminished after labour suggesting its gene expression is reduced or turned off during spontaneous labour and delivery.

* The chorion and the basal plate are additional sources for hPRL in the amniotic fluid but the placenta is not. Although the placental

Table 6.1

Production of human relaxin, prolactin and placental lactogen by intrauterine tissues

Tissue	RELAXIN		PROLACTIN		PLACENTAL LACTOGEN	
	I[a]	N[b]	I	N	I	N
Amnion	-	-	±	-	-	-
Chorion	+	+	±	+	+?	+
Decidua parietalis	++	+++	+++	+++	+?	+
Basal plate	+	++	++	++	+++	+++
Placental trophoblast	+	++	++	-	+++++	+++++

[a]Immunocytochemistry
[b]Northern analysis

trophoblast is not a site for hPRL synthesis as judged by immunocytochemistry, the positive signal at 0.9kb of poly(A)$^+$RNA with the 48-mer oligoprobe under moderately stringent conditions suggests that human placenta may express an incomplete mRNA for hPRL or a closely related mRNA to hPRL. Recently, prolactin-related genes have been shown to be expressed in the placenta of rodents (Ducworth et al., 1986) and cows (Schuler and Hurley, 1987).

* Extravillous chorionic cytotrophoblast and the placental basal plate are minor additional sources of hPL.

2. Conclusions and Caveats

The hypothesis developed by our laboratory is that relaxin is a paracrine/autocrine hormone in the human decidua and fetal membranes.

To establish this local role of relaxin, three criteria must be fulfilled: demonstration of local production in the tissues, detection of specific receptors and demonstration of biological effect(s) attributable to this hormone. Our data presented here clearly satisfy the first criterion. It has been previously shown that relaxin affects the secretion of plasminogen activator and collagenolytic enzyme activities of the amnion-chorion and thus relaxin may have a major role in connective tissue remodelling within the fetal membranes (Koay et al., 1986). However, little is known about the control of secretion of intrauterine relaxin. The presence of the relaxin receptor has been shown using particulate preparations of amnion and chorion cells (Koay et al, 1986), nevertheless further work on the characterization and

localization of the relaxin receptor is needed to provide conclusive evidence for a relaxin paracrine system.

The study of relaxin shown here is based only on two time frames, before and after labour and delivery. It will be important to look at the parameters of local hormones at different stages of pregnancy and perhaps an understanding may be useful in clinical problems of pregnancy such as premature delivery due to premature rupture of the fetal membranes.

The 48-mer oligoprobe to human relaxin was made, originally for reason of economy, from a region where H1 and H2 genes are identical, thus was not designed to provide information on whether one or both genes are expressed. There is consensus in the literature for H2 gene expression only. Our preliminary results using 24-mer oligoprobes suggested surprisingly that both genes H1 and H2 may be expressed. It is evident that more work is needed to define more precisely relaxin gene expression in these interesting tissues.

The production of hPRL and hPL by intrauterine tissues is presented here and their possible biological actions described in Chapter I. Synthesis in more than one site suggests that these two hormones may also function in the paracrine mode. Although, research on hPRL and hPL receptors are more advanced than in research on the relaxin receptor none has focussed on human intrauterine tissues and more research is needed in this area to fully explore hPRL and hPL as paracrine hormones.

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