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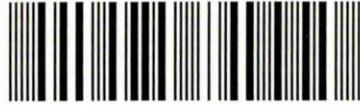
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**Cytokines and human endometrial function: abnormalities
in recurrent miscarriage women**

Beverley Anne Cork

A thesis submitted in partial fulfilment of the requirements of
Sheffield Hallam University for the degree of Doctor of
Philosophy.

May 2001



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Abbreviations

ACA	Anticardiolipin antibodies
ANOVA	Analysis of variance
APS	Antiphospholipid syndrome
CNTF	Ciliary neurotropic factor
DAB	Diaminobenzadine tetrachloride
DES	Diethylstilbestrol
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
ELISA	Enzyme linked immunosorbent assay
FSH	Follicle stimulating hormone
GM-CSF	Granulocyte macrophage colony stimulating factor
gp130	Glycoprotein 130
hCG	Human chorionic gonadotropin
IFN γ	Interferon gamma
IL-1 α	Interleukin-1 alpha
IL-1 β	Interleukin-1 beta
IL-1ra	Interleukin-1 receptor antagonist
IL-1RtI	Interleukin-1 receptor type I
IL-1RtII	Interleukin-1 receptor type II
IL-2	Interleukin-2
IL-3	Interleukin-3
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-6 R	Interleukin-6 receptor
IL-10	Interleukin-10
IL-11	Interleukin-11
IL-11R α	Interleukin-11 receptor alpha
IL-12	Interleukin-12
JAK	Janus kinase
LAC	Lupus anticoagulant

LGL	Large granular lymphocyte
LH	Luteinising hormone
LIF	Leukaemia inhibitory factor
LIF R	Leukaemia inhibitory factor receptor
LPD	Luteal phase defect
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MT-MMP	Membrane type matrix metalloproteinase
NFkB	Nuclear factor kappa B
NK	Natural killer
OSM	Oncostatin M
PAP	Peroxidase anti-peroxidase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PG	Prostaglandin
PP14	Placental protein 14
RM	Recurrent miscarriage
RT	Reverse transcriptase
RT-PCR	Reverse transcription polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SLE	Systemic lupus erythematosus
TBE	Tris borate EDTA buffer
TGFβ	Transforming growth factor beta
TIMP	Tissue inhibitor of metalloproteinase
TMED	N,N,N,N' - tetramethylethylenediamine
TNFα	Tumour necrosis factor alpha
TNFβ	Tumour necrosis factor beta
Tris	Trishydroxymethylaminomethane
UV	Ultra violet

Abstract

The human endometrium is the site of embryo implantation and is therefore responsible for providing a suitable environment for an embryo to grow and develop. This is achieved by the endometrium undergoing cyclical changes, under the control of steroid hormones. However, it is clear that steroid hormones are not the final effectors, but rather initiate a downstream cascade of molecular events through local autocrine and paracrine factors, such as cytokines. The role of cytokines in the human endometrium still remains to be determined, but they are thought to play an important role in the implantation process. This study has therefore focused on the expression of pro-inflammatory cytokines in the human endometrium and effects of these cytokines on endometrial function.

Immunocytochemistry was used to determine the expression of leukaemia inhibitory factor (LIF), interleukin-6 (IL-6), interleukin-1 α (IL-1 α), interleukin-1 β (IL-1 β) and tumour necrosis factor α (TNF α) in the human endometrium of normal fertile women throughout the menstrual cycle. The results showed that staining intensity for LIF and IL-6 increased in epithelial cells at the time of implantation. IL-1 α and IL-1 β remained relatively constant throughout the cycle, with a slight increase in epithelial IL-1 β at the time of implantation. The expression of TNF α could not be determined. Staining for LIF, IL-6, IL-1 α and IL-1 β was then repeated on endometrial sections from biopsies obtained from women who suffer recurrent miscarriage at the time of implantation and compared to the staining obtained in biopsies from normal fertile women. For all four cytokines, there were some biopsies from women who suffer recurrent miscarriage, where staining was significantly weaker than that seen in normal fertile women at the same time in the menstrual cycle. These results suggest that LIF, IL-6, IL-1 α and IL-1 β may therefore be important for successful implantation and subsequently successful pregnancy outcome.

Matrix metalloproteinases (MMPs) are postulated to be involved in the implantation process, as they are capable of digesting the components of the extracellular matrix. Recent studies have shown that cytokines may be involved in the regulation of MMPs in both the endometrium and the invading trophoblast cells. The effects of LIF, IL-6, IL-1 α and TNF α on endometrial MMP production *in vitro* were therefore

investigated. MMP-2 was produced by both cultured epithelial and stromal cells, MMP-9 was produced mainly by epithelial cells and MMP-7 was only produced by epithelial cells. Although LIF and IL-6 had no significant effect on endometrial MMP production, IL-1 α and TNF α did alter MMP-2, MMP-9 and MMP-7 production from both epithelial and stromal cells.

More recent studies have suggested the possible role of interleukin-11 (IL-11) in endometrial function, particularly decidualisation. Therefore the expression of both IL-11 and its receptor, IL-11R, was investigated in endometrial biopsies obtained from normal fertile women throughout the menstrual cycle. The results showed that both IL-11 and IL-11R were expressed throughout the menstrual cycle, predominantly by epithelial cells, however, stromal expression did increase slightly towards the end of the cycle.

The effects of cytokines on IL-11 production by cultured endometrial cells were also investigated. IL-1 α , TNF α and TGF β caused a significant increase in IL-11 production from both stromal and epithelial cells. Finally the effects of IL-11 on MMP-2, MMP-9, MMP-7, IL-1 β and TNF α produced by cultured endometrial cells were studied. No effect of IL-11 on MMP production was seen by either stromal or epithelial cells, but IL-11 did cause a concentration dependent decrease in TNF α production from cultured epithelial cells.

The results have increased our knowledge on the expression and function of endometrial pro-inflammatory cytokines and suggested that although endometrial LIF and IL-6 expression is greatest at the time of implantation and is decreased in women who suffer recurrent miscarriage, IL-1 and TNF α have a greater effect on endometrial function. IL-11 is also expressed by the endometrium and is affected by other cytokines. Its positioning within the cytokine networks, which could control endometrial function, requires further study.

Chapter 1

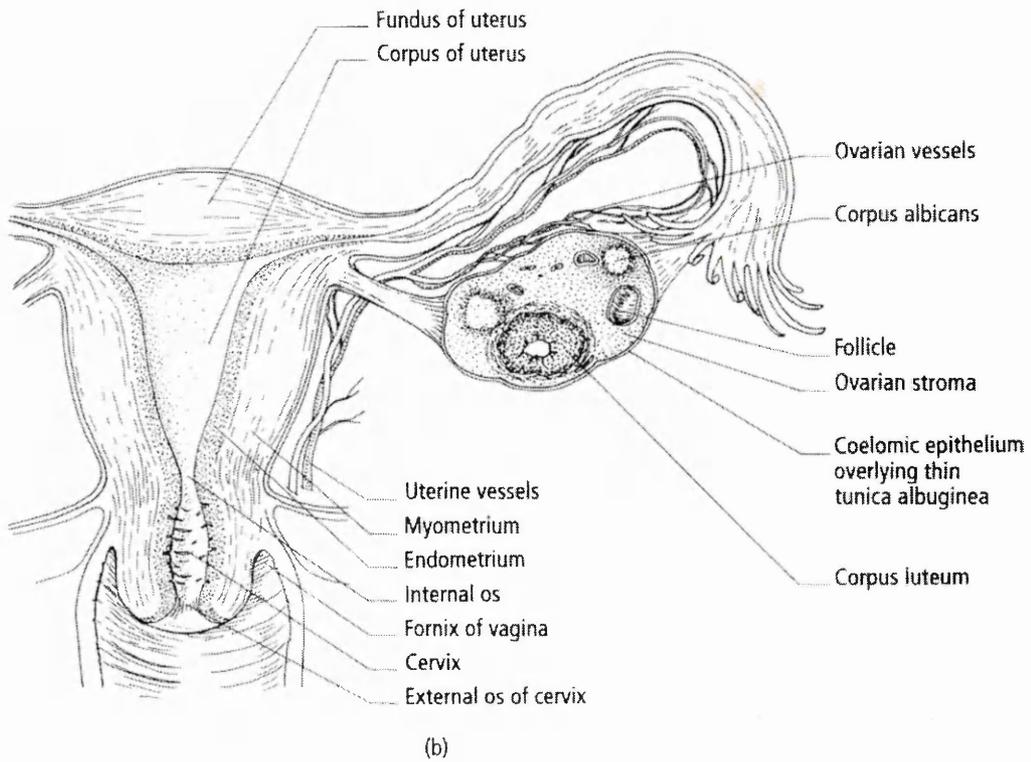
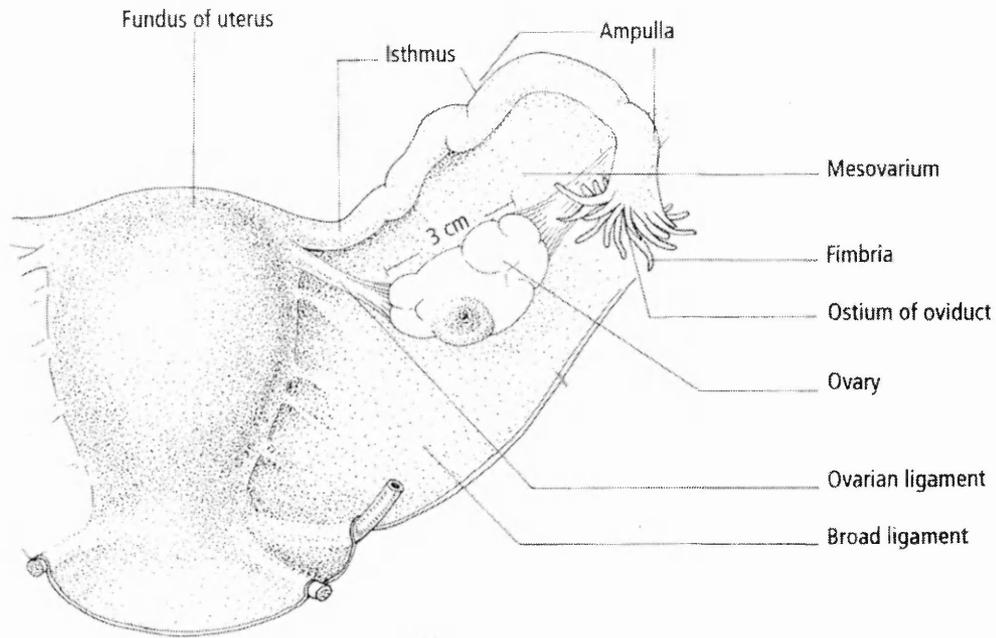
General Introduction

1.1 Female Reproductive Tract

The female reproductive tract has two distinct reproductive functions. Firstly, it must transport gametes to the site of fertilisation and secondly provide the site of implantation for the conceptus and a suitable environment for its subsequent development. There are four components to the human female reproductive tract, the anatomy of which is shown in figure 1.1. The ovaries are the centres of cyclicity, in that they release oocytes episodically at ovulation and are also responsible for the cyclic release of oestrogen and progesterone, two hormones that control female reproductive function. The fallopian tubes (or oviducts) consist of the fimbria, the ampulla and the isthmus, the function of which is to transport the oocytes from one of the ovaries to the uterus. The ampullary region of the fallopian tube is the site of fertilisation; as spermatozoa deposited in the vagina are transported up the female reproductive tract into the fallopian tube. The fallopian tube is also the site of early embryonic development as the resulting conceptus is transported to the uterus (Findlay, 1984; Johnson and Everitt, 1995).

The uterus is the site of implantation and placentation; it is here that growth of the embryo/fetus occurs. It consists of an upper expanded portion termed the body; a lower cylindrical part termed the cervix, and the fundus, which is the part of the body that extends above the point of entry of the fallopian tubes. The uterus is covered with a connective tissue layer called the peritoneum that extends laterally as the broad ligaments, from which the ovaries are suspended. The uterine wall consists of a thick smooth muscle layer, the myometrium and a vascular mucosal layer, the endometrium, which varies in thickness with the phases of the menstrual cycle. The final component of the female reproductive tract is the vagina, which along with the cervix is the site of deposition and capacitation of spermatozoa and is also responsible for the expulsion of the fully developed infant (Findlay, 1984).

Figure 1.1: Anatomy of the human female reproductive tract. Taken from Johnson and Everitt (1995).



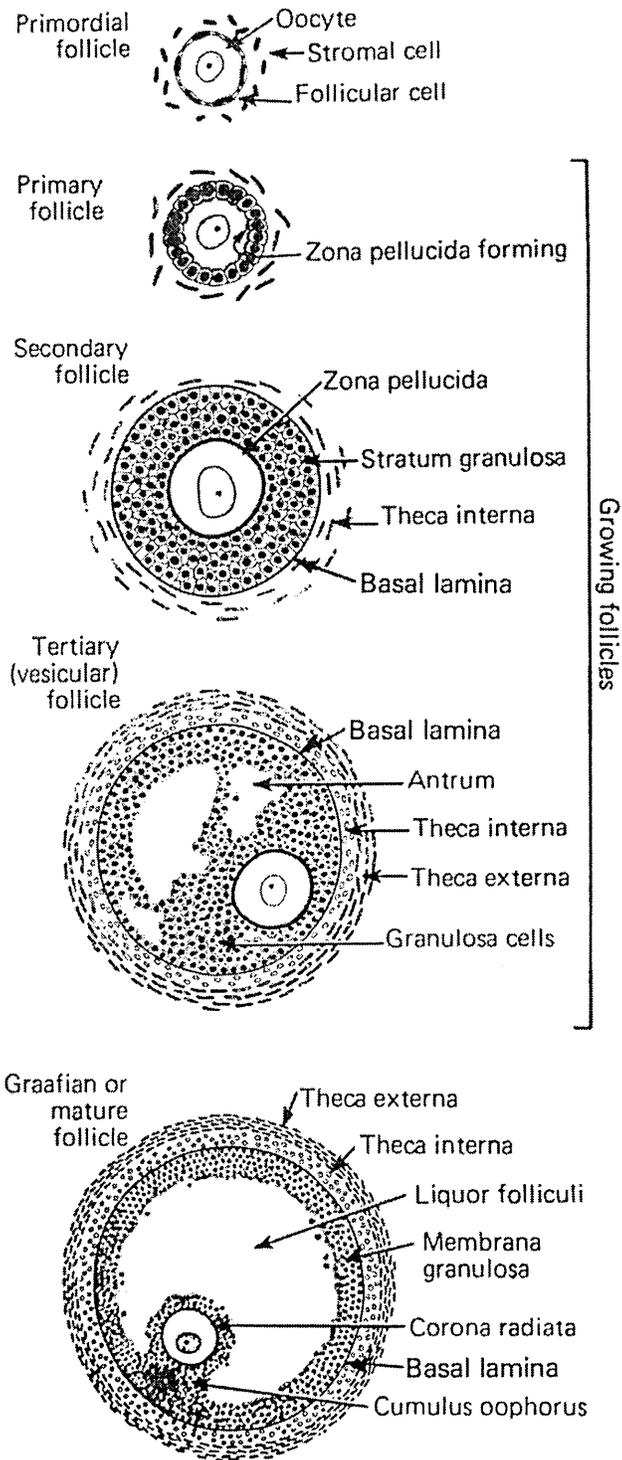
1.1.1 The ovary

A layer of low columnar epithelial cells, which lines a poorly defined layer of fibrous connective tissue called the tunica albuginea, covers the ovary. Within the ovary is an inner vascular medulla containing blood vessels, which branch off to form smaller blood vessels that then penetrate into the surrounding outer cortex. The cortex of the ovary consists of stromal cells and contains the primordial follicles, which constitute the fundamental functional units of the ovary (Sadow, 1980).

At birth each ovary contains approximately 2×10^6 primary oocytes, the majority of which undergo atresia during infancy. At puberty only 40,000 oocytes remain. They are surrounded by a layer of follicular cells, which are in turn surrounded by a layer of granulosa cells that secrete a basement membrane, the membrane propria, around the outside of the cellular unit which is now termed a primordial follicle. After puberty a small number of follicles are recruited daily into further growth, producing continuous development of follicles. It is not understood why there is daily recruitment of these primordial follicles or how the follicles that do begin to develop are selected. Before a primordial follicle can be released (at ovulation) it must pass through three stages of development (Findlay, 1984).

The first stage produces a primary or preantral follicle and is characterised by an increase in the size of the primary oocyte, secretion and formation of the zona pellucida by the oocyte and the development of thecal cells produced by the condensation of some of the surrounding stromal cells. The beginning of the next phase is characterised by the formation of vacuoles in the granulosa cells, which also begin to produce a viscous fluid known as follicular fluid. As the number of vacuoles increase, a cavity called the follicular antrum is formed. This increases in size and results in the oocyte being connected to the rim of the primordial granulosa cells by only a thin "stalk" of cells. The oocyte is however surrounded by a dense mass of granulosa cells called the cumulus oophorus (see figure 1.2). This maturing follicle also has two distinct thecal cell layers; the inner glandular theca interna and surrounding fibrous theca externa and is now termed the secondary or antral follicle. The initiation and early progress through the preantral stage is independent of any direct extra-ovarian controls but is probably under paracrine control and cytokines may be involved (Sadow, 1980).

Figure 1.2: Maturation of human ovarian follicles. Taken from Findlay (1984)



Further on in follicular development, external support is required and this is provided endocrinologically by the pituitary gland. If there is no hormonal influence at this stage in follicular development then the follicles will undergo atresia. The presence of luteinising hormone (LH) and follicular stimulating hormone (FSH) released from the pituitary are vital in preventing atresia (Johnson and Everitt, 1995).

During the late preantral and early antral phases of follicular development receptors for these hormones appear on the follicular cells. Receptors for LH appear on the theca interna cells, whereas FSH receptors appear on the granulosa cells. The binding of LH to receptors in the theca internal cells stimulates the production of androgens from these cells and there is also a release of oestrogens (Sadow, 1980). The oestrogens are produced in one of either two ways. A small amount of oestrogen is produced by de novo synthesis from acetate and cholesterol within the cell, but the majority is produced by aromatisation of androgens (Sadow, 1980). FSH binds to receptors on the granulosa cells and stimulates oestrogen production, however, this only occurs in the presence of exogenous androgens, which are then aromatised producing oestrogens. Oestrogen, progesterone and androgens are all released into the follicular fluid and oestrogens bind to receptors on granulosa cells, stimulating the proliferation of increased numbers of oestrogen receptors, therefore providing a positive feedback mechanism whereby oestrogen is stimulating an increase in oestrogen output (Sadow, 1980). As a result the most advanced follicles provide a surge in circulating oestrogen. As oestrogen levels increase, there is an increase in LH levels caused by a positive feedback of oestrogen on the pituitary and hypothalamus. The increase in LH is rapid and results in a surge in LH that peaks approximately 18 hours before ovulation (Johnson and Everitt, 1995). Ovulation occurs on approximately day 14 of the menstrual cycle. However, because the length of the follicular phase can vary between individuals, the exact day of ovulation is also termed day LH + 0 (i.e. no days after the LH surge). Subsequent days in the luteal phase are then referred to as day LH + the number of days after the LH surge. So if ovulation occurs on day 14 or LH + 0, then day 20 can be referred to more exactly as day LH + 6 (Li *et al.*, 1988).

The follicle undergoes final maturation to become a preovulatory or Graffian follicle. Oestrogen and FSH stimulate the appearance of LH binding sites on the outer layer of granulosa cells. This stage is critical for the expanded antral follicle to develop into the preovulatory phase of follicular growth (Sadow, 1980). The binding of LH to receptors on the granulosa cells causes two important processes to occur. Firstly it stimulates the terminal growth changes in the follicle and oocyte in the preovulatory follicle resulting in the oocyte rupturing through the follicle and being released, i.e. ovulation. Secondly, following the release of the oocyte, the follicle collapses. The granulosa cells are thrown into folds and bleeding occurs forming a clot in the centre. Capillaries from the theca interna infiltrate towards the centre and the granulosa cells proliferate. This whole process transforms the follicle into an endocrine organ called the corpus luteum, whose function is to produce progesterone (Johnson and Everitt, 1995).

The interval between successive ovulations is known as the ovarian cycle. The period before ovulation occurs is known as the follicular phase, due to oestrogens being secreted from the follicular cells. The period after ovulation is known as the luteal phase, due to progesterone being secreted from the corpus luteum (Johnson and Everitt, 1995).

1.1.2 The uterus

The human uterus is a pear-shaped organ with two uterine horns and consists of the fundus, body and cervix. The uterus is slightly flattened dorso-ventrally and thus contains a flattened uterine cavity (Sadow, 1980). The myometrium consists of bundles of smooth muscle fibres separated by vascular connective tissue. During menstruation the myometrium undergoes low frequency, high amplitude contractions. By day 6 of the cycle the contractions are more frequent and by the time of ovulation have become high frequency, low amplitude contractions. Towards the end of the cycle the contractions decrease in frequency and amplitude, before a rapid rise in amplitude just prior to menstruation. The pattern of contractile activity results from the combined effects of steroid hormones and prostaglandins (Findlay, 1984). The uterine cavity is lined by a mucous membrane, the endometrium, which is where the embryo implants.

1.1.3 The human endometrium

The human endometrium consists of two major cell types, epithelium and stroma. The epithelial layer is a simple columnar epithelium and is composed of a mixture of ciliated and secretory cells. It lines the uterine cavity and is invaginated, which results in numerous uterine glands, the morphology of which is continuous with the surface epithelium although there are fewer ciliated cells present. The stromal compartment of the endometrium can be divided into the stratum functionalis and stratum basalis regions (see figure 1.3). It consists mainly of stromal cells and also a population of leukocytes that are dispersed throughout the stromal compartment (Bulmer *et al.*, 1991).

Blood supply to the endometrium is provided via the uterine artery, which is a branch of the internal iliac artery. The uterine arteries, one from each side, run along the lateral walls of the uterus from the cervix upwards. The uterine arteries then branch off forming between 9 and 14 smaller arteries that penetrate the outer third of the myometrium. At this point the uterine arteries anastomose with the terminal branches of the ovarian arteries. From the anastomosis a series of arcuate arteries branch off, which run within the anterior and posterior myometrial walls thereby encircling it. The remaining myometrium is penetrated by radial arteries, which branch off the enveloping vasculature. As the radial arteries cross the myometrial-endometrial junction, they give off small straight branches called basal arteries, which are responsible for blood supply to the stratum basalis. The stratum functionalis requires a separate blood supply as it is shed during menstruation. This is provided by the spiral arteries, which branch off the basal arteries and pursue a winding course to reach the innermost endometrial zone, where they break up into a rich network of capillaries. Thin walled veins form an anastomosing network of sinusoids at all levels of the endometrium. Veins then follow the route of the arteries back to the uterine vein and eventually the internal iliac vein (Rogers, 1992).

1.1.4 The menstrual cycle

The ovarian cycle results in cyclic changes in steroid secretion that bring about changes to the endometrium (Figure 1.4). This cycle is termed the menstrual cycle and lasts for approximately 28 days, although any length between 25 and 35 days is

Figure 1.3: Morphology of the human uterus.
Taken from Sadow (1980)

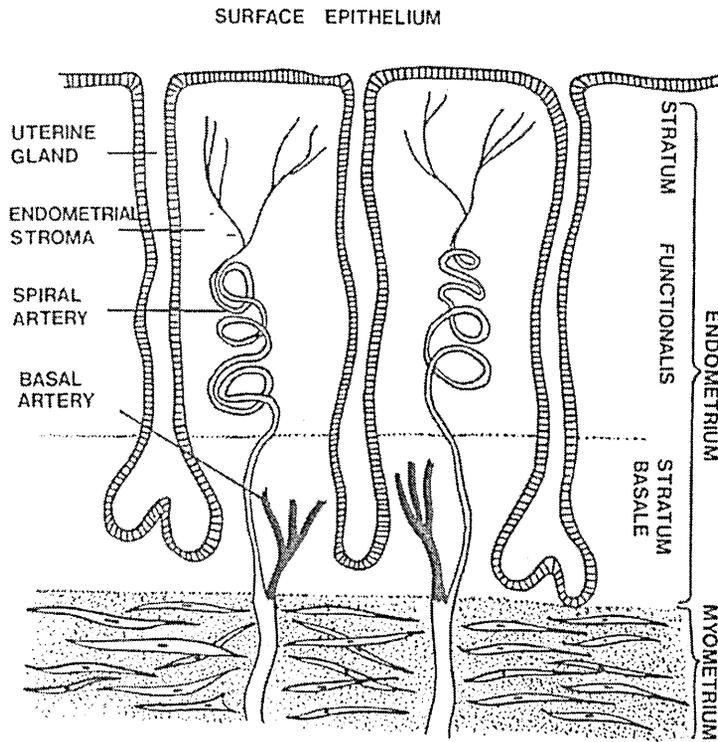
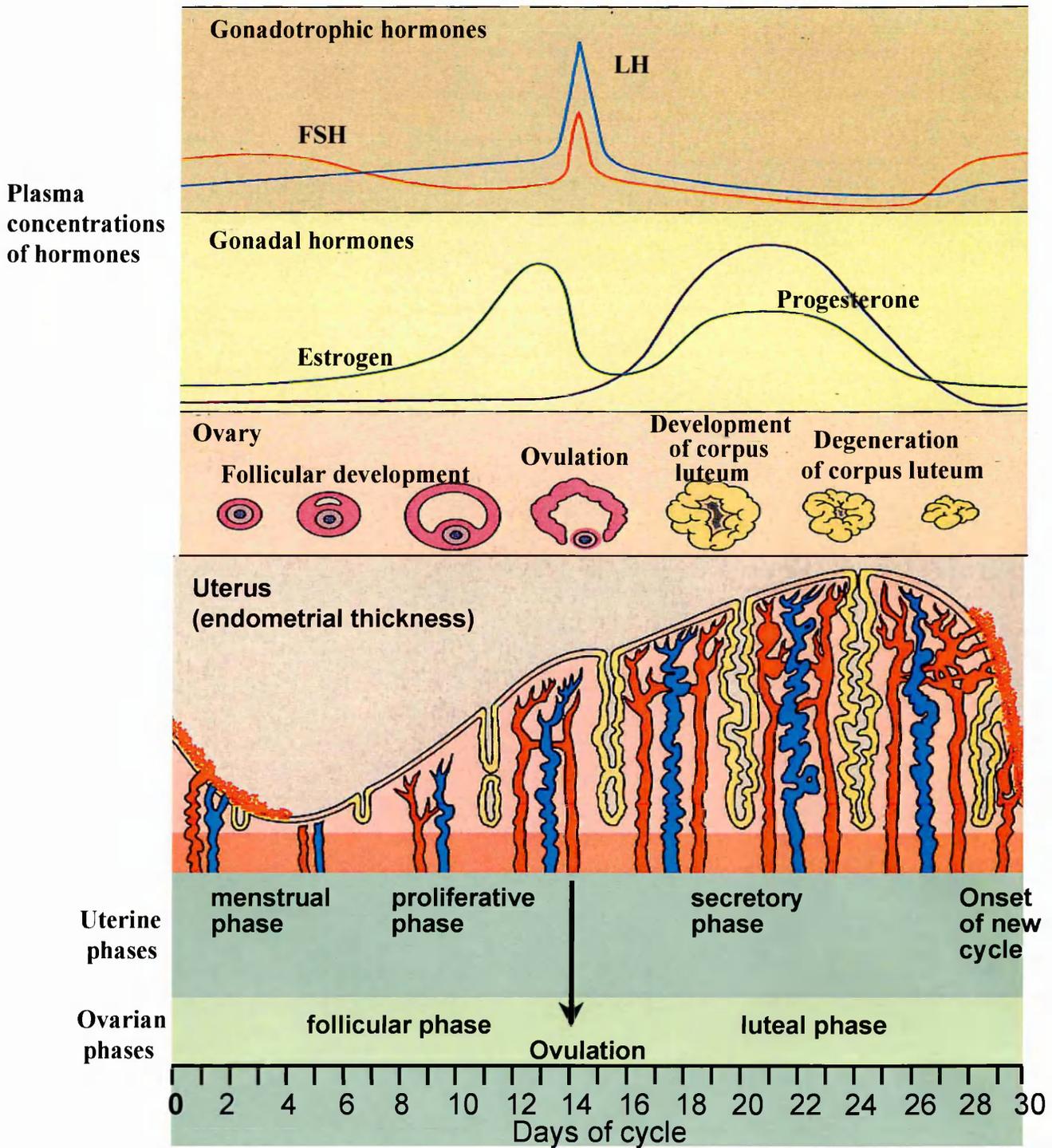


Figure 1.4: The menstrual cycle. Taken from Sherwood (1994)



considered to be normal. The menstrual cycle prepares the endometrium to receive the blastocyst and subsequently to provide a suitable environment for the continued growth and development of the fetus. The cycle is divided into two phases, firstly the proliferative or follicular phase (days 0-14), which is then followed by the secretory or luteal phase (days 15-28) (Sherwood, 1994; Johnson and Everitt, 1995).

The proliferative phase follows menstruation and during this period the endometrium is only 1-2 mm thick. Both the surface and glandular epithelium is made up of cuboidal cells and the glands are very narrow and collapsed. Throughout the follicular phase of the cycle there is marked proliferation of both epithelial and stromal cells under the influence of oestrogens secreted from the ovary, causing thickening of the stromal cell layer and an increase in surface area and metabolic activity of the surface epithelium, which becomes columnar. The endometrial glands increase in number and size, and epithelial secretions containing numerous proteins and proteolytic enzymes are increased, reaching a maximum at the time of the oestrogen surge (Johnson and Everitt, 1995). Oestrogens act on the proliferative endometrium by binding to oestrogen receptors that are abundant in uterine tissue. The main functions of oestrogen are to ensure proliferation of the endometrium and to "prime" the endometrium so that it will be capable of responding to progesterone during the secretory phase of the menstrual cycle. Priming of the endometrium by oestrogen results in an increase in the synthesis of intracellular progesterone receptors. At the beginning of the proliferative phase there are very few progesterone receptors present within the endometrium and as a result progesterone has little effect on the endometrium. However, following the surge in oestrogen and ovulation, the endometrium is receptive to progesterone and so the secretory phase of the menstrual cycle begins (Johnson and Everitt, 1995).

During the secretory phase of the menstrual cycle progesterone stimulates the synthesis of secretory material by the glands, which is rich in glycoproteins, sugars and amino acids (Johnson and Everitt, 1995). Effects of progesterone on the stromal cells are an increase in stromal proliferation resulting in the stromal cells becoming larger. Progesterone also stimulates full development of the spiral arteries. These changes in endometrial morphology prepare the endometrium for implantation of the blastocyst. If implantation does not occur, levels of the steroid hormones decrease

towards the end of the secretory phase, causing apoptotic cell death and a collapse of the secretory epithelium. The epithelial layer and stratum functionalis are shed via the cervix and vagina along with blood from the ruptured spiral arteries, which then contract to reduce bleeding. This process is known as menstruation and signifies the beginning of the next menstrual cycle (Johnson and Everitt, 1995).

1.1.5 Endometrial leukocyte populations

The human endometrium does not have a classical mucosal immune system; instead it contains a specialised population of endometrial leukocytes, which comprise 8-35% of the total endometrial cell number, depending on the time of the menstrual cycle (Johnson *et al.*, 1999). Leukocytes that are found in the endometrium include CD3⁺ T cells (approximately two thirds of which are also CD8⁺), CD14⁺ macrophages and a population of large granular lymphocytes (LGL's). Endometrial LGLs vary phenotypically from peripheral LGLs, in that they are mainly CD3⁻ CD16⁻ CD56⁺ with a small number of CD3⁻ CD16⁺ CD56⁺, and constitute the largest population of endometrial leukocytes. Studies have suggested the absence of B cells in the endometrium at any time in the menstrual cycle (Bulmer *et al.*, 1991).

During the proliferative and early secretory phases of the menstrual cycle leukocytes contribute to less than 10% of total endometrial cell number and this increases to greater than 20% by the late secretory phase. This increase in leukocyte number is largely due to an increase in the number of LGLs. The factors influencing this increase are poorly understood at present, but it is not thought that the numbers are increased due to an influx of leukocytes from the peripheral blood system (Bulmer *et al.*, 1991).

During the proliferative phase T cells make up approximately 45% of the endometrial leukocyte population. The number of T cells tends to remain constant throughout the menstrual cycle, however there is a decrease in the relative number of T cells during the secretory phase due to the increase in other leukocyte populations. As a result T cells represent the smallest number of endometrial leukocytes, during the secretory phase of the menstrual cycle (Vassiliadou and Bulmer, 1996). The number of endometrial macrophages is also relatively constant throughout the cycle with an increase being seen just prior to menstruation. Macrophages are generally

found scattered throughout the endometrium but can also be found within aggregates. They contain acid phosphatase, non-specific esterase and lysine. Within the endometrium they are thought to be involved in scavenging and degradative functions associated with menstruation. They are known to produce prostaglandin (PG) E₂ and PGF₂α and can also produce and respond to a wide range of cytokines (Johnson *et al.*, 1999).

The LGLs are the most abundant population of leukocytes during the secretory phase of the cycle. By the time of implantation they account for 70-80% of the endometrial leukocyte population and if conception occurs and the resulting blastocyst implants, the numbers increase further. As with macrophages, the LGLs are found scattered singly throughout the endometrium, but during the late secretory phase they tend to gather as aggregates particularly around the spiral arteries and endometrial glands. They possess granules that contain perforin and granzymes, which suggests that they may have the potential to provide an LGL-mediated cytolytic function. They express adhesion molecules (CD11a, CD11b and CD11c) and integrins (VLA-4), which may be involved in the control of LGL recruitment into the endometrium and in determining endometrial distribution. The dramatic increase in the numbers of LGLs during the secretory phase suggests that they may play a role in implantation and the establishment of pregnancy (King *et al.*, 1989).

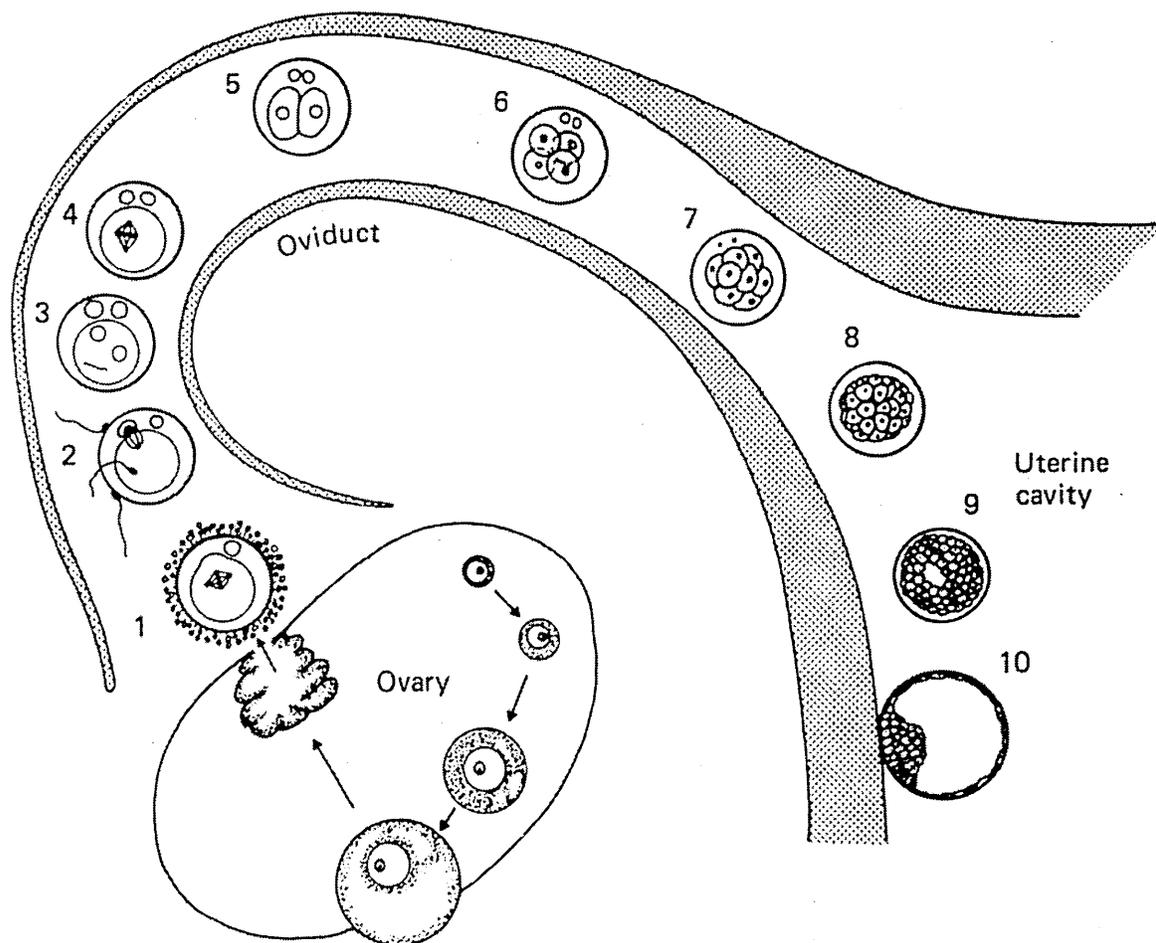
1.2 Implantation

For implantation to occur there must be a functional normal blastocyst, a receptive endometrium and successful communication between the blastocyst and endometrium. Development of the embryo to the blastocyst stage occurs independently of preparation of the endometrium, but at implantation the two become interdependent and the uterine environment regulates further embryonic development. The process of implantation is influenced by complex molecular interactions between the blastocyst and endometrial surface at the maternal-fetal interface (Psychoyos and Martel, 1985).

1.2.1 Blastocyst Development

Following release of the oocyte into the ampullary region of the fallopian tube, providing spermatozoa is present, fertilisation occurs, maternal and paternal chromosomes come together and a zygote is formed. Cleavage then occurs to form a two-cell stage embryo. The conceptus remains in the fallopian tube for 3-4 days where each of the two cells undergoes a series of divisions. Once it reaches the 8-16 cell stage the morphology of the conceptus changes; it undergoes compaction and this results in the formation of a morula. This is achieved by the time the conceptus has reached the uterine cavity (see figure 1.5). The process of compaction involves minimising intercellular contacts and transforming the morula to that of a highly polarised phenotype. The polarisation causes cell cleavage resulting in two cell types, which develop into the inner cell mass and the trophoctoderm cells. Once the distinction of these two cell types has been made, at approximately the 32-64 cell stage, the embryo is termed the blastocyst and is ready for implanting into the endometrium (Johnson and Everitt, 1995). The trophoctoderm forms an outer rim of cells, which constitute the first extra-embryonic tissue, giving rise to the trophoblast, an accessory fetal membrane. The trophoblastic cells are responsible for the initial contact at the maternal-fetal interface and they then continue to develop as the fetal component of the placenta. The trophoblastic cells surround the blastocoelic cavity which contains blastocoelic fluid and the inner cell mass. The inner cell mass is placed within one of the trophoblastic walls and it is this which develops into the embryo/fetus. Transfer from the fallopian tube to the uterus occurs at the late morula stage around 3-4 days after ovulation. The early blastocyst then floats freely within the uterine cavity for approximately 3-4 more days before implanting. Nutritional support for the blastocyst is provided by endometrial secretions stimulated by progesterone during the secretory phase of the menstrual cycle. Approximately 6 days after ovulation the zona pellucida comes away from the blastocyst, exposing trophoblastic cells. This process occurs due to proteolytic enzymes secreted from either the trophoblast itself or uterine secretions. The embryo is now able to implant into the epithelium. (Johnson and Everitt, 1995)

Figure 1.5: Diagrammatic representation of follicular growth, ovulation, fertilisation and embryonic development prior to implantation. Taken from Findley (1984).



Key

- | | |
|---|---|
| 1: Egg released from ovary with first polar body and second metaphase spindle | 5: 2-cell stage |
| 2: Sperm entry into egg, second polar body forming | 6: 4-cell stage |
| 3: Male and female pronucleus formation. Sperm tail in egg cytoplasm | 7: 8-cell stage |
| 4: First cleavage metaphase spindle | 8: Morula |
| | 9: Early blastocyst, blastocoele cavity forming |
| | 10: Blastocyst starting to implant |

1.2.2 Endometrial preparation for implantation

The human endometrium undergoes changes throughout the menstrual cycle in order to prepare for blastocyst implantation. These changes are driven by oestrogen and progesterone, which act on the endometrium and are thought to initiate a downstream cascade of molecular events via local paracrine and autocrine factors such as chemokines, cytokines, growth factors, adhesion molecules and invasive proteinases. The endometrium is only receptive to implantation for a limited period in the menstrual cycle. Psychoyos (1986) first discussed the concept of uterine receptivity and this period of receptivity is termed the "implantation window". In humans the implantation window is approximately 4 days long, between days 20 and 24 of the menstrual cycle, or days LH+7 to LH+10 (Bergh and Navot, 1992).

1.2.3 Morphological markers of implantation

Microscopical studies have shown that endometrial epithelial cells, in particular the secretory cells, change considerably during the secretory phase of the cycle (Nikas *et al.*, 1999a). During the early proliferative phase (days 4-7) the epithelial surface is regenerated following menstruation and is therefore very thin. Most glands are very short, straight and narrow and the stromal compartment is compact with spindle-shaped stromal cells with large nuclei. As development of the endometrium continues into the mid-proliferative phase (days 8-10) the surface epithelium becomes columnar, the glands become longer and there is evidence of stromal oedema. By the late proliferative phase (days 11-14) the glands become more tortuous showing active growth and pseudostratification of the epithelium. The stroma is moderately dense and actively growing (Noyes *et al.*, 1950).

The secretory epithelial cells are either elongated or polygonal and vary in size. The microvilli are short and slender then develop with progression of the proliferative phase (Nikas *et al.*, 1999a). By day 16, the glands increase in diameter and tortuosity. By day 17 the microvilli are long, thick and upright, and by day 18 the microvilli begin to appear swollen. On day 19, the microvilli decrease in number and length, fuse and disappear and are replaced by smooth membrane projections, which protrude and fold maximally forming pinopodes (Nikas *et al.*, 1999b). By day 20 there is a peak in the presence of intraluminal secretory material, microvilli are virtually absent and pinopodes are fully developed. The pinopodes only last for 24-

48 hours; by day 21 microvilli reappear on the cell membranes, which are now wrinkled in appearance due to regression of the pinopodes. A possible function of pinopodes is that they absorb molecules and fluids from the uterine lumen, facilitating the proximity between embryo and endometrium. They may also be involved in trophoblast adhesion and facilitate penetration of the implanting blastocyst into the underlying stroma, because it has been noted that the lateral cell-cell contacts between epithelial cells become looser in the presence of pinopodes (Nikas *et al.*, 1999b). From days 21-22 there is massive stromal oedema, the stromal cells increase in size and begin to appear dome-shaped. By day 23 the spiral arteries have become more prominent, indicating predecidualisation of the stromal cells. By day 24 there are collections of predecidual cells around the arterioles and stromal proliferation continues. Differentiation of the predecidua occurs around day 25 and by day 26 these cells appear as a solid sheet of well-developed decidual cells. If pregnancy has not occurred by day 24 glandular secretions become diminished and there is involution of the glandular epithelium. The glands become dilated; the previous tall, columnar epithelium is low. Day 27 is characterised by an increased population of polymorphonuclear leukocytes (Bulmer *et al.*, 1991) and areas of focal necrosis and haemorrhage that become apparent a few hours preceding menstruation (Noyes *et al.*, 1950).

1.2.4 Biochemical markers of implantation

1.2.4.1 Adhesion molecules

Biochemical markers indicating uterine receptivity include adhesion molecules and, in particular, integrins. Integrins consist of one α subunit and one non-covalently bound β subunit and these have been shown to be present within the endometrial epithelium (Lessey *et al.*, 1992; Tabibzadeh, 1992). Three integrin subunits are thought to be important during the period of receptivity. These are α_1 , α_4 and β_3 as there is co-expression of all three during the implantation window, but not at other times in the menstrual cycle (Lessey *et al.*, 2000). Expression of the α_1 and α_4 subunits is increased following ovulation with a later decrease in α_1 expression during the late secretory phase and β_3 expression is known to begin on day 19. The implanting blastocyst has the ability to modulate endometrial expression of these integrin subunits as it has been shown that a cytokine, interleukin-1 (IL-1), produced

by the blastocyst mediates the up regulation of β_3 in human endometrial epithelial cells (Simon *et al.*, 1998).

1.2.4.2 Steroid receptors

Expression of progesterone and oestrogen receptors may be used as markers for endometrial maturation as studies have found that these receptors are subject to fine hormonal control by ovarian steroid hormones (Garcia *et al.*, 1988; Snijders *et al.*, 1992). Receptor expression is maximal at the mid-cycle stage in response to increased oestradiol secretion. Expression then declines as progesterone-dependent down-regulation occurs. During the secretory phase the oestrogen receptor is down regulated rapidly in the stroma and more gradually in the glandular epithelium, whereas progesterone receptors decline rapidly in the glandular epithelium, but persist in the stroma (Garcia *et al.*, 1988; Snijders *et al.*, 1992). These alterations in steroid receptor expression could therefore be useful as markers for implantation.

1.2.4.3 Other molecules

Other molecules that could be potential markers of uterine receptivity are the mucin MUC-1 and the endometrial protein PP14 or glycodeilin. The function of MUC-1 in the human endometrium is unknown at present, however, it has been shown to be present in both glandular and luminal epithelium during the proliferative and secretory phases of the menstrual cycle (Hey *et al.*, 1995; Aplin, 1999). The expression of MUC-1 is up regulated in human endometrium during the peri-implantation period (Hey *et al.*, 1995), suggesting a possible role in implantation. Concentrations are increased in uterine flushings from normal fertile women between days LH+7 and LH+13 (Hey *et al.*, 1995) and there is strong MUC-1 activity present in the luminal epithelium at both the beginning and end of the receptive phase (Aplin, 1999). A possible function of MUC-1 is that it may act as a uterine barrier to implantation, requiring embryonic signals before it can be removed. As a result this may prevent any abnormal blastocysts, incapable of providing these signals, from implanting (Aplin, 1999).

Endometrial epithelial cells also produce glycodeilin, with maximal expression during the secretory phase of the menstrual cycle (Li *et al.*, 1993). Levels in endometrial

flushings show similar cyclical changes to that of MUC-1 with maximal levels being present between days LH+7 and LH+10 (Dalton *et al.*, 1995). The function of glycodefin is not known but there are some studies suggesting that it may have immunomodulatory properties (Pockley *et al.*, 1988).

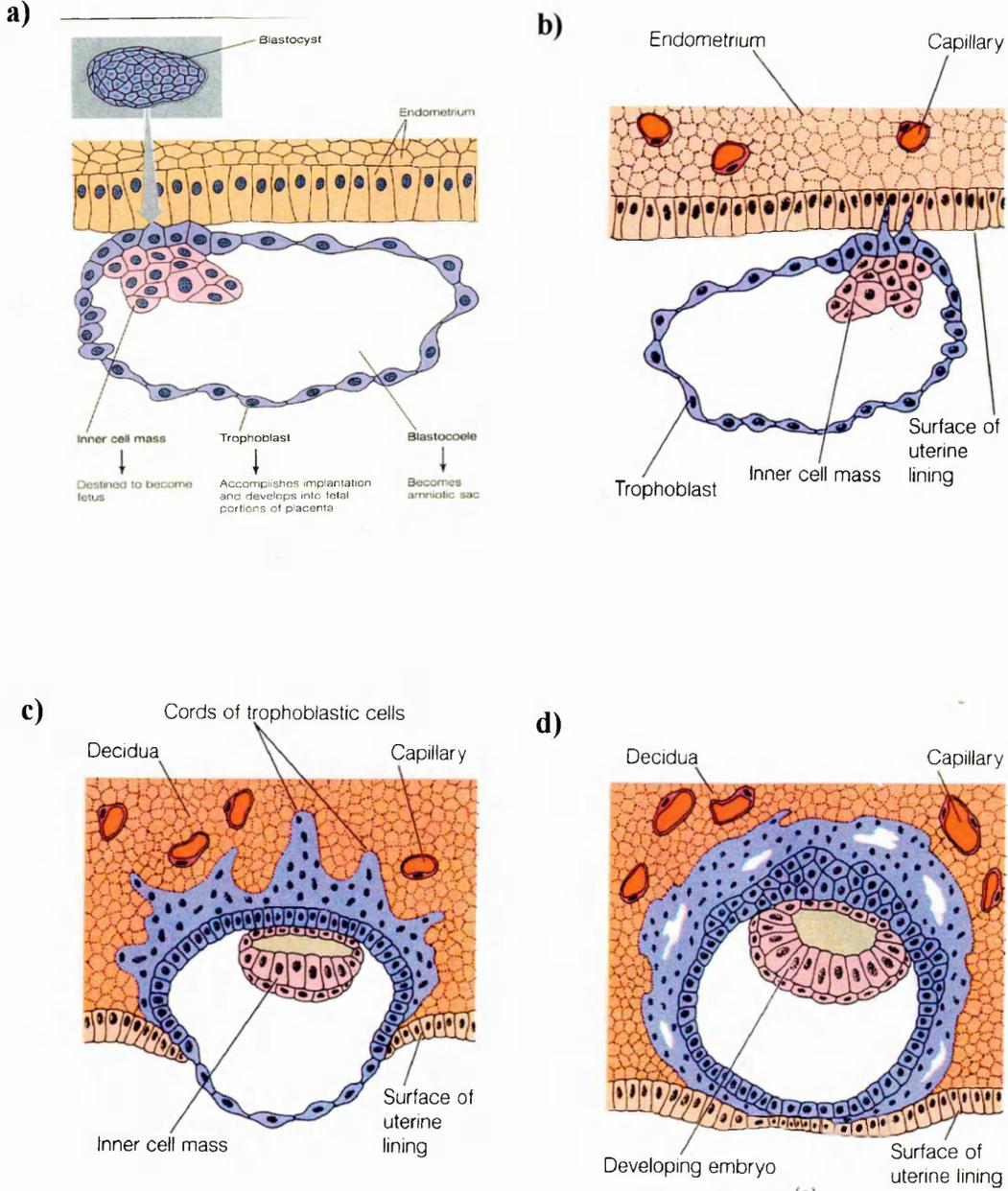
1.2.5 The process of implantation

Implantation consists of three separate phases. Firstly apposition of the blastocyst to the uterine epithelium, then adhesion and finally invasion through the epithelium into the underlying stromal compartment.

Apposition is the first contact between the cellular membranes of the trophoctoderm and the endometrial luminal epithelium. Apposition requires a direct contact between these two cell membranes and therefore must take place where the zona pellucida has ruptured or lysed (Psychoyos and Martel, 1985). It is thought that this cell contact is made possible by changes in cellular surface charge, which until apposition occurs, is negative on both surfaces. At this point there is no establishment of any visible connections between the blastocyst and endometrium and the blastocyst can be dislodged by washing of the uterine cavity (Bischof, 1997). Functional connections are established between the blastocyst and the endometrium during the adhesion phase of implantation. Cell surface glycoproteins, oligosaccharides and glycosaminoglycans in particular, are considered to be associated with attachment of the blastocyst to the endometrium in humans (Rohde and Carson, 1993). More recently studies have implicated cell adhesion molecules, such as E-cadherin, and integrins, such as $\alpha_6\beta_1$, to be involved in trophoblast adhesion to the human endometrial epithelium (Bischof, 1997).

After attachment, the blastocyst must invade the endometrium by penetrating the epithelial lining, basal lamina and underlying stroma. Within a few hours of attachment the epithelium beneath the blastocyst becomes eroded and trophoblastic processes protrude between the epithelial cells, isolating, dissolving and digesting them (Figure 1.6) (Sherwood, 1994). As trophoblastic cells invade the stromal compartment some of them fuse to form a syncytium and are referred to as syncytiotrophoblasts, whereas others retain their cellularity and are termed

Figure 1.6: Implantation of the blastocyst into the endometrium. (a) shows the attachment phase and (b), (c) and (d) show the stages of the invasion phase. Taken from Sherwood (1994).



cytotrophoblasts, and these continue to generate more trophoblastic cells. As the uterine glandular epithelium and underlying stroma is destroyed, the cells release large quantities of primary metabolic substrates providing nutrition for the implanting embryo (Johnson and Everitt, 1995).

1.2.6 Decidualisation

In humans interstitial implantation occurs, whereby the blastocyst implants deep into the maternal stroma and as this occurs the surface epithelium is restored above it. The blastocyst implants into undecidualised stroma; however, within a few days decidualisation occurs and extends throughout the endometrium (Bell, 1985).

Decidualisation is a differentiation of the fibroblast-like stromal cells into epitheloid-like cells. In humans a predecidual reaction will occur during the late secretory phase, even in the absence of implantation, therefore the initial reaction is not under embryonic influence (Noyes *et al.*, 1950). However, if implantation does occur then the reaction persists and the stromal cells become the decidua of pregnancy. During decidualisation progesterone acts on the oestrogen primed mid-luteal phase stromal cells causing numerous changes. The cells change shape, increase in size and there are development of organelles involved in protein synthesis. Ultrastructural studies on human decidual cells have shown that they possess all the characteristics of a secretory cell; euchromatic nucleus, numerous profiles of Golgi cisternae, dilated RER and dense membrane bound secretory granules (Kim *et al.*, 1998).

Decidualisation results in localised changes in the composition of the intercellular matrix (Aplin, 1996). There is also formation of desmosomes and gap junctions between adjacent cell walls, suggesting that decidualisation could facilitate the process of trophoblast invasion (Kim *et al.*, 1998). During decidualisation there is also an increase in endometrial leukocyte population, mainly due to an increase in LGLs and to a lesser extent the macrophages (King *et al.*, 2000). The exact function of the decidua is unclear, but it has been postulated that the roles probably include nourishing the developing embryo, protecting the maternal tissues from excessive trophoblast invasion and protecting the embryo from immunological rejections *in utero*. Once the decidualisation has been completed the decidual cells and trophoblastic cells begin to form the placenta.

1.2.7 Placentation

Following implantation the growing conceptus is provided with nutritional support from the decidual cells. However, as the embryo grows and differentiates this means of nutritional support is insufficient and the establishment of a placenta is required in order to provide the developing embryo/fetus with all its nutritional requirements. Formation of the placenta is completed during the first trimester of pregnancy and enables the growing fetus access to the maternal blood supply (Johnson and Everitt, 1995).

Invading trophoblast cells proliferate and differentiate into chorionic villi, which are composed of two cell layers, the cytotrophoblasts and syncytiotrophoblasts. Cytotrophoblasts are mitotically active mononuclear cells that form the inner layer of cells and the surrounding syncytiotrophoblasts are multinucleated masses of cells formed by the terminal differentiation and fusion of villous cytotrophoblasts (Boyd and Hamilton, 1970). The cytotrophoblasts at the tip of the villi proliferate and breach the syncytiotrophoblast layer to form extravillous cytotrophoblasts. A sub-population of the extravillous cytotrophoblast cells acquires an invasive phenotype (Bischof *et al.*, 1995a), which allows the cells to dissociate from the column and invade the decidua even deeper where they penetrate the upper portion of the myometrium and basal laminae of uterine arterioles. These invasive cytotrophoblasts form the anchoring villi and are responsible for anchoring the placenta to the decidua (Boyd and Hamilton, 1970). Another sub-population of chorionic villi is the floating villi, which float in the maternal blood mediating gas and nutrient exchange (Genbacev *et al.*, 1999). Syncytiotrophoblast cells bury through the decidua and destroy the walls of maternal uterine blood vessels converting them from muscular vessels to flaccid sinusoidal spaces that become filled with maternal blood. Groups of cytotrophoblasts from the cell columns invade the lumen of the endometrial spiral arteries as endovascular trophoblasts and this ensures that an adequate blood supply to the fetoplacental unit is established (Johnson and Everitt, 1995).

1.3 Recurrent Miscarriage

Miscarriage, early pregnancy loss or spontaneous abortion is defined as the termination of pregnancy before 20 weeks of gestation (dated from the last menstrual

period) or fetal weight being less than 500g and is the most common complication of pregnancy (Timbers and Feinberg, 1997). Approximately 15-20% of clinically established pregnancies abort within the first 20 weeks usually within the first trimester (weeks 1-12) (Stirrat, 1990). The true figure is likely to be much higher than this, nearer 70%, because there is a high rate of miscarriage within the first 2-4 weeks of pregnancy due to chromosomal abnormalities, that are not recognised because it is so early on in the pregnancy (Boklage, 1990). Because a large number of miscarriages occur due to chance, investigations into the cause of miscarriage are not usually carried out until the woman has been classified as suffering from recurrent miscarriage or recurrent spontaneous abortion. Recurrent miscarriage (RM) is defined as having 3 or more consecutive miscarriages and affects about 1% of the female population (Timbers and Feinberg, 1997). Clinical studies have shown that after 3 consecutive miscarriages then the risk of a further pregnancy loss is increased to 30-45% and the chance of pregnancy completing to term, resulting in a live birth is only 55-60% (Timbers and Feinberg, 1997). However, it is not the number of abortions that influences the diagnostic and therapeutic response to a couple suffering from RM, it is influenced by the age of the women, the level of anxiety of the couple and also any factors readily identifiable in the family/medical history (Timbers and Feinberg, 1997). There are many factors known to influence recurrent miscarriage.

1.3.1 Genetic Factors

Karyotyping of couples has shown that 3-8% of RM can be explained by a genetic abnormality. The most common cause of pregnancy loss, even in recurrent aborters, due to cytogenetic abnormalities is sporadic aneuploidies, the largest group of which is trisomic pregnancies. Other abnormalities include balanced translocation of part of a chromosome, sex chromosome mosaicism and ring chromosomes. Karyotyping shows a percentage of pregnancies lost to genetic abnormalities. However, karyotyping will not detect single gene defects, and therefore these could account for a number of RM (Timbers and Feinberg, 1997).

1.3.2 Anatomical factors

Approximately 5-10% of first trimester RMs are caused by abnormalities in uterine anatomy (Timbers and Feinberg, 1997). These abnormalities include both congenital

and acquired changes to normal uterine anatomy. Congenital Müllerian abnormalities such as a septate or bicornate uterus were originally thought to be associated with late second trimester miscarriages; however it is now recognised that these factors can also cause earlier miscarriages (Patton and Novy, 1988). The effect of congenital Müllerian abnormalities on recurrent miscarriage is not completely understood at present, but it is possible that they may result in compromised implantation and insufficient decidual and placental growth due to poor vascularisation of the endometrium (Portuondo *et al.*, 1986). Acquired Müllerian abnormalities include uterine synechiae (Asherman syndrome), submucosal leiomyomata and abnormalities seen following *in utero* exposure to diethylstilbestrol (DES). Uterine leiomyomas have varying effects on pregnancy loss depending on the size and location of the fibroid (Rock and Murphy, 1986). If submucosal and myometrial myomas are large enough there are alterations in endometrial vascularisation or a possible reduction in uterine size. *In utero* exposure to DES causes abnormalities associated with the upper genital tract. These only contribute to a small percentage of miscarriages; however, in females exposed to DES the first or second trimester miscarriage rate is 18-48% (Patton and Novy, 1988). Another factor that is thought to contribute to recurrent miscarriage in the late second trimester is cervical incompetence. This is characterised by painless dilation and prolapse with ballooning of the membranes into the vagina, followed by rupture of the membranes and expulsion of the immature fetus (Rock and Murphy, 1986).

At present there are no studies proving that surgical correction for uterine anomalies results in a successful outcome. However, 70-85% of women suffering from recurrent miscarriage that have undergone surgery for septate or bicornate uterus have had successful live births (Rock and Murphy, 1986).

1.3.3 Endocrinological factors

The most common endocrinological factor known to cause miscarriage is luteal phase defect (LPD) and this affects approximately 25% of women who suffer from recurrent miscarriage (Lee, 1987). LPD is a condition whereby progesterone production from the corpus luteum is diminished and results in inadequate endometrial development; therefore the endometrium is unable to support early pregnancy. Although LPD is well recognised there is no direct evidence that it is a

cause of recurrent miscarriage and there is no consensus regarding its underlying mechanisms, diagnosis or possible treatment. However, studies have shown that progesterone supplementation is likely to increase the chance of achieving successful pregnancy (Daya, 1989).

Other endocrine factors include thyroid disease and badly controlled diabetes mellitus. These conditions have been associated with an increased risk of fetal loss, but studies have failed to show a relationship between these conditions and recurrent miscarriage (Rock and Zancur, 1983).

1.3.4 Autoantibodies

The most recognised immunological causes of recurrent miscarriage are autoimmune reactions, where the cellular or humoral response is directed against a specific site within the host. The most common autoimmune disease is antiphospholipid syndrome (APS), which can occur as either a secondary condition in patients suffering from other immune disorders such as systemic lupus erythematosus (SLE), or as a primary condition in women with no previous recognised autoimmune disease (Branch *et al.*, 1992). APS is characterised by the presence of increased antiphospholipid antibodies and one or more of three clinical features; pregnancy loss, thrombosis and thrombocytopenia (Branch *et al.*, 1992). APS is directed against platelets and vascular epithelium and results in vascular damage, thrombosis and placental destruction. Lupus anticoagulant (LAC) and anticardiolipin antibodies (ACA) are the two major antiphospholipid antibodies present in APS. The presence of these antibodies has been shown to be associated with second trimester and occasional first trimester recurrent miscarriages (Dudley and Branch, 1989). APS can be treated with low dose aspirin, heparin, glucocorticoids and intravenous gamma globulin or a combination of these and has resulted in 55-85% successful pregnancy outcome (Rai *et al.*, 1997).

Other antibodies have been implicated as a possible cause of recurrent miscarriage, including antinuclear antibodies, thyroid antibodies and anti-SSA. However, further work is required before the effects of these antibodies on recurrent miscarriage can be understood (Timbers and Feinberg, 1997).

1.3.5 Immunological mechanisms

Other immunological influences on recurrent miscarriage include allogenic factors. The theory is that unexplained recurrent miscarriage may be due to an abnormal maternal immune response to fetal antigens of paternal origin. However, the mechanisms that allow maternal tolerance to the semiallogenic conceptus are not completely understood at present and as a result it is difficult to assess the association between allogenic immunological factors and recurrent miscarriage (Scott and Branch, 1994).

1.3.6 Microbiological factors

Infections are thought to be a possible cause of recurrent miscarriage, but studies have failed to show an increased incidence of infections in women suffering from recurrent miscarriage when compared to normal fertile women. The most common infection of the human female reproductive tract is *chlamydia trachomatis*, but studies have shown that it is unlikely to be a cause of recurrent miscarriage (Harrison *et al.*, 1983). Two other infections, *mycoplasma hominis* and *ureaplasma urealyticum*, although present in normal, asymptomatic women have been shown to be more frequent in women suffering from recurrent miscarriage (Stray-Pederson *et al.*, 1978). However, elimination of these mycoplasmas has not improved subsequent pregnancy outcome and they are therefore unlikely to be a significant cause of recurrent miscarriage.

Other possible causes include viral and parasitic infections such as cytomegalovirus and herpes simplex virus. Although there is no evidence that they are associated with recurrent miscarriage it is still considered that they may possibly be linked to first trimester losses (Timbers and Feinberg, 1997).

1.3.7 Unexplained RM

Even though the understanding of possible causes of recurrent miscarriage has improved greatly over the past twenty years, the majority (>50%) of recurrent miscarriage cases still remain unexplained (Timbers and Feinberg, 1997). A greater understanding of the paracrine and autocrine cascade of events that occurs during implantation may result in an explanation as to a possible cause for some of this group of recurrent miscarriage women. Recent evidence has suggested that along

with LPD other endometrial defects could provide an explanation for some unexplained recurrent miscarriages.

1.3.8 The endometrium and recurrent miscarriage

A recent study (Ogasawara *et al.*, 2000) found that the frequency of normal embryonic karyotype is significantly increased with the number of previous miscarriages, suggesting that the maternal factor of implantation and pregnancy becomes increasingly responsible for pregnancy failure as the number of previous miscarriages increases. Investigations into the cause of unexplained recurrent miscarriage have recently focused on these endometrial factors that may be involved in interactions at the maternal-fetal interface. Possible factors include morphological abnormalities (Serle *et al.*, 1994), altered endometrial secretions (Hey *et al.*, 1995; Dalton *et al.*, 1995), abnormal endometrial leukocyte distributions (Clifford *et al.*, 1999; Quenby *et al.*, 1999) and abnormal endometrial cytokine expression (Lim *et al.*, 2000).

Morphological evidence to support this has been provided by studies, which have shown that there was a high incidence of endometrial defect in women who suffer recurrent miscarriage (Tulppala *et al.*, 1991; Li *et al.*, 1998). Morphometrical analysis of the endometrium has also shown significant differences in a subgroup of women suffering from RM compared to normal fertile women (Serle *et al.*, 1994; Saleh *et al.*, 1995).

The use of immunohistochemistry has provided evidence that a possible cause of RM in some women may be due to changes in the composition of certain molecules in endometrial secretions. Expression of four mucin-related secretory epitopes is decreased in RM women (Searle *et al.*, 1994), and a decrease in epithelial secretion of MUC-1 in RM women has also been demonstrated (Hey *et al.*, 1995). The concentrations of MUC-1 and the endometrial secretory protein, placental protein 14 (PP14 or glycodelin) have also been shown to be decreased in the uterine flushings from women who suffer recurrent miscarriage compared to normal controls (Dalton *et al.*, 1995; Hey *et al.*, 1995; Aplin *et al.*, 1996). The plasma concentrations of glycodelin in RM women have also been investigated and found to be decreased when compared to normal controls (Tuppala *et al.*, 1995).

Changes in the population of leukocytes present in the endometrium, particularly during the secretory phase of the menstrual cycle, has also been suggested as a possible factor that may contribute to some unexplained recurrent miscarriages. Quenby *et al.* (1999) showed that there was a significant increase of endometrial leukocytes that were CD4⁺, CD14⁺, CD16⁺, CD56⁺ and MHC Class II⁺ in a group of RM women compared to controls. They also found that in this group of RM women, women who had a further subsequent miscarriage following the study, had an increased number of CD56⁺ staining cells than those whose subsequent pregnancy resulted in a live birth. The increase in endometrial CD56⁺ cells in RM women compared to controls has been confirmed by similar results from another group (Clifford *et al.*, 1999). This study also showed that there was no correlation between the number of CD56⁺ cells and other factors that may influence a further miscarriage such as; maternal age, number of previous miscarriages, past history of a live birth and time since last miscarriage.

The importance of the role of endometrial cytokines in implantation and pregnancy has also been suggested. Lim *et al.* (2000) showed, using RT-PCR, that endometrial expression of IFN γ , IL-2, IL-12 and TNF β was significantly increased in women suffering from RM when compared to normal fertile women, whereas the expression of IL-6 was significantly decreased. These results suggest that the endometrial cytokine profile is altered in some women who suffer unexplained RM and could therefore be a contributory factor to the cause of these miscarriages.

Results from the studies described above, support the notion that endometrial defects are associated with recurrent miscarriage, particularly where no other cause can be determined.

1.4 Cytokines

Cytokines are regulatory proteins secreted by cells in response to a variety of stimuli. They are a diverse group of molecules that are secreted into the extracellular medium and then influence target cells. Their action is similar to that of peptide hormones, except they are not produced by specific endocrine organs, but instead are produced

by more than one cell type in a number of different tissues. They were originally described as products of immune cells (Clemens, 1991). They generally act in a paracrine or autocrine manner but, although it is more unusual, they can also target cells in other parts of the body therefore acting in a similar way to endocrine stimulation produced by classical hormones (Hamblin, 1993).

Cytokines are relatively small molecules with a typical molecular mass of 15-30kDa. They can produce a wide variety of effects and most share common structural and functional features. Some cytokines are modified before they are secreted by the addition of a carbohydrate side chain and are therefore secreted as glycoproteins, whereas others are synthesised as large precursors and therefore require to be cleaved before they can become biologically active. The synthesis and secretion of cytokines is not constant, but instead dependent upon many factors that are capable of inducing and suppressing cytokine production (Clemens, 1991).

Cytokines have pleiotropic properties and are known to produce numerous physiological actions. The physiological roles of cytokines include cell proliferation and differentiation, regulation of hematopoiesis, immune responses, inflammatory responses and fever, control of host defences and cytotoxic/phagocytic cells, wound healing, tissue remodelling, bone formation and influences on cellular metabolism (Hamblin, 1993).

1.4.1 Cytokine receptors

Cytokines are all proteins/glycoproteins and cannot penetrate the plasma cell membrane. They must therefore exert their cellular functions via interactions with specific membrane-bound receptors. Generally cytokine receptors consist of three domains. Firstly the extracellular domain, which provides the binding site for the cytokine and also creates specificity for that particular ligand. Next is the transmembrane domain, which spans the phospholipid bilayer of the plasma membrane, and finally the intracellular or cytoplasmic domain that has either enzymatic activity or binds other molecules in order to deliver a signal inside the cell in response to binding of the cytokine ligand. The extracellular domains of cytokine receptors can be cleaved to produce soluble forms of the receptor (Hilton, 1994).

Most cytokine receptors signal through another molecule and this signalling molecule is often used by more than one cytokine. For example, gp130 is used by all members of the IL-6 family of cytokines, including LIF, IL-11, oncostatin M and IL-6 itself (Taga, 1997). Cytokine activity can be regulated by changes in cytokine concentration and also by modulation of receptors. Following binding of a cytokine to its receptor there is a decrease in the number of cell surface receptors caused by internalisation of the ligand-receptor complexes by endocytosis. This results in a temporary desensitisation to further stimulation and provides a mechanism whereby over-stimulation is prevented by internalisation, limiting the magnitude and duration of the response (Clemens, 1991). It also provides a possible mechanism by which the cytokine produces its effects. The specific responses of a cell to cytokines are controlled by signal transduction mechanisms. These pathways are central to the regulatory actions of cytokines.

1.4.2 T_H1 and T_H2 cytokines

CD4⁺ T-helper cells are a major source of cytokines in the immune system. The production of cytokines from CD4⁺ T-helper cells varies between two subgroups of T-helper cells. Cytokines that are produced by T helper type-1 cells (T_H1 cytokines) include IL-2, IFN γ and TNF β whereas T helper type-2 cells produce cytokines such as IL-4, IL-5, IL-6 and IL-10 (T_H2 cytokines). IFN γ and IL-2 promote the development of cytotoxic lymphocytes and NK cells, as well as inhibiting the development of B-cells in response to T_H2 cells, while IL-4, IL-5 and IL-6 are involved in B-cell development (Mosmann and Coffman, 1989).

1.4.3 Cytokines and pregnancy

The presence of numerous cytokines at the maternal-fetal interface has been shown and the receptors for these cytokines are present on trophoblast cells, suggesting that they may play a role in the growth and survival of the fetoplacental unit (Sharkey, 1998). The exact role of cytokines in maternal-fetal interactions is still poorly understood, particularly in humans, but experiments in mice led Wegmann *et al.* (1993) to propose that "since T_H1 cytokines compromise pregnancy and T_H2 cytokines are produced at the maternal-fetal interface, we hypothesise that these cytokines inhibit T_H1 responses, improving fetal survival but impairing responses

against some pathogens". This has led to numerous further studies into the role of cytokines at the maternal-fetal interface, particularly in mice.

Wegmann's initial experiments showed that injection of IL-10 into abortion-prone mice decreased the abortion rate (Chaouat *et al.*, 1990), while injection of IL-2 and IFN γ into normal mice increased the likelihood of abortion (Chaouat *et al.*, 1990). It was therefore considered possible that excessive T_H1-type cytokines could mediate a cellular response, which would lead to activation of the uterine NK cell population, whereas the T_H2-type cytokines from the fetoplacental unit could divert the maternal response away from T_H1-type responses.

It has also been shown that murine fetoplacental unit tissue *in vitro* spontaneously released IL-4, IL-5 and IL-10 during all three trimesters of pregnancy, whereas the only T_H1 -type cytokine present were small levels of IFN γ during the earliest phase of gestation (Wegmann *et al.*, 1993). These cytokine patterns reflect the T_H1 and T_H2 profiles defined by T-cell clones, however it is likely that at least some, if not the majority, of cytokines synthesised at the maternal-fetal interface are derived from non-T cells. Further studies have since provided more information for a possible role of cytokines at the maternal-fetal interface. IL-10 has been localised to the maternal decidua in mice on day 6 of pregnancy (Lin *et al.*, 1993). IFN γ has been shown to inhibit the secretion of GM-CSF from uterine epithelium (Robertson *et al.*, 1994). Also injections of TNF β , as well as IL-2 and IFN γ , terminated normal murine pregnancies (Chaouat *et al.*, 1990).

1.4.4 Evidence for the T_H1-T_H2 response in humans

The expression of T_H1 and T_H2-type cytokines at the materno-fetal interface has been studied in humans, to determine if the balance between these two groups of cytokines is critical for successful pregnancy outcome. As in mice, it was hypothesised that a decreased expression of T_H2-type cytokines would result in an increase of T_H1-type cytokines at the materno-fetal interface and would lead to impaired fetal growth (Hayes and Smith, 1997).

An increased expression of T_H2 cytokines has been reported in the endometrium, during the mid-secretory phase of the menstrual cycle (Krasnow *et al.*, 1996). This suggests that T_H2-type cytokines are important in the implantation process. There is also evidence to show that both T_H1 and T_H2-type cytokines are present in the developing placenta during human pregnancy (Vince and Johnson, 1996), which suggests that the balance between the expression of these cytokines is important in ensuring successful implantation and pregnancy. Haimovici *et al.* (1991) showed that IFN γ and TNF α inhibited embryonic and fetal development, as well as the proliferation of human trophoblast cell lines *in vitro*, providing further evidence that over expression of T_H1-type cytokines could be detrimental to successful implantation.

The role of T_H1 and T_H2-type cytokines in implantation has been investigated further by studies, which have looked at the expression of T_H1 and T_H2-type cytokines in women who suffer recurrent miscarriage. These studies have shown that there is a significant increase in T_H2-type cytokines and significant decrease in T_H1-type cytokines in normal fertile women when compared to women who suffer recurrent miscarriage (Vives *et al.*, 1999; Makhseed *et al.*, 1999; Lim *et al.*, 2000). Work has also demonstrated that the T_H2-type cytokine IL-4, upregulates production of LIF from decidual T cells, whereas T_H1-type cytokines downregulate LIF production (Piccinni *et al.*, 1998), which is important because LIF is thought to play a role in successful implantation. This study also showed that decidual T cell production of LIF and T_H2-type cytokines was decreased in women suffering recurrent miscarriages. Taken together, the results from these studies provide evidence to suggest that the expression of T_H1 and T_H2-type cytokines is important in human implantation and pregnancy and that it is the balance between T_H1 and T_H2-type cytokines, which is critical for implantation to be successful.

As well as T_H1 and T_H2-type cytokines there are a number of other cytokines that do not fit into these categories but may also have a role in successful pregnancy outcome.

1.5 Leukaemia inhibitory factor (LIF)

LIF is a secreted glycoprotein with a molecular mass of 32-62kDa depending on its state of glycosylation, which depends on the cellular source. The molecular mass following deglycosylation is 20-25kDa and glycosylation of LIF does not appear to affect the biological activity either *in vitro* or *in vivo*. LIF has an anti parallel up-up-down-down four-alpha-helix bundle topology, which is common to haemopoietic growth factors and contains 6 cysteine residues and 3 disulphide bonds (Robinson *et al.*, 1994). The LIF gene has 6290bp, consists of 3 exons and 2 introns (Stahl *et al.*, 1990) and is positioned on chromosome 22 at position 22q12 (Sutherland *et al.*, 1989), with transcription giving rise to 4.2Kbp mRNA (Stahl *et al.*, 1990). LIF was first described as a factor that induced the differentiation of murine myeloid leukaemic M1 cells into macrophages (Tomida *et al.*, 1984) and was later shown to inhibit the proliferation of M1 cells (Gearing *et al.*, 1987). The original source of LIF was alloreactive T cells, but it is now known that other cells are capable of producing LIF. The *in vitro* biological effects of LIF are numerous and include suppression of embryonic stem cell differentiation, enhancement of the survival and proliferation of primitive germ cells, stimulation of acute phase proteins, induction of a cholinergic phenotype in adrenergic neurones, stimulation of the proliferation of factor-dependent haemopoietic cells and enhancement of the generation of megakaryocytes by IL-3 (Hilton, 1992).

Signalling of LIF is mediated via interaction with a specific LIF receptor (LIF-R) (Gearing *et al.*, 1991). The signal is transduced through the 130-kDa membrane glycoprotein gp130, which associates with the LIF receptor following binding of LIF (Robinson *et al.*, 1994). Gp130 is a common signal transducing receptor component known to be involved in signalling from the IL-6 family of cytokines including LIF, IL-11, oncostatin M (OSM) and ciliary neurotrophic factor (CNTF), that are all pleiotropic and exhibit overlapping biological functions (Taga, 1997). Gp130 comprises a 597 amino acid extracellular domain, a 22 amino acid transmembrane domain and an intracellular domain of 277 amino acids (Hibi *et al.*, 1996). Gp130 has no LIF binding capacity by itself, but does play an important role in the formation of high-affinity binding sites by associating with the LIF/LIF-R complex in transduction of the LIF signal. Binding of LIF to LIF-R triggers the

heterodimerisation of the LIF/LIF-R complex with gp130 and together they initiate intracellular signalling, which involves activation of members of the JAK family of cytoplasmic tyrosine kinases (Lutticken, 1994; Stahl *et al.*, 1994). Although gp130 possesses no intrinsic tyrosine kinase domain, the dimerisation of gp130 leads to the activation of associated cytoplasmic tyrosine kinases and subsequent modification of transcription factors (Taga and Kishimoto, 1997).

1.5.1 Role of LIF in implantation

The importance of LIF in implantation was recognised in 1992 (Stewart *et al.*), who showed that knockout mice for the LIF gene produced normal blastocysts, but these blastocysts failed to implant. However, when LIF was administered to these mice the blastocysts implanted normally and developed fully to term, as they did when transferred to pseudopregnant mice. In mice, endometrial LIF expression is greatest in the endometrial glands, on day 4 of pregnancy, which is the time that implantation occurs (Bhatt *et al.*, 1991). The expression of LIF, LIF-R and gp130 has also been shown in the mouse uterus during pregnancy (Yang *et al.*, 1995) further suggesting a role for LIF in murine pregnancy.

1.5.2 Expression of LIF in humans

Expression of LIF by the human endometrium both *in vitro* (Chen *et al.*, 1995; Laird *et al.*, 1997) and *in vivo* has been shown, with an increase in expression during the mid-secretory phase of the cycle (Charnock-Jones *et al.*, 1994; Cullinan *et al.*, 1996; Laird *et al.*, 1997). LIF mRNA has also been localised to the endometrium (Kojima *et al.*, 1994; Vogiagis *et al.*, 1996) as has the LIF-R (Sharkey *et al.*, 1999). All of these studies have shown that expression of LIF is predominantly by endometrial epithelial cells, with little, if any production by stromal cells. Endometrial LIF production has also been shown to be increased by other cytokines (Arici *et al.*, 1997) and *in vivo* production has been shown to be stimulated by steroids such as progesterone (Cameron *et al.*, 1997).

LIF is also expressed by human decidua cells *in vitro* (Sharkey *et al.*, 1999; Hambartsoumian, 1998a) and production by these cells is also under the control of other cytokines and hormones (Sawai *et al.*, 1997). The importance of LIF in human implantation has been further suggested by studies of LIF in women with

reproductive failure. These studies have shown that endometrial LIF expression is decreased in women with unexplained infertility (Laird *et al.*, 1997; Hambartsoumian, 1998b; Giess *et al.*, 1999). One important possible role for LIF within human implantation may be in controlling trophoblast invasion. It has been shown that LIF inhibits the differentiation of cytotrophoblasts to an invasive phenotype by inhibiting the secretion of metalloproteinases and increasing the production of hCG and fibronectin (Bischof *et al.*, 1995a).

1.6 Interleukin-6 (IL-6)

Interleukin-6 is secreted as a glycoprotein with a molecular mass of 21-26kDa, depending upon its cellular source. IL-6 is a member of the same family of cytokines as LIF and has a similar structure. The precursor for IL-6 has 212 amino acids, with a signal sequence of 28 amino acids and the mature IL-6 molecule being a 184 amino acid protein with 2 glycosylation sites (Narazaki and Kishimoto, 1994a). The gene encoding IL-6, situated on chromosome 7 at position 7p21, is 5Kbp in length and consists of 5 exons and 4 introns. Transcription gives rise to 1.2Kb mRNA. The tertiary structure of IL-6 is a four-alpha-helix bundle containing four cysteine residues within the central portion and therefore 2 disulphide bridges (Narazaki and Kishimoto, 1994a). Numerous cells produce IL-6, including T and B-lymphocytes (following stimulation), monocytes, fibroblasts, bone marrow stromal cells, mesangial cells, keratinocytes and endothelial cells. IL-6 is one of the most ubiquitously active cytokines. It is a factor required for terminal differentiation of activated B cells and is also required by B cells to induce antibody secretion. It is a pro-inflammatory cytokine and acts on resting T cells as an activation factor (Taga and Kishimoto, 1997). Other biological actions include the stimulation of acute phase protein synthesis in hepatocytes, a growth factor for B cell tumours such as myelomas, plasmacytomas and hybridomas and the induction of cytotoxic T-lymphocyte differentiation (Hamblin, 1993). IL-6 also plays a central role in megakaryocyte maturation, neuronal differentiation and osteoclast activation (Narazaki and Kishimoto, 1994a).

IL-6 elicits its effect via interaction with the IL-6 receptor (IL-6R), a member of the immunoglobulin superfamily, and the signal transducing molecule gp130. IL-6R has

a molecular weight of approximately 80kDa and consists of 449 amino acids (Narazaki and Kishimoto, 1994b). Binding of IL-6 to IL-6R triggers the association of the IL-6/IL-6R complex with gp130, which then mediates IL-6 function via gp130 generating the IL-6 signal.

1.6.1 IL-6 and reproduction

Studies have shown that IL-6 is produced by the human endometrium *in vitro* and that production may be controlled by other cytokines and hormones (Tabibzadeh *et al.*, 1989; Laird *et al.* 1993; Tseng *et al.*, 1996). Epithelial cells are the major source of IL-6, but stromal cells prepared from the secretory phase of the menstrual cycle have also been shown to produce this cytokine (Laird *et al.*, 1993; Tabibzadeh *et al.*, 1995a). *In vivo* studies have shown that both IL-6 mRNA (Vandermolen and Yang, 1996) and protein (Tabibzadeh *et al.*, 1995a) is expressed in the endometrium and expression appears to increase at the time of implantation, particularly within the epithelial glands. Decidualised stromal cells also produce IL-6 (Montes *et al.*, 1995). The IL-6 receptor and gp130 have also been localised to the human endometrium (Tabibzadeh *et al.*, 1995a) and IL-6R has also been shown to be present in the human embryo (Sharkey, 1998).

1.7 Interleukin-1 (IL-1)

The interleukin-1 family consists of three polypeptides; IL-1 alpha (IL-1 α), IL-1 beta (IL-1 β) and IL-1 receptor antagonist (IL-1ra) that play a central role in the regulation of immune and inflammatory responses. All three cytokines have a molecular weight of approximately 17.5kDa and predominantly have a beta-sheet topology. The precursor forms of IL-1 α and IL-1 β have an amino acid length of 271 and 269 respectively, however, following proteolytic cleavage the mature forms are both 153 amino acids in length. IL-1ra is translated as a precursor with a conventional signal sequence of 25 amino acids and the mature form consisting of 152 amino acid residues. The precursor forms of IL-1 α and IL-1 β are not secreted, but instead accumulate in the cytoplasm where IL-1 α precursor is active but the IL-1 β precursor is not. The active form of IL-1 α is not secreted, whereas active IL-1 β is secreted (Mosley *et al.*, 1987). Both IL-1 α and IL-1 β are known to be produced by monocytes, keratinocytes, B lymphocytes, astrocytes, kidney mesangial cells and

endothelial cells, as well as activated macrophages, which also produce IL-1ra (Dower, 1992). However if the conditions are appropriate almost every cell in the body is capable of producing IL-1 (Clemens, 1991).

The gene encoding IL-1 α is approximately 11Kbp, has 7 exons and 6 introns, is situated at chromosome 2q13 and transcribes a 2.1Kbp mRNA. The gene encoding IL-1 β is 7.5Kbp, has 7 exons and 6 introns, is situated at 2q13-2q21 and has mRNA of approximately 1.6Kbp. IL-1ra gene is 15Kbp but with only four exons and three introns on chromosome 2q14-2q21 and gives rise to a 1.8Kbp mRNA (Dower and Sims, 1994).

The biological effects of IL-1 are numerous and diverse. IL-1 causes proliferation of fibroblasts and can also suppress the expression of matrix protein mRNAs in these cells. IL-1 also causes proliferation of smooth muscle cells and keratinocytes, induces TNF release and adhesion molecule expression and activation in endothelial cells, activates proton pumping in osteoclasts leading to bone resorption and induces metalloprotease secretion in chondrocytes. IL-1 can induce the secretion of acute phase proteins in hepatocytes; induce differentiation of B-cells and proliferation of T_H2 cells in combination with stimulation through the T cell antigen receptor. It can also induce proliferation of mature B-cells, induce cytokine secretion from monocytes and neutrophils and induce insulin secretion and cell death (over a slow period of time) in islet cells (Dinarello, 1991).

1.7.1 Interleukin -1 receptors

Interleukin-1 acts via two receptors, interleukin-1 receptor type I (IL-1RtI) and interleukin-1 receptor type II (IL-1RtII). IL-1RtI is the only functional receptor and IL-1RtII is thought to act as a decoy receptor (Stylianou *et al.*, 1992; Sims *et al.*, 1993), as IL-1 α and IL-1 β can bind to IL-1RtII but no signal is produced because the intracellular domain of this receptor is much shorter than that of IL-1RtI. Both IL-1 α and IL-1 β act as agonists for both receptor types, whereas IL-1ra acts as an antagonist for both receptors, possibly providing a mechanism whereby the biological effects of IL-1 can be suppressed. IL-1RtI consists of 569 amino acids made up of a 20 amino acid signal peptide, a 317 amino acid extracellular domain,

containing three immunoglobulin-like domains, a 22 amino acid transmembrane domain and a 210 amino acid intracellular domain. It has a molecular weight of 80kDa because all of its six potential glycosylation sites are used. The gene encoding IL-1RtI is situated on chromosome 2 at position 2q12 and transcribes a 5Kbp mRNA. IL-1RtII consists of 398 amino acids, consisting of a 13 amino acid signal peptide and a 334 amino acid extracellular domain, which shares 28% homology to the amino acid sequence of the IL-1RtI extracellular domain. It has a molecular weight of 60kDa and consists of a 22 amino acid transmembrane domain and a 29 amino acid intracellular tail, both of which show no similarity to these regions of the IL-1RtI. The lower molecular weight is due to a shorter cytoplasmic region. The gene encoding IL-1RtII is situated on chromosome 2 at position 2q12-2q13 (Sims and Dower, 1994).

The mature forms of IL-1 have affinity for both receptors, although IL-1 α binding has the highest affinity for IL-1RtI and IL-1 β binding has highest affinity for IL-1RtII. The precursor form of IL-1 β does not bind to IL-1RtI, which explains why proIL-1 β has no biological activity. However, the receptor binding and biological activities of the mature and pro forms of IL-1 α appear indistinguishable (Mosley *et al.*, 1987). The effects of both IL-1 α and IL-1 β are similar despite having only 28% homology, but their similarity in biological activity is due to the fact that they both signal through the same receptors.

1.7.2 IL-1 in reproduction

The IL-1 family of cytokines has been shown to be expressed by the human endometrium and they may have a role to play in implantation. Fukuda *et al.* (1995) demonstrated, using ELISA and RT-PCR, that all three members of the IL-1 family of cytokines were expressed by human endometrial cells *in vitro*. Expression of IL-1 α , IL-1 β and IL-1 α protein has also been demonstrated in the human endometrium *in vivo* (Tabibzadeh and Sun, 1992; Kauma *et al.*, 1990; Simon *et al.*, 1993a; Simon *et al.*, 1994a; Simon *et al.*, 1995a). Work in mice has shown that implantation is prevented by blocking IL-1RtI with IL-1 α , suggesting that IL-1 plays an important role in the implantation process (Simon *et al.*, 1994b). IL-1RtI has been shown to be present in the human endometrium throughout the menstrual cycle (Simon *et al.*,

1993a; Simon *et al.*, 1993b) and also within decidual glands and syncytiotrophoblast cells (Simon *et al.*, 1994a). Maternal decidua and villous cytotrophoblast and syncytiotrophoblast cells are also known to express IL-1 β and IL-1ra (Simon *et al.*, 1994a). *In vitro* studies have also shown that IL-1 is capable of stimulating epithelial and stromal IL-6 production (Tabibzadeh *et al.*, 1989; Laird *et al.*, 1994; Vandermolen and Yang, 1996) and stromal and decidual LIF production (Arici *et al.*, 1995; Sawai *et al.*, 1997). This, taken together with the findings that the complete IL-1 family is expressed by the human embryo (De los Santos *et al.*, 1996), supports an autocrine/paracrine role for the IL-1 system in human implantation.

1.8 Tumour Necrosis Factor - alpha (TNF α)

TNF α is a 157 amino acid long polypeptide that is not glycosylated in humans. It has a molecular weight of 17kDa under denaturing conditions but exists as a trimer with a molecular weight of 45-55kDa under native conditions (Aggarwal and Reddy, 1994). It has a trimeric crystal structure consisting of an antiparallel beta-sheet sandwich with edge-to-face "jelly roll" structural motif (Aggarwal, 1992). The gene encoding TNF α is approximately 3.6Kbp with 4 exons and 3 introns, is situated on chromosome 6p21.1-6p21.3 and transcribes a 1.7Kbp mRNA. TNF α was originally identified as a factor produced in *bacillus Calmette-Guerin* primed mice in response to endotoxin and found to be responsible for necrosis of various tumours *in vivo* and is cytotoxic for transformed cell lines *in vitro* (Hamblin, 1993). It is produced by a wide variety of cells in response to bacterial, viral and various other parasitic infections. The major source of TNF α is from macrophages, but it is also produced by other cells including natural cytotoxic cells, T and B-lymphocytes, granulocytes, fibroblasts, mast cells, smooth muscle cells, breast, ovarian and glial tumour cells, astrocytes, Kupffer cells, adipocytes and granulosa cells (Aggarwal and Reddy, 1994).

Biological actions of TNF α include protection of cells from viral infection, activation of granulocytes and macrophages, promotion of bone resorption by osteoclasts and inhibition of collagen synthesis. TNF α also induces cytokine secretion (in particular IL-2 and IL-6) and is known to co-stimulate T cell proliferation and enhance B-cell differentiation and immunoglobulin secretion. It also activates vascular endothelial

cells, promotes angiogenesis, stimulates proliferation of fibroblasts, stimulates acute phase protein synthesis by hepatocytes and induces fever (Hamblin, 1993).

1.8.1 TNF α receptors

There are two receptors for TNF α , TNF α receptor type 1 or type B (p60) and TNF receptor type 2 or type A (p80). The p80 receptor is 461 amino acids in length and consists of a 235 amino acid extracellular domain, a 30 amino acid transmembrane domain and a 174 amino acid intracellular domain. The gene is 17Kbp long, has 10 exons and 9 introns, is situated on chromosome 12p13 and its mRNA is 3Kbp long. The p60 receptor is 455 amino acids long, consisting of a 182 amino acid extracellular domain, a 21 amino acid transmembrane domain and a 233 amino acid intracellular domain. Its gene is situated on chromosome 12p1 and transcribes a 4.5Kbp mRNA. The p60 receptor is primarily expressed on epithelial cells whereas the p80 receptor is primarily expressed on myeloid cells. The extracellular domains of both receptors are rich in cysteine and they have a 25% homologous amino acid sequence. In contrast, the intracellular domains have no homology to each other, or to any other receptors. The majority of functions are mediated through the p60 receptor and this may be due to the fact that the intracellular domain of p60, and not that of p80, contains potential phosphorylation sites for cAMP-dependent protein kinase, protein kinase C and tyrosine kinases. Following binding of TNF α to its receptor it is quickly internalised and degraded. Although it is not clear which signals are mediated through each receptor, it is known that both p60 and p80 activate the transcriptional factor nuclear factor kappa B (NF κ B) (Aggarwal and Reddy, 1994).

1.8.2 TNF α and endometrial function

TNF α has been shown to be present in the human endometrium *in vitro* (Tabibzadeh, 1991). However there are contrasting reports about its presence in the stromal and epithelial compartments throughout the menstrual cycle. Two studies have shown that expression of both mRNA and protein is strong in the stromal compartment during the proliferative phase and becomes variable during the secretory phase. Whereas there was no TNF α present in the glandular epithelium in the proliferative or early secretory phase, but expression was seen during the mid-late secretory phases (Tabibzadeh, 1991; Phillippeaux and Piguet, 1993). However, other studies

have detected TNF α mRNA and protein throughout the menstrual cycle with glandular epithelial TNF α mRNA being high during the proliferative phase, then decreasing in the early proliferative phase before increasing again during the mid-late secretory phases (Hunt *et al.*, 1992; von Wolff *et al.*, 2000). This pattern of TNF α expression has also been shown by cultured endometrial epithelial cells *in vitro* (Laird *et al.*, 1996). Both TNF α receptors (p60 and p80) have been shown to be present in the human endometrium throughout the menstrual cycle (Tabibzadeh *et al.* 1995b) suggesting that TNF α is functional within the endometrium. A role for endometrial TNF α in implantation and formation of the fetoplacental unit has been further implicated by work that has shown that TNF α mRNA is decreased in women who suffer RM (von Wolff *et al.*, 2000). TNF α production by human endometrial epithelial cells is influenced by IL-1 and steroids (Laird *et al.*, 1996) suggesting that it may have a role in the mediation between cytokines and steroids in the endometrium.

1.9 Interleukin-11 (IL-11)

Interleukin 11 is a multifunctional cytokine with a molecular weight of 19kDa and is 178 amino acids in length. The IL-11 precursor is 199 amino acids in length and has a molecular mass of 23kDa (Trepicchio and Dorner, 1998). IL-11 has a similar four-alpha-helix bundle topology to IL-6 and LIF. It lacks any glycosylation sites or cysteine residues, but is a highly stable molecule (Du and Williams, 1997). IL-11 was first identified as a soluble factor released by PU-34 primate bone marrow stromal cells that has the ability to stimulate the proliferation of IL-6 dependent cells (Paul *et al.*, 1990). Human IL-11 cDNA was later cloned from a human fetal lung fibroblast cell line (Paul and Schendel, 1992). The gene for human IL-11 is 7Kbp in size, contains 5 exons and 4 introns and is located on chromosome 19q13.3-19q13.4. Transcription gives rise to two mRNA transcripts of 2.5Kbp and 1.5Kbp (Paul *et al.*, 1990; Du and Williams, 1997), the difference resulting from the presence of additional 3' noncoding sequences within the larger transcript.

1.9.1 IL-11 receptor alpha

IL-11 is another cytokine that signals through gp130 in association with its own receptor interleukin-11 receptor alpha (IL-11R α). IL-11R α is a 422 amino acid

protein that has a genomic region of 9Kbp, consists of 12 exons and 12 introns and is located on chromosome 9p13 (Du and Williams, 1997). IL-11 has similar biological actions to IL-6 in that it acts synergistically with other cytokines (in particular IL-3) in supporting growth of primitive haemopoietic progenitors (Musashi *et al.*, 1991), directly promotes megakaryocyte maturation (Burstein *et al.*, 1992), acts with IL-3 in promoting megakaryocyte colony formation (Yonemura *et al.*, 1992), promotes synthesis of acute phase proteins in hepatocytes and inhibits adipocyte development (Kawashima *et al.*, 1991). Also, like IL-6 but unlike LIF, IL-11 does not prevent embryonic stem cell development but does promote haemopoietic differentiation by developing embryoid bodies in culture (Keller *et al.*, 1993). However, in contrast to IL-6 it does not interact with T-cells (Clark, 1994).

1.9.2 IL-11 and endometrial function

Studies in mice showing that IL-11R α is essential for normal decidual development have suggested that IL-11 is important in implantation and pregnancy (Bilinski *et al.*, 1998; Robb *et al.*, 1998). Female mice with either an inactive or null mutation for the IL-11R α chain are fertile and their blastocysts implant and elicit an initial decidual response. However only small decidua form, which subsequently degrade and lead to unsuccessful implantation and therefore pregnancy. These results suggest the importance of IL-11 in murine endometrial function.

1.10 Matrix metalloproteinases (MMPs)

Matrix metalloproteinases are a family of zinc dependent enzymes that are capable of degrading all components of the extracellular matrix (ECM). They are secreted in a latent form and are then activated by proteolytic cleavage. They can be divided into subgroups depending on their size and substrate specificity into collagenases, gelatinases, stromelysins and membrane-type MMPs (Salamonsen, 1996).

The collagenases include: MMP-1 (interstitial collagenase 1), MMP-8 (interstitial collagenase 2) and MMP-13 (collagenase 3). The gelatinases are: MMP-2 (72kD-metalloproteinase, gelatinase A) and MMP-9 (92kD-metalloproteinase, gelatinase B). The stromelysins include: MMP-3 (stromelysin 1), MMP-10 (stromelysin 2),

MMP-11 (stromelysin 3), MMP-12 (metalloelastase) and MMP-7 (matrilysin) and the membrane type MMPs are: MMP-14 (MT1-MMP), MMP-15 (MT2-MMP), MMP-16 (MT3-MMP) and MMP-17 (MT4-MMP) (Birkedal-Hansen *et al.*, 1993).

1.10.1 Structure of matrix metalloproteinases

All MMPs contain three functional domains, the pre domain at the amino-terminal, followed by the pro domain and then the catalytic domain. With the exception of MMP-7, all other MMPs also contain a fourth functional domain, the haemopexin-like domain, at the carboxy-terminal (Hulboy *et al.*, 1997). The pre domain is the leader sequence, which signals for cellular export. The pro domain consists of 80-90 amino acids and contains a cysteine residue within a highly conserved sequence (PRCGVPDV), adjacent to the active site, which binds zinc. It is the cleavage and removal of this domain that activates the latent, or proMMPs, into their active form (Matrisian, 1990). The catalytic domain contains conserved histidine residues and is the zinc-binding domain. The haemopexin-like domain is involved in mediating associations with ECM components and inhibitors (Hulboy *et al.*, 1997). There is also a short, but variable, hinge region present in MMPs that connects the haemopexin-like domain to the catalytic domain, which has been shown to play a role in substrate binding and in interactions with inhibitors (Massova *et al.*, 1998).

The gelatinases (MMP-2 and MMP-9) possess fibronectin-like sequences within their catalytic domain, which facilitate gelatin binding by these enzymes (Hulboy *et al.*, 1997). The MT-MMPs contain an extra domain, consisting of approximately 25 amino acids, near the carboxy-terminal that localises the enzymes to the plasma membrane (Massova *et al.*, 1998).

1.10.2 Control of MMP activity

MMP activity is regulated at multiple levels including transcription, secretion, activation and inhibition. Inhibition is accomplished by a family of tissue inhibitors of metalloproteinases (TIMPs), of which there are four, TIMP-1, TIMP-2, TIMP-3 and TIMP-4. In general they are widely expressed and are frequently regulated in coordination with MMPs (Hulboy *et al.*, 1997). TIMP-1 is a ubiquitous 28.5 kDa secreted glycoprotein that forms tight stoichiometric non-covalent complexes with the active forms of all MMPs and also with proMMP-2. TIMP-2 is a 21.5 kDa non-

glycosylated protein that has 40% homology to TIMP-1 and similar inhibitory activity against active MMPs, but it preferentially binds proMMP-2. TIMP-3 is a 24 kDa glycoprotein that is different to other TIMPs in that it has an affinity for the ECM (Zhang and Salamonsen, 1997). TIMP-3 and TIMP-4 are not as well characterised at present, but have been shown to inhibit MMP activity (Hulbooy *et al.*, 1997).

Transcription of some MMPs, in particular MMP-1, MMP-3, MMP-7 and MMP-9 is regulated by growth factors, cytokines and hormones (Birkedal Hansen *et al.*, 1993). Most cytokines and growth factors tested have been shown to induce MMP transcription, with the exception of TGF β , which decreases transcription (Birkedal Hansen *et al.*, 1993). Glucocorticoids inhibit MMP synthesis by decreasing transcription (Salamonsen, 1996), and progesterone has been shown to decrease proMMP-1, proMMP-3 and proMMP-7, but increase TIMP-1 and TIMP-2 in progesterone dependent tissues (Salamonsen, 1996). Thyroid hormone, angiotensin II and FSH have all been implicated in the control of MMP activation (Salamonsen, 1996).

1.10.3 MMP production by the endometrium

The presence of MMPs within the endometrium is well documented. MMP-7 has been shown to be present in epithelial cells during the proliferative, late secretory and menstrual phases of the menstrual cycle (Rogers *et al.*, 1993; Rogers *et al.*, 1994), but is not produced by stromal cells. The expression of MMP-2 and MMP-9 in both epithelial and stromal cells *in vivo* and *in vitro* has been reported (Martelli *et al.*, 1993; Rogers *et al.*, 1994; Freitas *et al.*, 1999; Skinner *et al.*, 1999). Other studies have shown the presence of MMP-1 (Salamonsen, 1994; Marbaix *et al.*, 1995), MMP-3 (Jeziorska *et al.*, 1996), MMP-10 and MMP-11 (Rogers *et al.*, 1994) in the endometrium. The exact role of each of these MMPs in endometrial function remains unclear, but increased levels of some MMPs are seen at the time of menstruation suggesting that they may play a role in the degradation of the endometrium at this time (Salamonsen, 1994).

1.10.4 MMPs and implantation

MMPs are also postulated to be involved in the endometrial remodelling associated with embryo implantation. MMP-9 production by the endometrium is increased during the mid-secretory phase of the menstrual cycle (Skinner *et al.*, 1999), which coincides with the implantation window (Johannisson, 1991). Although the expression of TIMPs by the endometrium does not alter dramatically through the menstrual cycle (Zhang and Salamonsen, 1997) the expression of TIMPs is increased in decidualised stromal cells (Zhang and Salamonsen, 1997). The invasive trophoblast cells invading the maternal decidual cells also produce MMP-9 (Bischof *et al.*, 1995a;), MMP-2 (Bischof *et al.*, 1991; Fernandez *et al.*, 1992) and MMP-14 (Nawrocki *et al.*, 1995; Hurskainen *et al.*, 1996). This is in contrast to the non-invasive trophoblast cells, which do not produce MMPs. These results suggest that MMPs do play an important role in the implantation process.

1.10.5 Control of endometrial MMP production

The control of MMP production by human endometrium is less well understood. Several studies have suggested that progesterone and oestrogen suppress MMP production (Osteen *et al.*, 1994; Osteen *et al.*, 1997; Singer *et al.*, 1997; Skinner *et al.*, 1999) and that the reduction of steroid hormone levels at the end of the cycle results in the increased MMP production associated with menstruation. More recent work has suggested that the expression of endometrial and trophoblastic MMPs are regulated by various cytokines (Rawdanowicz *et al.*, 1994; Bischof *et al.*, 1995a; Singer *et al.*, 1999; Meisser *et al.*, 1999a), particularly IL-1, TNF- α and LIF. These cytokines in particular are known to be important in the control of endometrial function and embryo implantation (Stewart *et al.*, 1992; Hunt *et al.*, 1992; Sharkey, 1998; Simon *et al.*, 1998); thus one of their roles in implantation may be to control MMP production.

Chapter 2

Materials and Methods

2.1 Human subjects

2.1.1 Normal fertile women

Endometrial biopsies were obtained, using a Sharman's curette (Dawns Surgical Ltd, Sheffield, U.K.), from women attending the Jessop Hospital for Women, Sheffield, U.K. for non-endometrial pathology. All subjects gave their informed consent to participate in the study and local ethical committee approval was obtained. All of the women were aged between 25 and 40 and had regular menstrual cycles of between 25 and 35 days.

Endocrinological investigation showed evidence of ovulation. All subjects had normal uterine anatomy, had no steroid treatment for at least 2 months prior to the study and were all of proven fertility. The day of the cycle in which the biopsy was taken was calculated from the date of the last menstrual period.

2.1.2 Women with unexplained recurrent miscarriage

Endometrial biopsies were obtained, using a Pipelle sampler (Unimar, Nevilly-en-Thelle, France), from women attending the recurrent miscarriage clinic at Jessop Hospital for Women, Sheffield, U.K. All subjects gave their informed consent to participate in the study and local ethical committee approval was obtained. All women had suffered at least three or more consecutive miscarriages, were aged between 25 and 40 and had menstrual cycles of between 25 and 35 days. They all had normal uterine anatomy and showed no evidence of anticardiolipin or lupus antibodies. The day in the cycle that the biopsies were obtained were all calculated from the day of the LH surge and were all obtained between days LH+6 and LH+10.

2.1.3 Collection of biopsies

Endometrial biopsy samples were collected directly from the operating theatre or outpatients clinic and taken to the laboratory. Samples used for immunocytochemistry or RT-PCR analysis were snap frozen in liquid nitrogen.

Samples used for cell culture were collected in Hanks balanced salt solution (Sigma, Poole, U.K.) and used immediately.

2.2 Cryostat sectioning

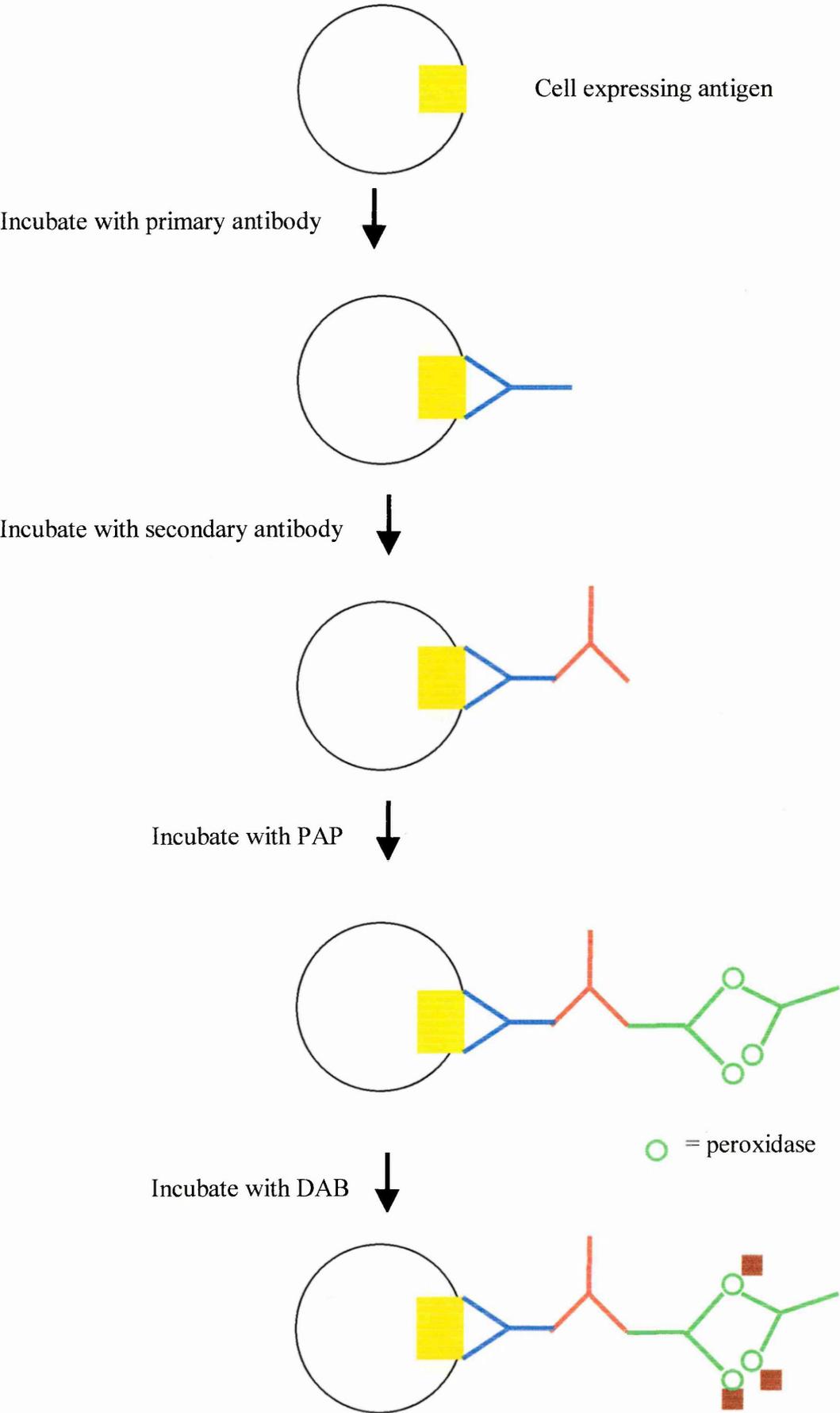
All reagents were obtained from Sigma, Poole, U.K. Biopsies were transported from storage in liquid nitrogen to ensure that they did not thaw. A Leica cryostat was used to cut the sections and a temperature of -20°C was maintained throughout the entire procedure. Each biopsy was removed from liquid nitrogen and attached to a holder using Cryo-M-Bed embedding compound (Bright Instrument Company Ltd, Huntington, U.K.). Once the embedding compound had frozen, serial cryostat sections of $15\ \mu\text{m}$ were obtained from each biopsy. Serial sections were placed on each microscope slide and the duplicate section used as a negative control. After cutting, the sections were immediately fixed in 3.7% (w/v) paraformaldehyde in phosphate buffered saline (PBS) for 15 min at room temperature. The sections were then washed twice for 5 min in PBS and fixed in methanol for 4 min then acetone for 2 min, both at -20°C , before being washed again for $2 \times 5\text{min}$ in PBS and stored in 8% (w/v) sucrose solution at -20°C until they were used.

2.3 Immunocytochemistry

2.3.1 Principles of immunocytochemistry

Immunocytochemistry uses antibodies to specifically bind to antigens present within a cell. A substrate is then used to visualise where the antibodies have bound. The immunostaining technique used in these studies is known as the PAP procedure (figure 2.1). Sections of tissue are incubated with a primary antibody, which will bind specifically to any antigen that is present within the cells. The sections are then incubated with a secondary antibody specific for the primary antibody in order to amplify the binding. Peroxidase anti-peroxidase (PAP) is then applied to the sections, which will bind to the secondary antibody. The sections are then incubated with a substrate that can be identified due to its colour, which in this case was diaminobenzadinetetrachloride (DAB) that produces a brown colouring. This step allows the binding to be visualised. A counterstain is then used to visualise any cells that do not contain the antigen.

Figure 2.1: Principles of immunocytochemistry using the PAP procedure



2.3.2 Immunocytochemistry procedure

All reagents were obtained from DAKO, Ely, U.K., unless otherwise stated. Sections were washed twice for 5 min in PBS to remove sucrose solution and then quenched in 3% (v/v) hydrogen peroxide (Sigma, Poole, U.K.) in methanol (Sigma, Poole, U.K.) for 10min at room temperature, before being blocked with 10% (v/v) normal rabbit serum in PBS (Sigma, Poole, U.K.) for 30 min at room temperature. Sections were then incubated with a primary antibody for each of the cytokines either overnight at +4°C or for 1h at room temperature. The sections were then incubated with secondary antibody for 30 min at room temperature. Primary and secondary antibodies were all diluted in 10% normal rabbit serum.

Sections were then incubated with a 1:100 dilution of mouse or goat peroxidase-anti-peroxidase (PAP), in PBS, for 30 min at room temperature. Both the secondary antibody and PAP stages were then repeated when staining for LIF, IL-6 IL-11 and IL-11R α , in order to amplify the staining. Diaminobenzadine tetrachloride (DAB) substrate (Vector Laboratories, Peterborough, U.K.) was then used to visualise antibody binding and the sections counterstained with haematoxylin (Vector, U.K.). Sections were then dehydrated through 50%, 70% and 90% alcohol (BDH, Poole, U.K.) for 5min each and then 95% and 2 x absolute alcohol for 10 min each before being cleared in Xylene (BDH, Poole, U.K.) overnight and mounted with a cover slip using DePeX (BDH, Poole, U.K.). All incubations were carried out in a humid chamber and two five-minute washes in PBS were carried out between each of the incubations. A negative control was run parallel to each section where the primary antibody had previously been neutralised following an overnight incubation with a blocking peptide (Santa Cruz Biotechnology, Inc., U.K.). For each antibody staining was repeated on at least 3 different sections from each biopsy.

2.4 Quantification of staining

Two methods were used to quantify the amount and intensity of staining obtained using immunocytochemistry.

2.4.1 Semi-quantitative analysis

Two investigators, independent of one another, assessed all staining semi-quantitatively. Intensity of staining was graded on a scale of 0 (-) to 4 (++++), where – represented negative staining, -/+ represented negative to weak staining, + represented weak staining, +/+ represented weak to moderate staining, ++ represented moderate staining, ++/+ represented moderate to strong staining, +++ represented strong staining, +++/+ represented strong to very strong staining and ++++ represented very strong staining. Overall there was good agreement on the grade given for each section by each assessor. On the rare occasion that differences occurred these sections were re-examined and a grade was obtained by consensus.

2.4.2 Quantification using image-capture analysis

Staining for LIF, IL-6 and IL-1 was also assessed using digital image software. For each section five fields were captured using a Neotech Image Grabber (version 1.2). Three epithelial and three stromal areas were then marked in each field and the average intensity for each area calculated using Jandel Sigma Scan Image Measurement Software. The average intensity was calculated as the total intensity of the area divided by the number of pixels that the area covered. This provided fifteen intensities for epithelial staining and fifteen intensities for stromal staining in each section. The mean intensity for epithelial and stromal staining was then calculated for each section. This method is described in more detail in chapter 3.

2.5 Tissue culture

All materials were obtained from Sigma, U.K. unless otherwise stated. Endometrial epithelial and stromal cells were prepared and cultured using an established method (Laird *et al.*, 1993; Laird *et al.*, 1994; Laird *et al.*, 1997). Endometrial tissue was collected immediately into Hanks balanced salt solution containing streptomycin (100µg/ml) and penicillin (100µg/ml). The tissue was then chopped finely and incubated in Dulbecco's modified Eagle's medium containing 0.1% (w/v) type 1a collagenase (DMEMC), for 45 min at 37°C with gentle pipetting every 15 min to disperse the cells. The cell suspension was then centrifuged for 10 min at 100 x g, leaving a pellet containing epithelial cells and supernatant containing the stromal cells. The epithelial cells were then incubated and pipetted for a further 45 min at

37°C in DMEM before repeating the centrifugation. Following centrifugation the epithelial cells were resuspended in 2 ml DMEM (containing 2% (w/v) glutamine, streptomycin/penicillin (100 µg/ml) and 10% (v/v) foetal bovine serum) (CDMEM). The supernatants containing stromal cells were centrifuged for 5 min at 300 x g. The resulting supernatant was discarded and the pellet of stromal cells was resuspended in 2 ml CDMEM.

For each cell type a density gradient was performed where the cell suspensions were layered over 8 ml CDMEM and left for 30 min, allowing the larger epithelial cells and gland fragments to fall to the lower 2 ml and the smaller stromal cells to remain in suspension in the upper 8 ml. For the epithelial cells the upper 8 ml was discarded and the lower 2 ml used for cell culture, whereas for the stromal cells the upper 8 ml were used for cell culture and the lower 2 ml discarded. Each cell type was plated out at a density of 1×10^5 cells/ml into 96 well plates, to study the effects of cytokines on cultured cells, or 2.5×10^5 cells/ml into tissue culture flasks, for mRNA studies using RT-PCR analysis. Cells were then incubated at 37°C in an atmosphere containing 5% CO₂ and 95% air until confluent. This was usually 48 h when cultured in plates and 5-7 days when cultured in flasks.

When the stromal and epithelial cells cultured in 96 well plates reached confluency media was replaced with CDMEM containing either no additions (controls) or cytokines (0.1-10 ng/ml) in replicate wells for each concentration. After a further period of 48 h the media was removed and stored at -20°C for analysis by zymography or ELISA. The number of plates of stromal and epithelial cells prepared from a single biopsy varied depending on the size of the biopsy; between 1-3 plates of epithelial and 1-2 plates of stromal cells were normally obtained for each biopsy. Therefore, cells prepared from a single biopsy could be incubated with more than one different type of cytokine.

2.6 Preparation of endometrial biopsy material and cultured endometrial cells for analysis of cytokines using RT-PCR

2.6.1 Principles of RT-PCR

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) is a two-step process that can be used to identify the presence of messenger RNA (mRNA). The first step uses reverse transcriptase (RT) to make a DNA sequence that is complementary (cDNA) in base sequence to the mRNA. This step is required because the polymerase used in PCR will only amplify DNA. PCR is then used to selectively amplify the defined cDNA produced from the RT step. To enable specific amplification of cDNA, two oligonucleotide primer sequences are designed that, when added to the cDNA, will bind specifically to complementary DNA sequences immediately flanking the desired target region. The new DNA strands, which are complementary to the original cDNA, are synthesised in the presence of DNA polymerase and four deoxynucleoside triphosphates (dATP, dCTP, dGTP and dTTP). This technique is termed "chain reaction" because the newly synthesised DNA strands then act as a template for further DNA synthesis in subsequent cycles. After about 30 cycles, the amount of DNA synthesised is about 10^5 copies of the original cDNA sequence targeted by the primers, which is then enough to visualise as a band of a specific size when run on agarose gel electrophoresis.

2.6.2 Preparation of cells for RNA extraction

Endometrial cells were grown in flasks as described, until confluent. Once confluent the media was removed from the cells and PBS added to the flask to wash the cells. The PBS was then removed and cell dissociation solution (containing EDTA, glycerol and sodium citrate in PBS) (Sigma, Poole, U.K.) added for 10 min at 37°C. The cell dissociation fluid is a gentler method than trypsin and EDTA for removing cells. The cell dissociation solution was then discarded and CDMEM was pipetted gently over the cells removing them from the bottom of the flask into cell suspension. The cell suspension was then centrifuged at 300 x g for 5 min and the supernatant discarded.

2.6.3 Preparation of endometrial biopsies for RNA extraction

Endometrial biopsies were treated with a dismembrator, where tissue and a ball bearing are placed inside a Teflon tube, which is held tightly in place and shaken vigorously until the tissue is broken up. This ensured the biopsies were broken up, without thawing, in preparation for the RNA extraction. Thawing would cause mRNA breakdown because of the action of RNAases. The biopsies were moved directly from liquid nitrogen into Teflon tubes that had been chilled in liquid nitrogen. A ball bearing, also chilled in liquid nitrogen, was also added to the tube and the top replaced. The tube was then placed in the dismembrator and vigorously shaken for approximately 1 min. 1 ml of TRIzol reagent (Sigma, Poole, U.K.) was then added to the homogenised biopsy and mixed thoroughly before being frozen and stored in liquid nitrogen.

2.6.4 RNA extraction

Following preparation, 1 ml of TRIzol Reagent was added to the endometrial cell pellets and left on ice for 5 min. Endometrial biopsies previously prepared with TRIzol reagent were thawed on ice. 100 µl of ice-cold chloroform was then added to the tubes and mixed well before sitting on ice for 10 min. The tubes were then centrifuged at 12000 rpm for 15 min at +4°C. The top aqueous layer (approximately 500 µl) was then removed and placed into a new tube with an equal amount of ice-cold isopropanol and left on ice for 1 h. The centrifugation was then repeated at 12000 rpm for 15 min at +4°C, the supernatant was discarded and the pellet was resuspended in 250 µl 80% ethanol. The resuspended pellets were then centrifuged at 12000 rpm for 5 min at +4°C and the supernatant removed. The pellets were then air-dried for approximately 10 min and resuspended in 20-50 µl sterile H₂O.

2.6.5 Analysis of endometrial mRNA

The RNA concentration for each sample was calculated by measuring the optical density at 260 nm. The resulting figure was then put into the following equation to give the RNA concentration:

$$\text{RNA concentration } (\mu\text{g}/\mu\text{l}) = (\text{optical density at 260 nm} \times \text{dilution factor}) \times 0.04$$

The samples were then diluted to ensure an RNA concentration of approximately 1 $\mu\text{g}/\mu\text{l}$ was obtained.

The ratio of the optical density at 260 nm:optical density at 280 nm was also calculated. This value reflected the purity of the mRNA in the sample, with a value of 2.0 being the purest.

2.7 Reverse Transcriptase – Polymerase Chain Reaction (RT-PCR)

2.7.1 RT

All materials were obtained from Gibco, U.K. For each sample a 36 μl mastermix consisting of 8 μl 5 x RT buffer, 8 μl 5 x KCl, 16 μl dNTP's (containing equal amounts of dGTP, dATP, dCTP and dTTP), 1.2 μl oligo dT, 1.2 μl RT and 1.6 μl sterile H_2O was added to 4 μl of RNA (1 $\mu\text{g}/\mu\text{l}$). The mixture was then overlaid with 40 μl of mineral oil and thermocycled at 37.5°C for 1 h, 99°C for 5 min and finally 4°C. The samples were then removed and stored at -20°C. Two controls were run parallel to each sample. The first contained only sterile H_2O in replacement of RNA (negative control) and the second contained sterile H_2O in replacement of the RT enzyme (no RT control).

2.7.2 PCR

All materials were obtained from Gibco, U.K. PCR was used to detect any mRNA present in the endometrial cells and biopsies for LIF, IL-6, IL-1 β and TNF α . Initial analysis was carried out using the housekeeping gene 7B6, to detect whether the RNA extraction was successful and if there was any genomic DNA contamination and also to ensure that adequate mRNA had been obtained. All primers were synthesised in-house. The sequences for all primers, with the exception of those for LIF, were obtained from previous studies (Francis and Duff, 1993; Estdale *et al.*, 1996). The primer sequences for LIF were designed using Oligo™ Primer Analysis Software (version 3.4). This software enables the generation of primer sequences at specific points along the DNA sequence of interest and subsequent calculation of the optimum annealing temperature for the PCR reaction. Furthermore it predicts the potentially negative effects of complementary annealing between primers and the

possibility of primer hairpin loops forming. The sequences for all of the primers used are as follows:

7B6	Forward	5' AGC CGT AGA CGG AAC TTC CA 3'
	Reverse	5' CTA AAA CAG CGG AAG CGG T 3'
LIF	Forward	5' GAG TTG TGC CCC TGC TGT TG 3'
	Reverse	5' ATC CCT CCG TTC ACA GCA CA 3'
IL-6	Forward	5' CCA CAC AGA CAG CCA CTC ACC 3'
	Reverse	5' GGC TTG TTC CTC ACT ACT CTC 3'
IL-1 β	Forward	5' CGA CAC ATG GCA TAA CGA GC 3'
	Reverse	5' CAT CTT TCA ACA CGC AGG AC 3'
TNF α	Forward	5' GAG TGA CAA GCC TGT AGC CC 3'
	Reverse	5' CCC AGA TAG ATG GGC TCA TA 3'

All primers were used at a concentration of 20 μ M. For each sample a 20 μ l mastermix was added to 5 μ l cDNA. However, the amounts of reagents varied for each PCR. The mastermix for 7B6 consisted of 15.25 μ l sterile H₂O, 2.5 μ l 10 x PCR buffer, 0.5 μ l MgCl₂, 0.75 μ l forward primer, 0.75 μ l reverse primer and 0.25 μ l Taq polymerase. The mastermixes for LIF and IL-6 consisted of 15.85 μ l sterile H₂O, 2.5 μ l 10 x PCR buffer, 0.4 μ l MgCl₂, 0.5 μ l forward primer, 0.5 μ l reverse primer and 0.25 μ l Taq polymerase and the mastermixes for IL-1 β and TNF α consisted of 15.75 μ l sterile H₂O, 2.5 μ l 10 x PCR buffer, 0.5 μ l MgCl₂, 0.5 μ l forward primer, 0.5 μ l reverse primer and 0.25 μ l Taq polymerase. The samples containing the mastermix were then overlaid with 40 μ l mineral oil and thermocycled. For each pair of primers the thermocycle program was optimised. This involved repeating the program using different annealing temperatures and variations in the number of cycles used in each program.

The optimum thermocycle programs used were as follows:

	<u>7B6</u>	<u>LIF</u>	<u>IL-6</u>	<u>IL-1β</u>	<u>TNFα</u>
1. Denaturation of DNA					
5 min	95°C	95°C	95°C	95°C	95°C

	<u>7B6</u>	<u>LIF</u>	<u>IL-6</u>	<u>IL-1β</u>	<u>TNFα</u>
2. Denaturation of DNA					
1min	95°C	95°C	95°C	95°C	95°C
3. Primer reannealing					
1min	57°C	62°C	53°C	55.9°C	59.9°C
4. DNA synthesis					
1min	72°C	72°C	72°C	72°C	72°C
Cycles of 2, 3 and 4	x35	x35	x35	x35	x35
5. Completion of synthesis					
5min	72°C	72°C	72°C	72°C	72°C
6. Completion of annealing					
5min	55°C	55°C	55°C	55°C	55°C
7. Holding Temperature	4°C	4°C	4°C	4°C	4°C

2.7.3 Electrophoresis

Agarose gel electrophoresis was used to separate the PCR products on the basis of fragment size. 1g agarose was dissolved in 50ml 1 x TBE (10.8% w/v Tris, 5.5% w/v boric acid and 0.93% EDTA) and 2 μ l ethidium bromide added. The gel mixture was then poured into the gel plate and left to set around a comb, giving a set number of wells that are required. The gel was then placed in a tank containing 1 x TBE and the PCR products added to the wells. Each PCR product (10 μ l) was mixed with 6 μ l loading buffer, and 10 μ l of this mixture added to the appropriate well. A marker [ϕ F174-HaeIII] (Sigma, Poole, U.K.) was run parallel to the PCR products in every gel, and was used to determine the size of any product seen. Gels were run at 100-110V for 40-50 min.

The gels were visualised under an ultraviolet trans-illuminator. Bands could be seen due to the ethidium bromide being intercalated within the DNA fragments and fluorescing under the UV light. Images were recorded using a Polaroid film camera.

2.8 Analysis of MMP secretion into media

MMP production by cultured stromal and epithelial cells was assessed using zymography and ELISA.

2.8.1 Zymography

Zymography is a technique that can identify enzymes by separating them according to their size, using SDS-PAGE (Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis). SDS is an anionic detergent that disrupts nearly all non-covalent interactions in naïve proteins. Following denaturation with SDS, the larger the protein is, the more SDS they bind and the more negatively charged they become. An electrical current is passed through the gel and proteins are fractionated according to their overall charge, the smaller the protein, the further it will travel down the gel. In zymography a protein substrate specific for the enzyme that is to be detected, is incorporated into the gel. MMP-2 and MMP-9 are gelatinases, so gelatin is added to the gel. Once the gel has been run, the SDS is washed out of the gel to prevent the enzymes from being denatured and the gel is incubated overnight. This allows the gelatinases to digest the gelatin, leaving an area where the gelatin has been digested. When the gel is then stained with Coomassie blue, all of the gel is dyed blue except the areas where there is no gelatin present, indicating the presence of the gelatinases.

All materials were obtained from Sigma, UK., unless otherwise stated. A 7% acrylamide lower gel solution was prepared containing 1.4 ml Bis (2.6% w/v) acrylamide (40% w/v) (BDH, Poole, U.K.), 1.8 ml Tris buffer solution (1.5M Tris, 0.4% SDS, pH 8.8), 0.8 ml gelatin (1% w/v) and 4 ml distilled H₂O. 15 µl TMED and 50µl ammonium persulphate (10% w/v) was then added and the gel solution poured between two glass plates. TMED and ammonium persulphate cause the Bis acrylamide to polymerise forming a polyacrylamide gel matrix. Once the gel has set, an upper gel solution was poured on top of the lower gel. The upper gel solution contained 0.59 ml Bis (2.6% w/v) acrylamide (40% w/v), 0.75 ml Tris buffer solution (0.5M Tris, 0.4% SDS, pH 6.8). 1.69 ml distilled H₂O, 15 µl TMED and 50 µl ammonium persulphate (10% w/v). A comb with 10 wells was then placed in the top of the gel and the gel left to set around it. Once the gel had set, the comb was removed leaving 10 wells at the top of the gel. The gels (still in glass plates) were

then placed into a tank containing a running buffer (2M glycine, 1% SDS, 0.25M Tris HCl).

5µl of conditioned cell culture media was added to 5µl of non-reducing buffer and added to each well. A molecular weight marker was run parallel to each of the samples containing bands between 205kDa and 36kDa. Supernatants from the D3X human melanoma cell line (a gift from Dr A. Cross, Sheffield Hallam University) were used as a positive control for MMP-2 and MMP-9 and were also run in each gel. After electrophoresis the gels were incubated in 2.5% (v/v) Triton-X-100 for 3 x 30min washes at 37°C before being incubated in 0.25 M Tris buffer containing 1M NaCl and 25mM CaCl₂ for 24h. The gels were then stained with 0.5% (w/v) Coomassie blue R-250 in 7% (v/v) acetic acid, 50% (v/v) methanol and destained in 7% (v/v) acetic acid, 30% (v/v) methanol. Individual gels were run with supernatants taken from cells incubated with each concentration of cytokine. Zymography was repeated at least 3 times for each of twelve different experiments to ensure a consistent result was obtained.

2.8.2 Densitometry

Bands representing MMP-2 and MMP-9 in the zymography gels were measured quantitatively using densitometric analysis. The net intensity of each band was measured using Kodak Digital Science™ 1D Image Analysis Software. The intensity of the band for each different concentration of cytokine was compared to the control band on each gel. As intensities for the control band varied greatly between experiments the results were recorded as a percentage change from the control value. For each of the cytokines tested a minimum of 3 gels for each experiment were run. This was repeated for each of the 3 different biopsies and the mean results for each cytokine concentration was calculated.

2.8.3 ELISA for MMPs

The amounts of MMP-2, MMP-9 and MMP-7 produced by cultured endometrial epithelial and stromal cells was measured quantitatively using commercially available BIOTRA ELISA kits (Amersham Life Sciences, U.K.) according to the manufacturer's instructions. The MMP-2 assay kit detected latent, active and TIMP-

bound forms of MMP-2 while the MMP-9 kit did not detect active MMP-9 but did detect latent and TIMP-bound forms and the MMP-7 kit only detected the latent form. The sensitivity of the assays was 0.37 ng/ml for MMP-2, 0.6 ng/ml for MMP-9 and 0.16 ng/ml for MMP-7. The intra-assay variation range was 5.7%, 5.2% and 3.5% while the inter-assay variation was 10.0%, 8.8% and 6.9% for MMP-2, MMP-9 and MMP-7 respectively. As these experiments were carried out in CDMEM containing non-heat inactivated serum, CDMEM was run as a control parallel to the samples tested using ELISA. Samples were diluted 1:50 for MMP-2 and 1:20 for MMP-7 in assay buffer to ensure the metalloproteinase concentration was within the range of the standard curve. The absorbance was measured using a Wallac 1420 multilabel counter at 450nm, as instructed by the manufacturer. ELISA measurements were performed on each of four replicate samples for both epithelial and stromal cell cultures, for each concentration of cytokine.

2.9 ELISA for IL-11

The amount of IL-11 produced from cultured epithelial and stromal cells was measured quantitatively using a commercially available Quantikine® ELISA kit (R+D Systems, Abingdon, U.K.). The kit used an anti-human IL-11 antibody conjugated to horseradish peroxidase to detect IL-11 and the colour reagent used was tetramethylbenzidine. The sensitivity of the assay was 8.0 pg/ml with an intra-assay variation range of 2.4% and an inter-assay variation of 6.9%. Again, CDMEM was run as a parallel control to the samples tested using ELISA. Epithelial samples were diluted 1:10 and stromal samples diluted 1:5 in calibration diluent to ensure the cytokine concentration was within the range of the standard curve. The absorbance was measured at 450 nm and 550 nm using a Wallac 1420 multilabel counter, as instructed by the manufacturer. The total values were calculated as absorbance at 450 nm minus the absorbance at 550 nm in order to correct for optical imperfections in the plate.

2.10 ELISA for TNF α and IL-1 β

The amount of TNF α and IL-1 β produced from cultured epithelial and stromal cells was measured quantitatively using commercially available DuoSet ELISA Development systems (R+D Systems, Abingdon, U.K.). A 96 well plate was coated

with the capture antibody provided (mouse anti-human TNF α or IL-1 β) and incubated overnight at room temperature. The wells were then washed with wash buffer (0.05% Tween 20 in PBS) before adding 300 μ l block buffer (1% BSA, 5% sucrose in PBS with 0.05% NaN₃) into each well for 1h at room temperature. Following another wash, 100 μ l of cell culture supernatant was then added to each well and incubated for 2 h at room temperature. For TNF α the cell culture supernatants were diluted 1:50 in assay diluent to ensure that the values fell on the standard curve. The wells were then washed again and 100 μ l of detection antibody (biotinylated goat anti-human TNF α or IL-1 β) was added to each well and incubated for 2 h at room temperature. The wells were then washed again, before adding 100 μ l of streptavidin (conjugated to horseradish-peroxidase) to each well for 20 min at room temperature. Following a final wash, 100 μ l of substrate solution (1:1 mixture of H₂O₂ and tetramethylbenzidine) was added to each well for 20 min at room temperature before adding 50 μ l of stop solution (2M H₂SO₄). The absorbance was measured at 450 nm using a Wallac 1420 multilabel counter. Absorbance at 550nm was not measured in this case as it was not advised by the manufacturer. Excellent standard curves and duplication of results was obtained by measurement at 450nm only.

2.11 Bioassay for TNF α

TNF α production from cultured epithelial and stromal cells was also measured quantitatively using cytotoxic bioassay. This assay utilises the cytotoxic action of TNF α on pre-sensitised murine aneuploid fibrosarcoma cells (L929). These cells are sensitised to TNF α by treatment with actinomycin D.

L929 cells were plated into the wells of a 96-well plate at 2×10^5 cells/ml. The cells were then incubated at 37°C in an atmosphere of 5% CO₂ for 24 h, until confluency had almost been reached. Nine TNF α standards between 0.91 and 0.0018 ng/ml were prepared and 100 μ l of each added to 3 replicate wells. Supernatants from cultured epithelial cells were added in duplicate. The supernatants were diluted 1:5 in cell culture media to ensure that the values fell on the standard curve. 4 μ l of actinomycin D (0.1 mg/ml) was then added to every well to give a final concentration of 2 μ g/ml

in each well. The plates were then incubated for a further 24 h at 37°C under an atmosphere of 5% CO₂. The cell media was then removed from each well and the cells washed with 200 µl PBS. Cells were then fixed with 200 µl methanol for 15 min. The methanol was then removed and the plates air dried. The plates were then stained with crystal violet (0.1% solution in 200 mM boric acid), 200 µl/well for 20 min. The plates were then washed three times in distilled H₂O and the stained cell layer solubilised in 50 µl 10% (v/v) glacial acetic acid. The plate was then incubated for 30 min in a gas incubator to achieve dissolution. The absorbance of each well was then read at 570 nm.

The absorbance readings were inversely proportional to the amount of TNFα present. Higher amounts of TNFα caused a greater cytotoxic effect on the cells and therefore less crystal violet staining was seen, due to the presence of fewer cells, resulting in a lower absorbance reading.

2.12 Statistical analysis

Data was expressed as group means ± SEM (n = 4, number of replicate wells from a single experiment). Results shown are those obtained from cells prepared from a single biopsy and are typical of those obtained on each of the 3 occasions that the experiment was repeated. Differences in the amounts of MMP produced in the presence of different cytokines were analysed using ANOVA.

Chapter 3

Endometrial cytokine expression in normal fertile women

3.1 Introduction

The exact role of cytokines in human endometrial function still remains to be determined, but it is thought that the paracrine and autocrine action of cytokines provides local specificity to the regulation of implantation and uterine function. The actions of cytokines allows different cell types within the endometrium to have separate but co-ordinated growth and differentiation and also provides a means of mediating the embryonic-maternal interactions required for successful development and implantation of a blastocyst. Although many cytokines have been shown to be present within the endometrium this work has focused on LIF, IL-6, IL-1 and TNF α .

3.1.1 Leukaemia Inhibitory Factor

LIF has been shown to be essential for implantation in mice (Stewart *et al.*, 1992) and more recent work has suggested that it may also be important in implantation in humans. LIF is known to be expressed by endometrial epithelial and stromal cells (Charnock-Jones *et al.*, 1994; Vogiagis *et al.*, 1996; Cullinan *et al.*, 1996), decidual cells (Sawai *et al.*, 1997) and villous and extravillous trophoblast cells (Sharkey *et al.*, 1999). It has also been detected in endometrial flushings (Laird *et al.*, 1997) and mRNA for both LIF and its receptor has been identified in pre-implantation blastocysts (Charnock-Jones *et al.*, 1994; Chen *et al.*, 1999) suggesting an important role of LIF in human implantation.

3.1.2 Interleukin-6

IL-6 has also been shown to be present in human endometrial epithelial and stromal cells (Tabibzadeh *et al.*, 1995a; Vandermolen and Yang., 1996; Laird *et al.*, 1993), but expression is greater in epithelium. IL-6 is also produced by decidual cells (Montes *et al.*, 1995) and the IL-6 receptor is present in both the human embryo and

glandular epithelium with mRNA expression being increased at the time of implantation (Sharkey, 1998).

3.1.3 Interleukin-1

IL-1 α and IL-1 β protein and mRNA have been shown to be expressed by human endometrial cells (Kauma *et al.*, 1990; Tabibzadeh and Sun, 1992; Simon *et al.*, 1993a), decidual cells (Simon *et al.*, 1994a), placental cells (Simon *et al.*, 1994a; Simon *et al.*, 1995b) and pre-implantation embryos (De los Santos *et al.*, 1996). The antagonist for these cytokines, IL-1ra, has also been reported to be produced by endometrial cells (Fukuda *et al.*, 1995; Simon *et al.*, 1995a), decidual cells (Simon *et al.*, 1994a), trophoblast cells (Simon *et al.*, 1994a) and pre-implantation embryos (De los Santos *et al.*, 1996). It has also been shown that endometrial cells (Simon *et al.*, 1993a; Simon *et al.*, 1993b), decidual and trophoblast cells (Simon *et al.*, 1994a) and embryos (De los Santos *et al.*, 1996) express the functional receptor IL-1R tI. Overall this work has demonstrated that the IL-1 system is present in both embryonic and maternal tissues, particularly around the time of implantation, and this may also be involved in signalling between the embryo and endometrium.

3.1.4 Tumour Necrosis Factor

TNF α is another cytokine that has been localised in and produced by the human endometrium (Hunt *et al.*, 1992; Phillippeaux and Piguet, 1993; Tabibzadeh, 1991, Laird *et al.*, 1996). The receptors for TNF α are also expressed by the human endometrium (Tabibzadeh *et al.*, 1995b).

We wanted to confirm the pattern of expression of these cytokines in the endometrium, throughout the menstrual cycle *in vivo*. This could then be compared to cytokine expression in women with impaired fertility, such as recurrent miscarriage, and used to determine if abnormal cytokine expression could be a contributing factor to unsuccessful pregnancy in these women. The aim of this study was therefore to determine the pattern of protein and mRNA expression of LIF, IL-6, IL-1 α , IL-1 β and TNF α throughout the menstrual cycle using immunocytochemistry and RT-PCR.

3.2 Materials and Methods

3.2.1 Endometrial sections

Cryostat sections were obtained from endometrial biopsies from normal fertile women as described in the materials and methods section. Staining obtained for LIF was performed on sections from biopsies from 26 different women during the early proliferative (n=2), mid proliferative (n=1), late proliferative (n=2), early secretory (n=10), mid secretory (n=8) and late secretory (n=3) phases of the menstrual cycle. Staining for IL-6 was performed on sections from biopsies from 24 different women obtained during the early proliferative (n=1), mid proliferative (n=4), late proliferative (n=4), early secretory (n=4), mid secretory (n=9) and late secretory (n=2) phases of the menstrual cycle. Staining obtained for IL-1 α was performed on sections from biopsies from 19 different women during the early proliferative (n=1), mid proliferative (n=2), late proliferative (n=5), early secretory (n=3), mid secretory (n=7) and late secretory (n=1) phases of the menstrual cycle. Staining obtained for IL-1 β was performed on sections from biopsies from 24 different women during the mid-proliferative (n=4), late proliferative (n=4), early secretory (n=4), mid secretory (n=10) and late secretory (n=2) phases of the menstrual cycle. Staining obtained for TNF α was performed on sections from biopsies from 10 different women during the mid-proliferative (n=2), late proliferative (n=2), early secretory (n=2), mid secretory (n=2) and late secretory (n=2) phases of the menstrual cycle.

Dating of the biopsies was calculated from the time of the last menstrual period.

Biopsies obtained between days 0-4 were termed the early proliferative phase; days 5-9, the mid-proliferative phase; days 10-14, the late proliferative phase; days 15-19 the early secretory phase; days 20-24, the mid-secretory phase and days 25+, the late secretory phase of the menstrual cycle.

3.2.2 Immunocytochemistry

All sections were stained according to the protocol described in the materials and methods section. The protocol for each antibody was optimised, by initially using varying concentrations of both primary and secondary antibody to identify the optimal combination for visualisation of the antigen. The primary and secondary

antibodies used in these experiments were sold specifically for immunocytochemical studies and are summarised in tables 3.1 and 3.2 respectively. The binding was visualised using PAP antibodies and DAB substrate.

3.2.3 Quantification of staining

All staining was assessed semi-quantitatively as described in chapter 2.

The intensity of staining for LIF, IL-6, IL-1 α and IL-1 β was also assessed using a quantitative image analysis method that was developed as part of my studies. This method of analysis was used in an attempt to provide a more robust method for quantification of immunocytochemical staining.

From one section from each biopsy, 5 fields were chosen at random and digitally captured using Neotech Image Grabber (version 1.2). The images were then converted into greyscale images before the intensity of staining could be measured. This was necessary because the software used to measure the intensity of staining could not differentiate between different colours. Therefore, all colour was converted into greyscale so that the intensity could be determined. The overall intensity observed in positive staining therefore included intensities from both positive staining (i.e. from DAB substrate) and the counterstain (i.e. haematoxylin). The intensity of staining seen in the negative controls, where only haematoxylin is present, was therefore subtracted from the total intensity seen in the positive sections, in order to correct for this. For each field, 3 areas containing glandular epithelial cells and 3 areas containing stromal cells were highlighted and the intensity of each area measured using Jandel Sigma Scan image measurement software. This produced intensity values for 15 glandular epithelial and 15 stromal areas from each section. Each area was chosen at random and encompassed between 5 and 20 cells. Glandular epithelial and stromal staining was then assessed separately. For each cell type the average intensity of each area was calculated by dividing the total intensity of the area by the number of pixels present within the selected area. Intensity was measured on a scale of 0-255, where 0=black and 255=white. The average intensity

Table 3.1: Primary antibodies used when staining for LIF, IL-6, IL-1 α , IL-1 β and TNF α with concentrations and incubation times used for each antibody. The antibodies were obtained from R+D Systems, Abingdon, U.K. (♦), Genzyme Diagnostics, West Malling, U.K. (♥) and Santa Cruz Technology Inc, U.K. (♣).

	Antibody	Concentration	Incubation
LIF	Goat anti-human LIF ♦	1:50 (2 μ g/ml)	24h
IL-6	Mouse anti-human IL-6 ♥	1:20 (50 μ g/ml)	24h
IL-1α	Goat anti-human IL-1 α ♣	1:100 (2 μ g/ml)	24h
IL-1β	Goat anti-human IL-1 β ♣	1:100 (2 μ g/ml)	24h
TNFα	Mouse anti-human TNF α ♣	1:50 (4 μ g/ml)	48h

Table 3.2 : Secondary antibodies used when staining for LIF, IL-6, IL-1 α , IL-1 β and TNF α with concentrations and incubation times for each antibody. All antibodies were obtained from DAKO (Ely, U.K.).

	Antibody	Concentration	Incubation
LIF	Rabbit anti-goat Ig	1:200 (9 μ g/ml)	30min
IL-6	Rabbit anti-mouse Ig	1:25 (140 μ g/ml)	30min
IL-1α	Rabbit anti-goat Ig	1:100 (18 μ g/ml)	30min
IL-1β	Rabbit anti-goat Ig	1:100 (18 μ g/ml)	30min
TNFα	Rabbit anti-mouse Ig	1:25 (140 μ g/ml)	30min

results were then inverted by subtracting each value from 255 to allow higher results to represent higher intensities of staining. This also prevented the values becoming negative once the background staining was subtracted from the intensity values. This was repeated on the negative controls run parallel to each section, in each of the experiments. For LIF and IL-6, all positive sections and negative controls had been counterstained with haematoxylin, so the staining on the negative controls was quantified so that it could then be used to adjust the positive staining results to allow for background staining. For IL-1 α and IL-1 β intensity of staining on two types of negative control needed to be quantified. Firstly where haematoxylin had been used, to allow for background in the positive staining when a counterstain was used and also where no haematoxylin had been used to allow for background when no counterstain had been used on the positive sections.

For each cytokine, the mean average intensity of the negative controls was calculated (mean of the 15 average intensities obtained from each of the 15 areas). This mean intensity was then subtracted from each of the 15 results from the positive staining results for each section. The mean intensity and standard error of the mean was then calculated from these 15 results for each section/biopsy and plotted on a graph. To determine if there was any significant difference in staining throughout the cycle, the mean results from each biopsy were divided into the phases of the cycle that they were obtained from and assessed using ANOVA.

3.2.4 RT-PCR

RT-PCR analysis for expression of LIF, IL-6, IL-1 β and TNF α mRNA in endometrial biopsies was attempted. We first performed this on cultured stromal and epithelial cells, as this has previously been successful for IL-6, IL-1 β and TNF α (Estdale *et al.*, 1996).

3.3 Results

3.3.1 Semi-quantification of staining

3.3.1.1 Leukaemia inhibitory factor

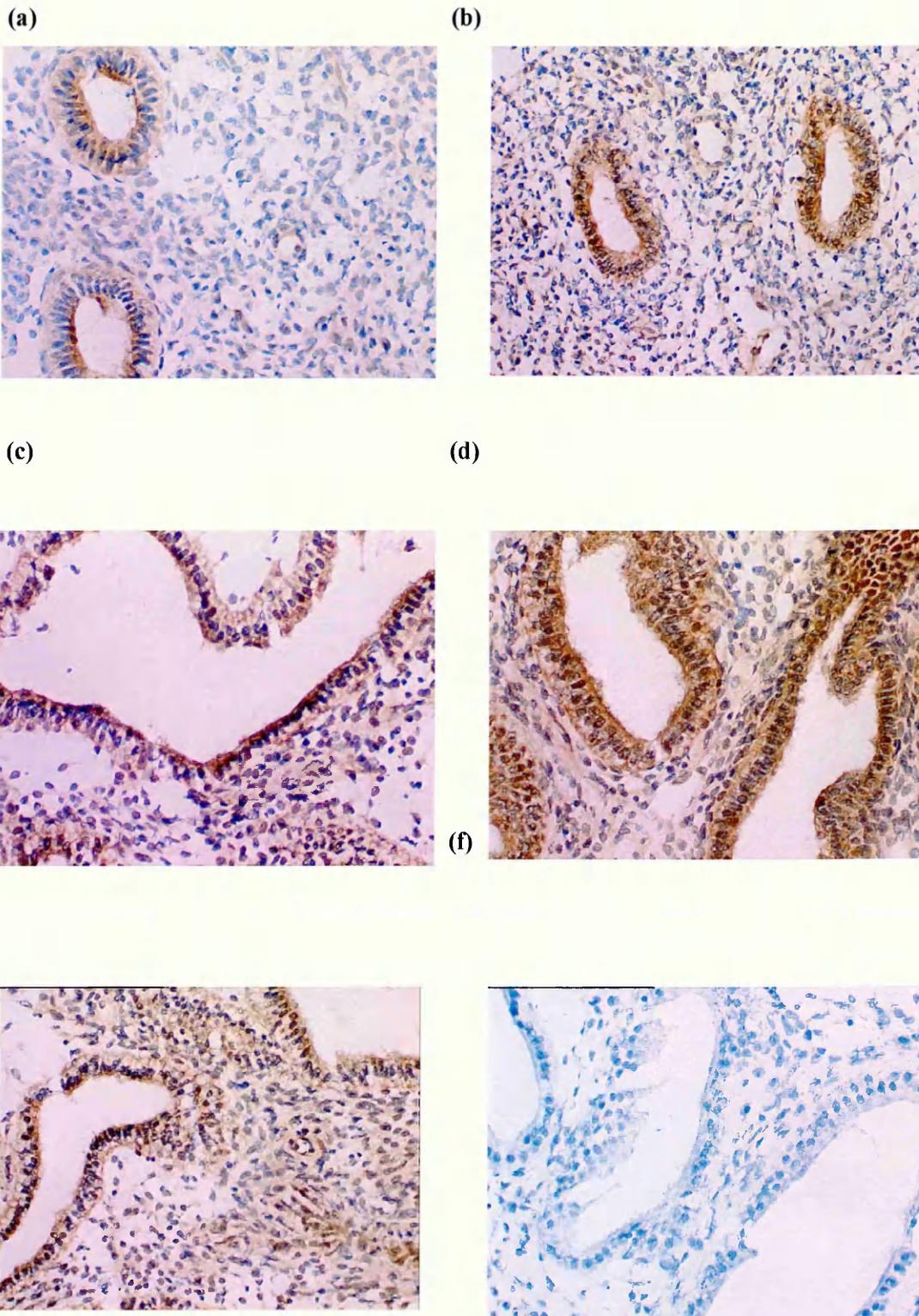
Figure 3.1 shows examples of LIF staining in sections obtained during the mid-proliferative (figure 3.1a), late proliferative (figure 3.1b), early secretory (figure 3.1c), mid-secretory (figure 3.1d) and late secretory (figure 3.1e) phases of the menstrual cycle. A negative control is included, where a serial section had been incubated with primary antibody, which had been neutralised before being added to the section (figure 3.1f). The results show that, throughout the cycle, epithelial staining was more intense than that seen in the stromal compartment of the endometrium. Glandular epithelial staining was moderate in the early-mid proliferative phases of the menstrual cycle (figure 3.1a), but increased in intensity throughout the late proliferative (figure 3.1b) and secretory phases (figure 3.1c,d and e) of the menstrual cycle, peaking during the mid-secretory phase (figure 3.1d), at the time of implantation. Luminal epithelium was not present in every section, but when it was present showed similar staining to that seen in the glandular epithelium. Stromal intensity was relatively weak and remained relatively constant throughout the menstrual cycle, but overall showed greater intensity during the secretory phase compared to the proliferative phase of the menstrual cycle.

Table 3.3 summarises the semi-quantitative analysis for LIF throughout the menstrual cycle in the sections from biopsies from 26 different normal fertile women.

3.3.1.2 Interleukin-6

Figure 3.2 shows IL-6 staining in sections obtained from endometrial biopsies during the mid-proliferative (figure 3.2a), late proliferative (figure 3.2b), early secretory (figure 3.2c), mid-secretory (figure 3.2d) and late secretory (figure 3.2e) phases of the menstrual cycle. A negative control is included, where a serial section had been incubated with primary antibody, which had been neutralised before being added to

Figure 3.1: Immunocytochemical staining for LIF in sections obtained from endometrial biopsies during the (a) mid-proliferative, (b) late proliferative, (c) early secretory, (d) mid-secretory and (e) late secretory phases of the menstrual cycle. A negative control is also included (f).



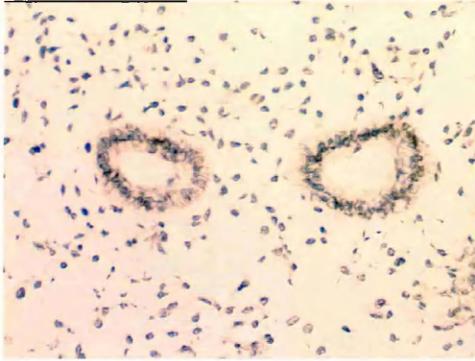
Magnification = X400

Table 3.3: Semi quantitative analysis of staining intensity for LIF in 26 endometrial biopsies throughout the menstrual cycle.

Biopsy	Day of cycle	Epithelial staining	Stromal staining
1	3	++	-/+
2	3	++/+++	-/+
3	5	+++	+
4	12	+++	+
5	12	++/+++	+
6	15	++	-/+
7	15	++/+++	+
8	15	+++	+/++
9	16	+++	-/+
10	16	+ / ++	-/+
11	18	+++	++
12	18	+++ / +++++	-/+
13	18	+++ / +++++	++
14	19	+++ / +++++	-/+
15	19	++ / +++	+/++
16	20	+++	++
17	20	++ / +++	+
18	21	+++	++
19	21	+++	+
20	21	+++ / +++++	+/++
21	22	++++	+/++
22	23	++++	+
23	24	++++	+
24	26	+++	+/++
25	29	++ / +++	+/++
26	30	++	-/+

Figure 3.2: Immunocytochemical staining for IL-6 in sections obtained from endometrial biopsies during the (a) mid-proliferative, (b) late proliferative, (c) early secretory, (d) mid-secretory and (e) late secretory phases of the menstrual cycle. A negative control is also shown (f).

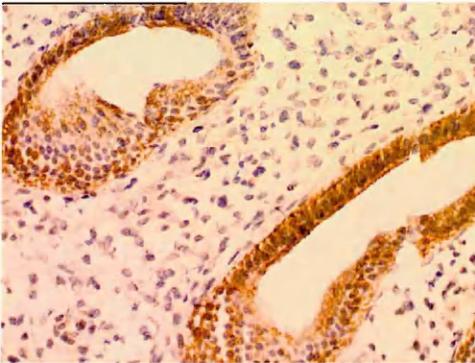
(a)



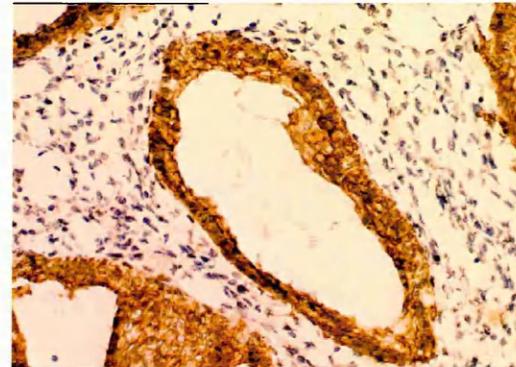
(b)



(c)



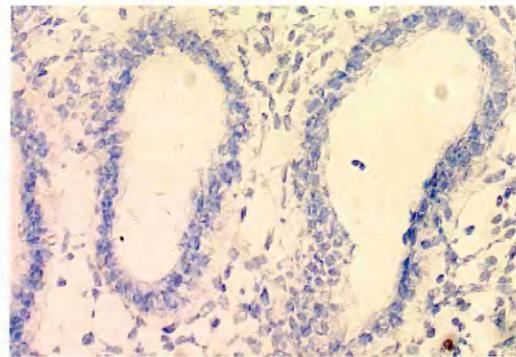
(d)



(e)



(f)



Magnification = X400

the section (figure 3.2f). The pattern of staining through the cycle, was similar to that seen for LIF. Glandular epithelial staining was weak in the early-mid proliferative phases of the menstrual cycle (figure 3.2a), but increased in intensity throughout the late proliferative (figure 3.2b) and secretory phases (figure 3.2c,d and e) of the menstrual cycle, peaking during the mid-secretory phase (figure 3.2d). Luminal epithelium was not present in every section, but when it was present showed similar staining to that seen in the glandular epithelium. Only very pale staining was seen in the stromal compartment at any time in the menstrual cycle, and no attempt was made to analyse this semi-quantitatively.

Table 3.4 summarises the semi-quantitative analysis of IL-6 in the sections from biopsies from 24 different normal fertile women.

3.3.1.3 Interleukin-1 α

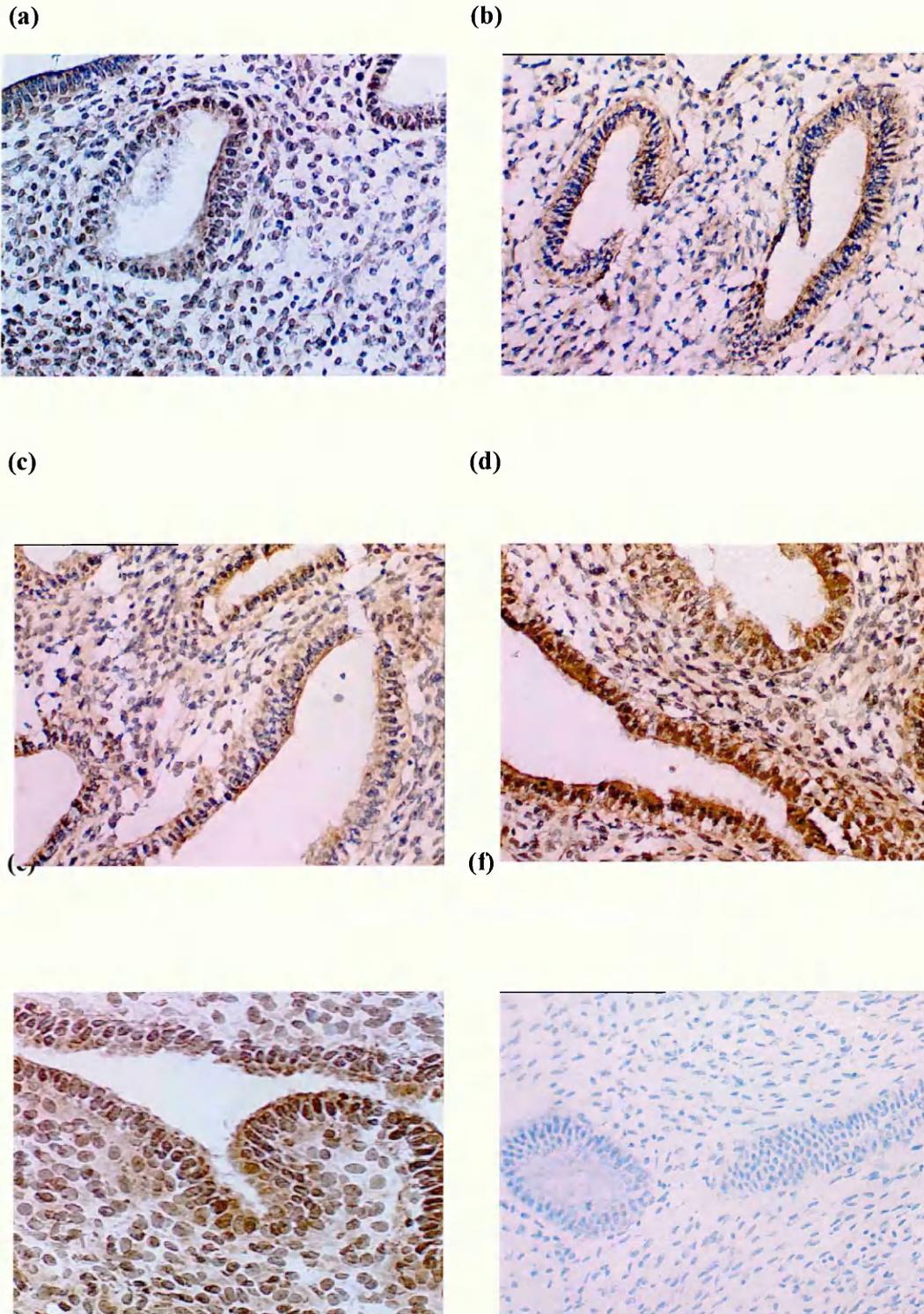
Figure 3.3 shows examples of IL-1 α staining in sections obtained during the mid-proliferative (figure 3.3a), late proliferative (figure 3.3b), early secretory (figure 3.3c), mid-secretory (figure 3.3d) and late secretory (figure 3.3e) phases of the menstrual cycle. A negative control is included, where a serial section had been incubated with primary antibody, which had been neutralised before being added to the section (figure 3.3f). The results show that IL-1 α was expressed by epithelial and stromal endometrial cells. Glandular epithelial staining remained relatively constant throughout the menstrual cycle, with a slight increase in intensity during the secretory phase (figure 3.3c,d and e). Luminal epithelium was not present in every section, but when it was present showed increased staining to that seen in the glandular epithelium (figure 3.3a). Stromal intensity was moderate throughout the menstrual cycle, but increased in intensity during the secretory phase, in particular the late secretory phase (figure 3.3e).

Table 3.5 summarises the semi-quantitative analysis for IL-1 α in the sections from biopsies from 19 different normal fertile women.

Table 3.4: Semi quantitative analysis of staining intensity for IL-6 in 24 endometrial biopsies throughout the menstrual cycle.

Biopsy	Day of cycle	Epithelial staining	Stromal staining
1	3	+	-
2	5	+	-
3	6	+	-
4	7	+	-
5	9	+	-
6	10	+	-
7	12	++	-
8	13	++	-
9	14	++	-
10	15	++	-
11	16	+++	-
12	18	+++	-
13	19	+++	-
14	20	+++ /++++	-
15	21	+++ /++++	-
16	22	+++ /++++	-
17	23	+++ /++++	-
18	23	++++	-
19	23	++++	-
20	24	++++	-
21	24	++++	-
22	24	++++	-
23	29	++ /+++	-
24	31	++ /+++	-

Figure 3.3: Immunocytochemical staining for IL-1 α in sections obtained from endometrial biopsies during the (a) mid-proliferative, (b) late proliferative, (c) early secretory, (d) mid-secretory and (e) late secretory phases of the menstrual cycle. A negative control is also shown (f).



Magnification = X400

Table 3.5: Semi quantitative analysis of staining intensity for IL-1 α in 19 endometrial biopsies throughout the menstrual cycle.

Biopsy	Day of cycle	Epithelial staining	Stromal staining
1	3	++/+++	+
2	7	+++	+ /+++
3	8	+++	+ /+++
4	10	++/+++	+ /+++
5	11	+++	+ /+++
6	12	++/+++	+ /+++
7	14	+++ /++++	+ /+++
8	14	+++	+
9	16	+++ /++++	+ /+++
10	19	+++	++
11	19	+++	++
12	20	+++	++
13	20	+++	+ /+++
14	20	+++	++
15	21	+++	+ /+++
16	21	+++	++
17	23	++++	++ /++++
18	24	+++ /++++	++ /++++
19	31	+++	++ /++++

3.3.1.4 Interleukin-1 β

Figure 3.4 shows examples of IL-1 β staining in sections obtained during the mid-proliferative (figure 3.4a), late proliferative (figure 3.4b), early secretory (figure 3.4c), mid-secretory (figure 3.4d) and late secretory (figure 3.4e) phases of the menstrual cycle. A negative control is included, where a serial section had been incubated with primary antibody, which had been neutralised before being added to the section (figure 3.4f). The results show that throughout the cycle, epithelial staining was more intense than that seen in the stromal compartment of the endometrium. Glandular epithelial staining was strong in the early-mid proliferative phases of the menstrual cycle (figure 3.4a) and remained relatively constant throughout the late proliferative (figure 3.4b) and secretory phases (figure 3.4c,d and e) of the menstrual cycle, but did show highest intensity during the mid-secretory phase (figure 3.4d). Luminal epithelium was not present in every section, but when it was present, showed similar staining to that seen in the glandular epithelium (figure 3.4b and d). Stromal intensity was moderate throughout the menstrual cycle, but was strong in the late secretory phase.

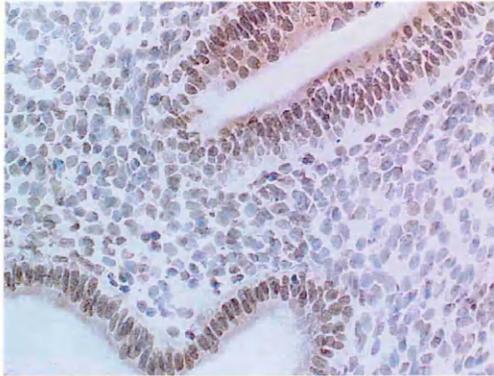
Table 3.6 summarises the semi-quantitative analysis for IL-1 β in the sections from biopsies from 24 different normal fertile women.

3.3.1.5 Tumour necrosis factor α

The expression of TNF α in sections from endometrial biopsies could not be determined using immunocytochemistry. Varying concentrations of primary and secondary antibodies were used, and for different incubation times. However, the staining obtained was very non-specific (figure 3.5a) and this could not be reduced, even after numerous attempts to try and decrease the non-specific staining. This included using different concentrations of the primary and secondary antibodies, increasing the time that the sections were quenched and increasing the number of washes between each step of the protocol. The negative control for TNF α , where the antibody had been neutralised prior to incubation on the section, also showed non-specific binding (figure 3.5b) confirming that the staining which was obtained in the positive sections was non-specific.

Figure 3.4: Immunocytochemical staining for IL-1 β in sections obtained from endometrial biopsies during the (a) mid-proliferative, (b) late proliferative, (c) early secretory, (d) mid-secretory and (e) late secretory phases of the menstrual cycle. A negative control is also shown (f).

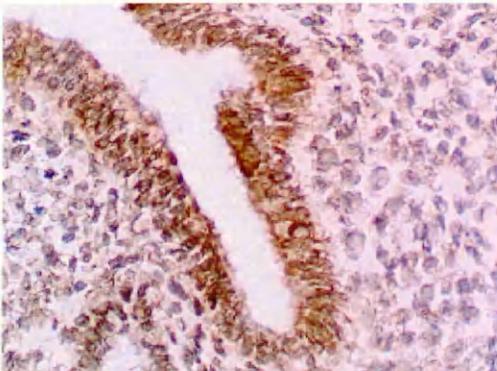
(a)



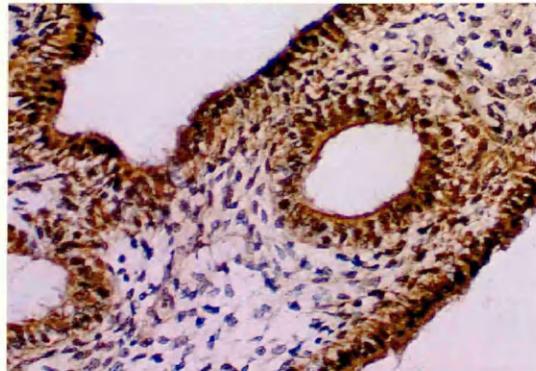
(b)



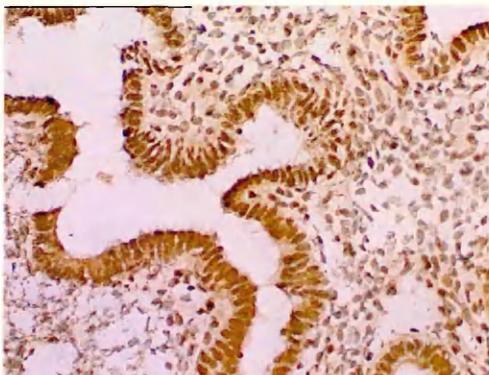
(c)



(d)



(e)



(f)

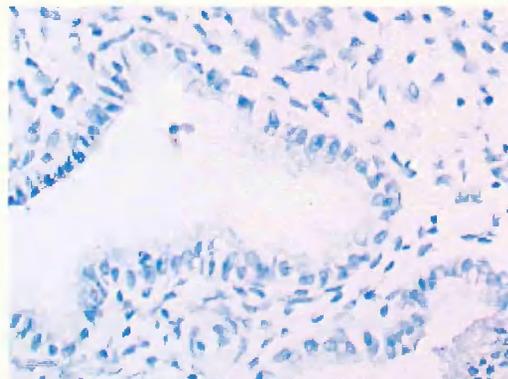
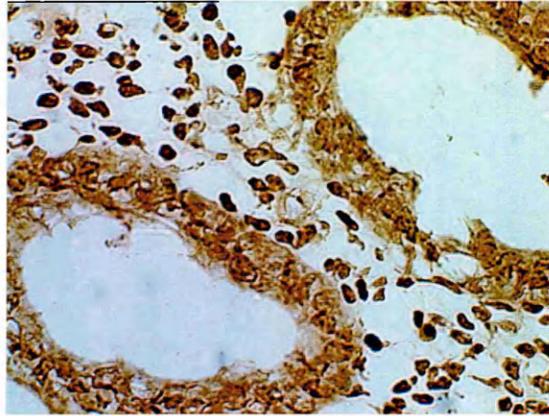


Table 3.6: Semi quantitative analysis of staining intensity for IL-1 β in 24 endometrial biopsies throughout the menstrual cycle.

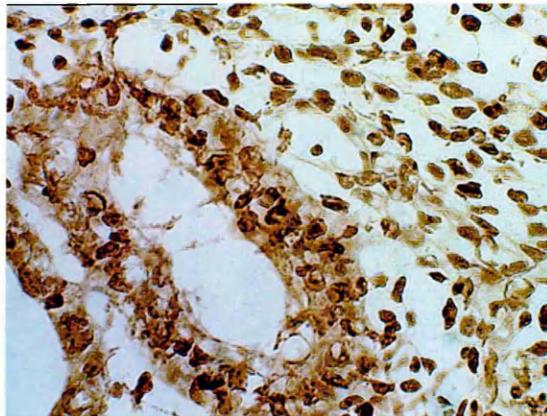
Biopsy	Day of cycle	Epithelial staining	Stromal staining
1	5	++++	+
2	5	+++	+
3	7	+++	+ / ++
4	8	+++	++
5	10	++++	++
6	10	+++	++
7	12	+++	++
8	14	+++	++
9	15	+++	++
10	15	+++	+
11	16	+++ / +++++	+ / ++
12	19	+++	++
13	20	+++	+ / ++
14	20	++++	+
15	20	+++ / +++++	+
16	21	++++	++
17	21	++++	+ / ++
18	21	++++	+ / ++
19	21	++++	+
20	22	++++	+
21	23	+++	++
22	24	+++	++
23	25	+++	+++
24	31	+++	++ / +++++

Figure 3.5: Examples of immunocytochemical staining for TNF α . Staining for TNF α is shown in (a) and (b) shows a negative control.

(a)



(b)



Magnification = X400

3.3.2 Quantification of results

Quantification of the staining did not confirm the pattern of cytokine expression seen when using the semi-quantitative method. This lack of correlation was demonstrated by the results obtained from quantification of immunocytochemical staining for LIF in sections from biopsies obtained from 26 different normal fertile women using the method that was developed. Intensity of epithelial LIF staining is shown in figure 3.6 (a). These results suggest that epithelial staining for LIF varies throughout the menstrual cycle, but the pattern of expression does not confirm that obtained when using semi-quantitative analysis. The staining intensity readings were relatively constant in biopsies obtained throughout the cycle, but is increased on day 21 of the cycle in two biopsies and is low in the late secretory phase. Analysis of staining intensity in this way showed no significant difference in epithelial staining in biopsies grouped according to the phases of the menstrual cycle (figure 3.6b).

Figure 3.7 (a) shows the staining intensities for LIF in stromal cells from 26 different normal fertile women throughout the menstrual cycle, obtained using quantitative analysis. The values obtained for the staining intensity are lower in the stromal than in the epithelial compartment of the endometrium at all times in the menstrual cycle, as was suggested by the semi-quantitative method. Overall these results suggest that staining is slightly higher in the proliferative phase of the menstrual cycle compared to the secretory phase. Figure 3.7 (b) suggests that the staining in the mid-proliferative phase is significantly higher than in the other phases. However, this result is unfairly influenced by the single high reading of day 5 of the cycle.

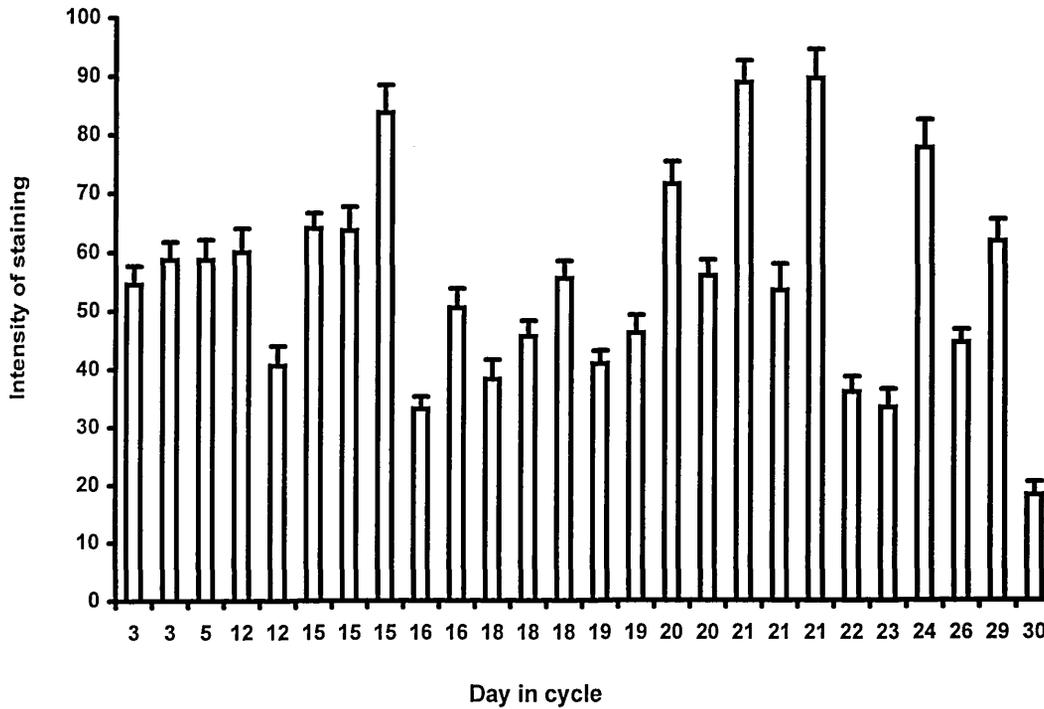
These results suggest that this quantitative method is not suitable as a means of measuring staining intensity. The problems shown in the results for LIF are typical of that seen when using this method to quantify the staining for IL-6, IL-1 α and IL-1 β and therefore the results of the quantification for IL-6, IL-1 α and IL-1 β staining intensity have not been included in this work.

3.3.3 RT-PCR analysis

Table 3.7 shows the optical density readings and A260/280 ratios for RNA extracted from 8 epithelial and 5 stromal cell cultures and 8 endometrial biopsies. The

Figure 3.6: Quantitative analysis of staining intensity for epithelial LIF in sections from biopsies from 26 different women throughout the menstrual cycle, (a) individually and (b) grouped into phases of the menstrual cycle. EP = early proliferative, MP = mid proliferative, LP = late proliferative, ES = early secretory, MS = mid secretory and LS = late secretory phases of the menstrual cycle.

(a)



(b)

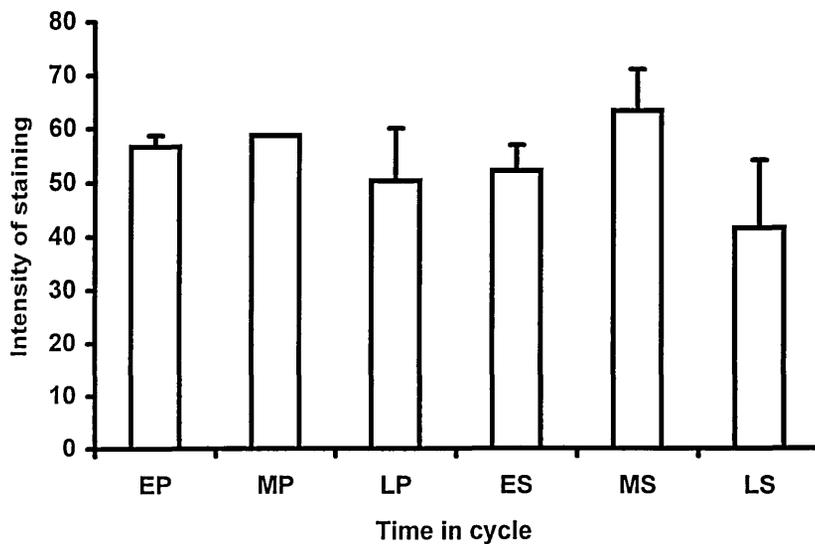
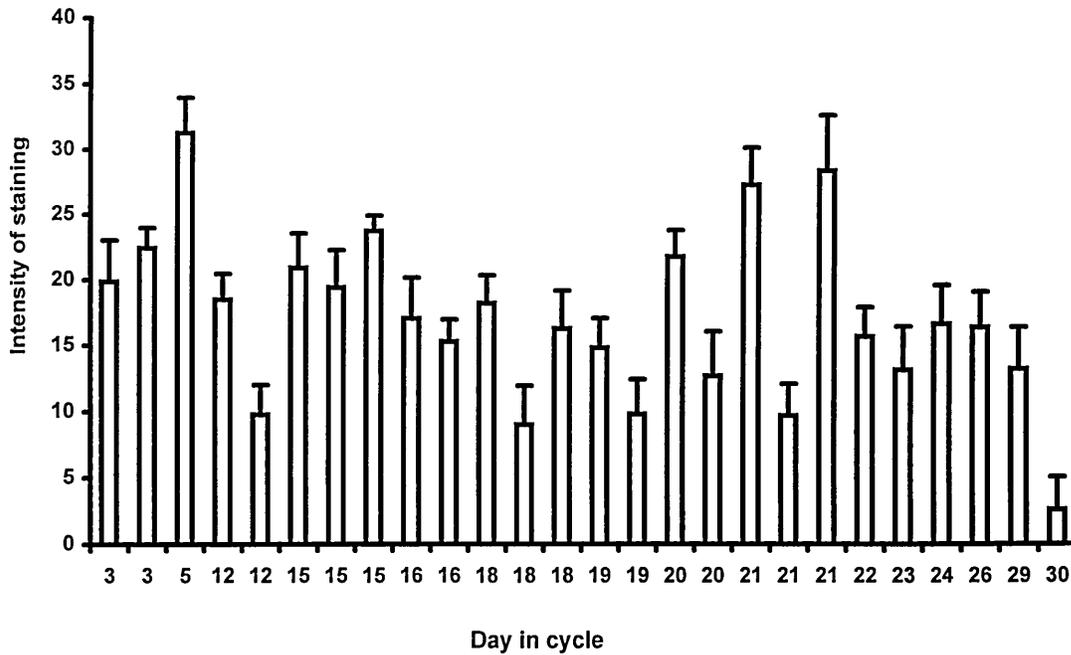


Figure 3.7: Quantitative analysis of staining intensity for stromal LIF in sections from biopsies from 26 different women throughout the menstrual cycle, (a) individually and (b) grouped into phases of the menstrual cycle. EP = early proliferative, MP = mid proliferative, LP = late proliferative, ES = early secretory, MS = mid secretory and LS = late secretory phases of the menstrual cycle.

(a)



(b)

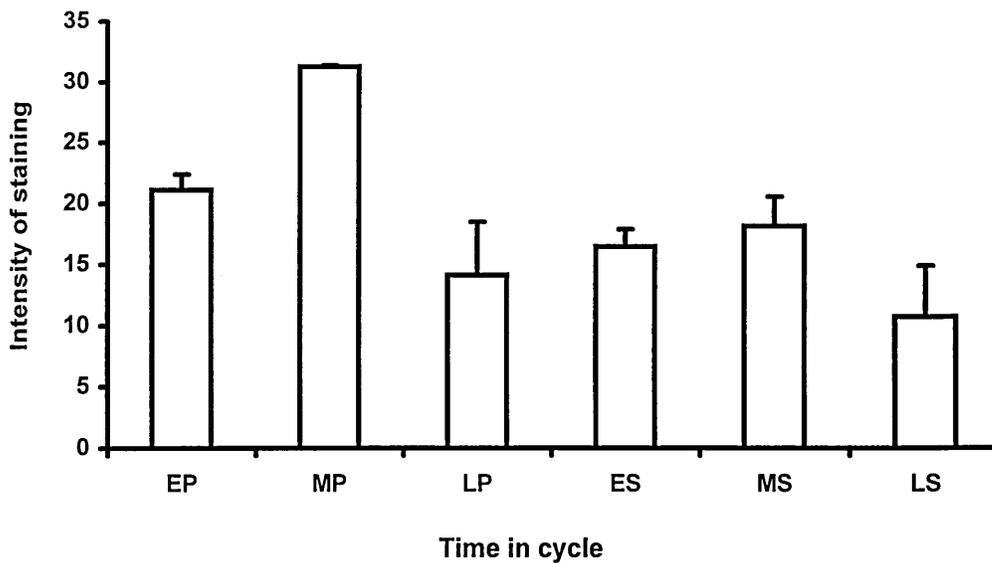


Table 3.7: Optical density readings and absorbance at 260nm and 280nm ratios for RNA extracted from 8 epithelial and 5 stromal cell cultures and 8 endometrial biopsy samples

	Optical density reading	A260:280
Epithelial cell cultures		
1	0.376	1.736
2	0.276	1.775
3	0.391	1.663
4	0.607	1.354
5	0.178	1.84
6	0.183	1.813
7	0.147	1.844
8	0.119	1.821
Stromal cell cultures		
1	0.063	1.839
2	0.558	1.048
3	0.359	1.096
4	0.615	1.053
5	0.555	1.053
Endometrial biopsies		
1	0.284	1.427
2	0.587	1.875
3	0.194	1.552
4	0.2	1.6
5	0.163	1.304
6	0.147	1.869
7	0.255	1.877
8	0.317	1.744

absorbance readings suggested that RNA was successfully extracted from the majority of both cell cultures and endometrial biopsies. However, the A260/280 ratios were quite low ($<1.8\mu\text{g}/\mu\text{l}$) for a lot of the samples suggesting that RNA was not as pure as it could be. It is likely that genomic DNA is also present in these samples, which would account for the impurity of some of the samples. RT-PCR for the housekeeping gene 7B6 confirmed that RNA extraction had been successful. Figure 3.8 shows the RT-PCR product representing 7B6, in cDNA obtained from cultured epithelial cells (lane 3), stromal cells (lane 4) and in endometrial biopsies (lanes 5, 6 and 7). Lanes 8-12 are negative controls where no RT was added to the PCR mastermix, however, bands are present in these lanes at approximately 1300bp and these bands are likely to represent products from genomic DNA.

In contrast to this, only limited success was achieved in the analysis of mRNA for LIF, IL-6, IL-1 β and TNF α in the mRNA extracted from both cultured endometrial cells and biopsy samples, by RT-PCR, despite numerous attempts to optimise primer concentrations, annealing temperatures and MgCl₂ concentrations. Figure 3.9 (a) shows that a PCR product of the expected size for LIF was seen in mRNA prepared from cultured epithelial cells. However, this could not be repeated for mRNA extracted from 8 endometrial biopsy samples obtained at any time in the menstrual cycle. The higher molecular weight bands are again likely to represent products from genomic DNA.

Figure 3.9 (b) again shows that a PCR product could be obtained for IL-6 from mRNA prepared from cultured epithelial cells, but that when the same reaction was carried out with mRNA extracted from endometrial biopsies, no specific bands were seen. There are numerous non-specific bands present in this gel, which suggest that contamination of the mastermix, via impurities in one of the components of the mastermix, has occurred.

Figure 3.10 shows that, as for LIF and IL-6, PCR products for IL-1 β and TNF α could be obtained from cultured endometrial epithelial cells and bands were also obtained from mRNA extracted from at least 2 of the endometrial biopsies. However, consistent positive results for all four cytokines were only obtained for a very few

Figure 3.8 : Gel showing bands for 7B6 in cDNA from cultured epithelial cells (lane 3), cultured stromal cells (lane 4) and endometrial biopsies (lanes 5, 6 and 7). Lane 1 contains the marker, lane 2 is the negative control (no RNA added and lanes 8-12 are negative controls (no RT added).

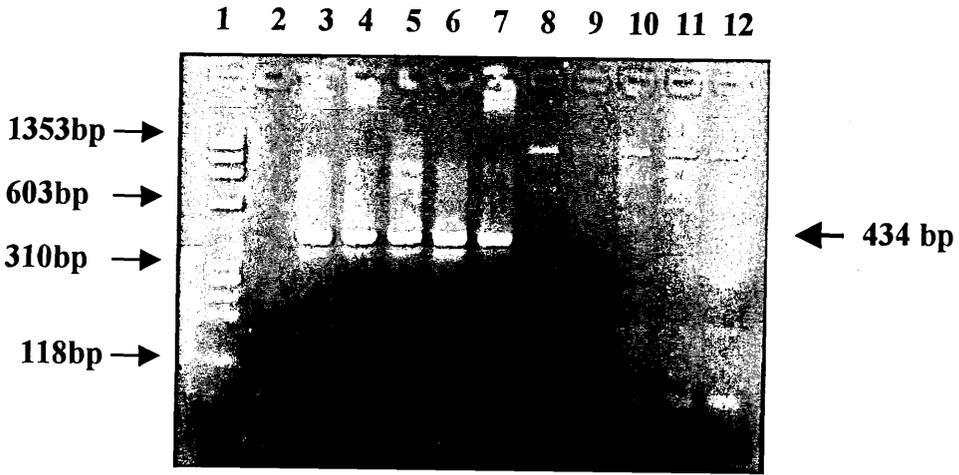
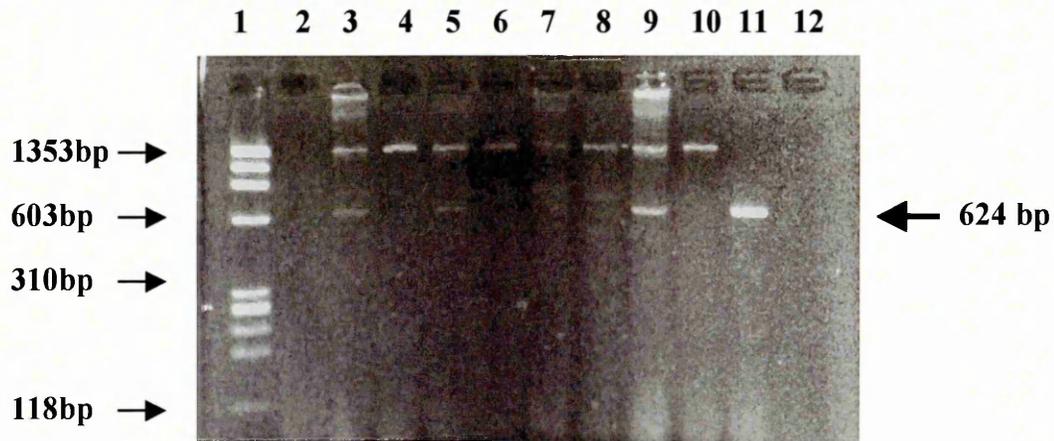


Figure 3.9 : Gels to show PCR products for (a) LIF and (b) IL-6. In both gels, lane 1 = marker, lane 2 = negative control. In figure (a) lanes 3, 5, 7, 9 and 11 = cultured epithelial cells and lanes 4, 6, 8, 10 and 12 = negative controls (no RT). In figure (b) lanes 3 and 4 = cultured epithelial cells and lanes 5-12 = endometrial biopsies. The expected band sizes are arrowed.

(a)



(b)

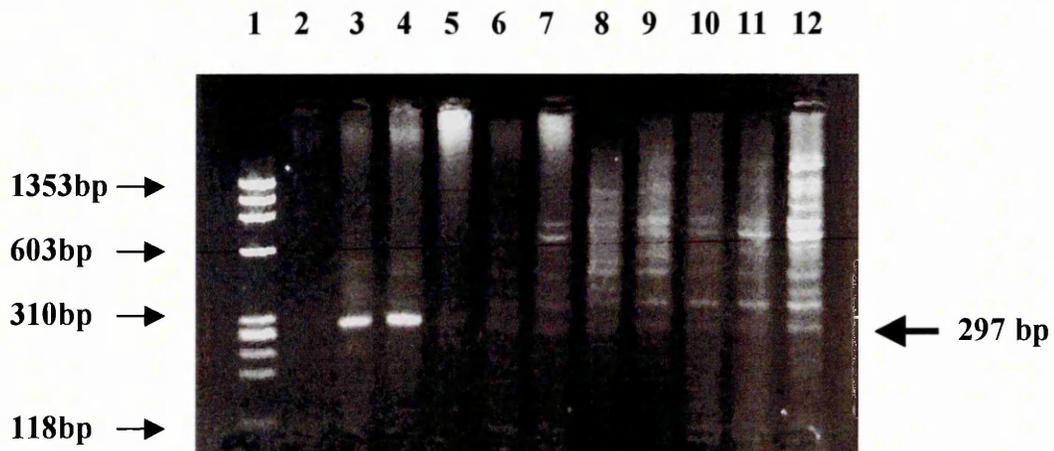
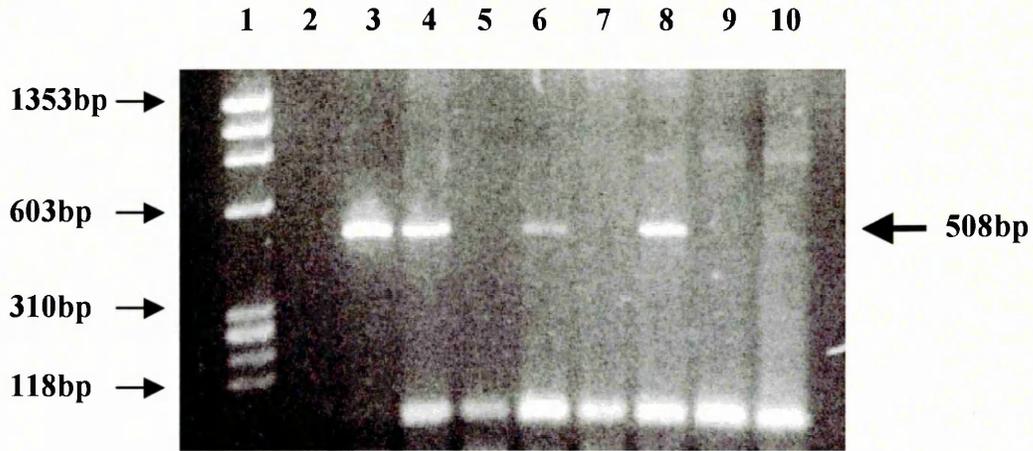


Figure 3.10: Gels to show PCR products for (a) IL-1 β and (b) TNF α . In both gels lane 1 = marker, lane 2 = negative control, lanes 3 and 4 = cultured epithelial cells and lanes 5-10 = different endometrial biopsies.

(a)



(b)



biopsies and cultured cells, despite the RT-PCR reactions being repeated numerous times.

Figure 3.10 (a) shows a band for IL-1 β present in samples from cultured epithelial cells and also 2 of the endometrial biopsies. The higher molecular weight bands in lanes 8-10 are likely to represent products from genomic DNA and the lower molecular weight bands in lanes 4-10 are a result of dimerisation of the primers.

Figure 3.10 (b) shows bands representing TNF α mRNA at 436bp. Again the higher molecular weight bands are likely to represent products from genomic DNA. The other bands that are present in some lanes at lower molecular weights are non-specific bands could be a result of contamination or the primers binding non-specifically.

3.4 Discussion

The control of human endometrial function by steroids is well understood, however it is now believed that the actions of local autocrine/paracrine factors needs to be determined in order to understand the exact mechanisms involved in human endometrial function. To gain further insights into the potential autocrine and paracrine regulation of endometrial function, in particular implantation, the expression of the cytokines LIF, IL-6, IL-1 and TNF α has been studied in the normal human endometrium throughout the menstrual cycle. The aim was to develop immunocytochemical and RT-PCR methods using endometrium from normal fertile women, so that we could then compare expression in women who suffer recurrent miscarriage.

3.4.1 RT-PCR

It was hoped that use of RT-PCR would show the presence of mRNA transcripts for LIF, IL-6, IL-1 β and TNF α in endometrial biopsies obtained throughout the menstrual cycle. Similar methods for analysis of cytokine mRNA in cultured epithelial cells had previously been used in the laboratory (Estdale *et al.*, 1996). Consistent evidence for the presence of mRNA for these cytokines in endometrial biopsy samples could not be obtained. However, RT-PCR for 7B6 on RNA extracted from both cultured endometrial cells and endometrial biopsies was successful. This suggests that the RNA extraction procedure used did result in good quality RNA being obtained from both the cultured cells and the biopsies. Thus it would appear that the RT-PCR technique was inadequate. This is however unlikely, because some PCR products for LIF, IL-1 β and TNF α , particularly from cultured endometrial epithelial cells, were obtained, which would not have happened if the PCR method was not working. Attempts were made to optimise the PCR conditions used for all primers. This included optimising the concentrations of primers, magnesium and Taq polymerase in each of the mastermixes, as well as optimising the annealing temperature and number of cycles used for each reaction. The sequences of the primers were all checked to ensure that they were suitable for the reactions. However, PCR using nested primers was not attempted and this may have produced more specific results. The most probable explanation for the inconsistency of the

results is that the RNA quality extracted from the endometrial biopsies was not good enough to detect these cytokines. The reason for successful RT-PCR for 7B6 and failure for the cytokines may be due to differences in abundance of mRNA in the tissue. Previous studies have shown that it is possible to analyse cytokine mRNA from endometrial biopsies obtained from the operating theatre at Jessop Hospital for Women (Lim *et al.*, 2000). However, the subsequent analysis was not carried out in our laboratory and although similar methods are described, there may be subtle differences. A modification of our collection procedure for endometrial biopsies and RNA extraction procedure would be required to proceed further with this type of analysis. This might include addition of RNAase inhibitors at an early stage of RNA extraction or even during collection of the sample.

3.4.2 Quantification of staining

The method used in this study to quantify the intensity of staining obtained has been developed specifically for this study. It was hoped that it would provide a more robust numerical method for comparing staining intensities in different biopsy samples than the semi-quantitative scoring systems that have traditionally been used. It would also allow statistical comparison between staining seen at different times in the cycle. However the attempt to quantify staining intensity in this way was not successful.

One of the reasons for the lack of success with the quantitative method is likely to be a result of using a counterstain. Although this allows visualisation of cells that do not stain positively, it provides background intensity that interferes with the measurement of the positive brown staining, even after the results have been corrected for negative staining, by subtracting the intensity of the negative sections. The quantification of staining was also carried out on sections that were not run in parallel to one another. Therefore inter experimental variations could also be a factor influencing the results. Another reason is the need to convert the image to greyscale for analysis. An image analysis method that detects specific colour would be a better method to use. Another difficulty is the variation in cellular density. A more intense signal is obtained when the cells are closely packed together. Hence a comparison of the densely packed epithelial cells and sparsely packed stromal cells is difficult.

Although the results achieved using this method of quantification were invalid, this method is one that could prove to be useful for future work. However, it would be essential to ensure that no counterstain was included in the immunocytochemistry. Also, the results should be analysed at the end of each separate staining run and not compared between different slides prepared on different occasions, because although the results may show similar staining patterns, the intensity of the staining, even the counterstain alone, can vary between different experiments.

3.4.3 Leukaemia Inhibitory Factor

LIF is a cytokine that has previously been shown to be essential for implantation in mice (Stewart *et al.*, 1992). It has therefore been postulated that LIF may be an important factor in human implantation. The results from this study clearly show that LIF is expressed by the human endometrium throughout the menstrual cycle. Analysis of the staining semi-quantitatively shows that epithelial LIF protein expression is higher than stromal expression throughout the menstrual cycle agreeing with previous observations by other groups (Charnock-Jones *et al.*, 1994; Chen *et al.*, 1995; Arici *et al.*, 1995). Glandular epithelial expression is moderate to strong during the proliferative phase and increases in intensity during the secretory phase, peaking at the time of implantation, whereas stromal staining was weak to moderate throughout the cycle, with little cyclical variation. This pattern of expression is similar to that reported by Vogiagis *et al.* (1996), Cullinan *et al.* (1996) and Yang *et al.* (1996), although Yang *et al.* (1996) detected only minimal stromal LIF expression during the late proliferative and secretory phases of the menstrual cycle. Another study (Charnock-Jones *et al.*, 1994) also showed that expression of LIF protein was maximal in the epithelium during the mid-late secretory phase, but did not detect any LIF in the epithelial cells during the proliferative phase and found no stromal expression throughout the menstrual cycle. These differences may be accounted for by the fact that goat anti-human LIF antibodies from different manufacturers were used in each study. In this study, luminal epithelium was not present in all sections, but when present LIF expression was similar to that seen in the glandular epithelium which again agrees with previous results (Vogiagis *et al.*, 1996). The most important observation from this and previous studies is the increased expression of epithelial

LIF during the mid-secretory phase, at the time when implantation occurs in humans (Charnock-Jones *et al.*, 1994; Kojima *et al.*, 1994; Arici *et al.*, 1995; Vogiagis *et al.*, 1996; Laird *et al.*, 1997). LIF receptor mRNA has been identified in luminal epithelium throughout the menstrual cycle (Cullinan *et al.*, 1996) and also in pre-implantation blastocysts (Charnock-Jones *et al.*, 1994), suggesting that human blastocysts are capable of responding to a maternal LIF signal prior to implantation. Taken together, these results suggest that LIF does play a role in human endometrial function and is likely to be important in the human implantation process.

3.4.4 Interleukin-6

The function of IL-6 in implantation in mice has not yet been determined, however, it is not considered as important as LIF, as knockout mice for the IL-6 gene have been shown to have normal fertility (Poli *et al.*, 1994; Kopf *et al.*, 1994). However, IL-6 does share the signal transducing receptor component gp130 with LIF and it may therefore play a similar role to LIF in human endometrial function. The results from this study show that IL-6 is expressed in the endometrium throughout the menstrual cycle. Analysis using the semi-quantitative method showed that epithelial expression varied considerably with the time in the cycle. Expression was very low in the early-mid proliferative phases and increased gradually throughout the cycle, with maximal intensity during the mid-late secretory phases. When luminal epithelium was present within the sections, expression of IL-6 was similar to that seen in the glandular epithelium. This study confirms the presence of IL-6 within the human endometrium as shown previously, using RT-PCR (Tabibzadeh and Sun, 1992; Vandermolen and Yang, 1996) and immunocytochemistry (Tabibzadeh *et al.*, 1995a). Tabibzadeh *et al.* (1995a) showed a similar pattern of staining for IL-6 as shown in this study, with predominant epithelial expression throughout the cycle and increased expression at the time of implantation. In addition, they showed that IL-6 was expressed in the upper functionalis of the stromal cells, but only during the late secretory phase of the menstrual cycle. Production of IL-6 by endometrial cells in culture has previously been reported (Laird *et al.*, 1993) and also showed that stromal IL-6 was only detected in supernatants of cells prepared from late secretory endometrium. Only two biopsies were obtained from the late secretory phase in this study, which may explain why no IL-6 staining was observed. The increased

expression of IL-6 at the time of implantation suggests that IL-6 may also play a role in implantation.

3.4.5 Interleukin-1

The interleukin-1 system is known to be present in both fetal and maternal tissues at the time of implantation (Simon *et al.*, 1996). The results from this study agree with these previous studies and show that both IL-1 α and IL-1 β are expressed by the human endometrium throughout the menstrual cycle. The results of the semi-quantitative analysis showed that both epithelial and stromal cells express IL-1 α with little variation throughout the cycle. However, epithelial expression is more intense than stromal expression at all times in the menstrual cycle, except the late secretory phase when IL-1 α expression appears to increase in the stromal cells. Expression of IL-1 β is similar to that of IL-1 α , in that, it is expressed throughout the menstrual cycle, by both epithelial and stromal cells. As with IL-1 α , expression of IL-1 β is more intense in epithelial cells than stromal cells at all times, except the late secretory phase when stromal expression is similar to that in the epithelium. Epithelial IL-1 β expression, although strong throughout the cycle, did appear to be greatest during the mid-secretory phase of the menstrual cycle. IL-1 β is the secreted form of IL-1, while IL-1 α is thought to be retained within the cell (Dower and Sims, 1994). The technique of immunocytochemistry will not detect the secreted form of IL-1 β ; therefore a similar expression of IL-1 α and IL-1 β is to be expected. These results agree with previous work that has shown ubiquitous expression of IL-1 α and IL-1 β mRNA in epithelial and stromal cells throughout the menstrual cycle (Tabibzadeh and Sun, 1992). The mRNA for IL-1RtI is expressed in the endometrium throughout the menstrual cycle, reaching maximal levels at the early and late secretory phases and epithelial mRNA expression of IL-1 β is increased during the secretory phase (Simon *et al.*, 1993a). De los Santos *et al.* (1996) demonstrated the presence of the complete IL-1 system in pre-implantation embryos. Further work from this group (Simon *et al.*, 1998), has demonstrated that embryonic IL-1 may interact with its endometrial receptor to upregulate the expression of the integrin β_3 , which is thought to be a marker for uterine receptivity (Lessey *et al.*, 1992; Lessey *et al.*, 1994). Taken together these results suggest the importance of the

IL-1 system in human endometrial function and the increased expression of epithelial IL-1 β at the time of implantation, seen in this study, provides further evidence for the importance of this cytokine system, particularly during implantation.

3.4.6 Tumour Necrosis Factor α

In this study, the expression of TNF α by endometrial cells could not be determined using immunocytochemistry. Although some of the staining observed may have indicated the presence of TNF α within endometrial sections, there was also a lot of non-specific staining seen, which despite much effort, could not be removed. The reason for this is unclear as neutralisation of the primary antibody for use as a negative control was attempted, but still resulted in non-specific staining. In an attempt to remove the non-specific staining, the concentrations of both primary and secondary antibodies were reduced, the concentration of peroxidase used to quench the sections was increased and the length and number of washes between each step in the protocol was increased. No attempt was made to localise TNF α using other anti-human TNF α antibodies due to time constrictions, but it is likely that the use of other TNF α antibodies would have produced more specific results.

Chapter 4

Endometrial cytokine expression in RM women

4.1 Introduction

Previous reports from our laboratory have shown that a proportion of women who suffer recurrent miscarriage have an abnormal endometrial development (Dalton *et al.*, 1995; Hey *et al.*, 1995). Several studies have shown the importance of cytokines in the development of the feto-placental unit and the prevention of miscarriage. Therefore it is possible that abnormal endometrial cytokine production during the peri-implantation period may lead to subsequent abnormal development of the feto-placental unit and miscarriage. Relatively few studies have examined the expression of endometrial cytokines in women who suffer recurrent miscarriages and compared the findings to those seen in normal fertile women. Decreased endometrial expression of IL-1 β and IL-6 mRNA transcripts during the mid-secretory phase of the menstrual cycle has been reported in women with habitual abortion, when compared to normal fertile women (von Wolff *et al.*, 2000). Another study has shown that endometrial expression of IFN γ , IL-2 and IL-12 mRNA and protein is increased in women with recurrent miscarriage when compared to normal fertile women, whereas IL-6 expression is significantly decreased (Lim *et al.*, 2000). Altered IFN γ , IL-10, TNF α and TGF β mRNA expression by decidual and trophoblast cells in women who had a successful pregnancy outcome compared to those who have had a failed pregnancy has also been reported (Vives *et al.*, 1999). LIF has also been implicated as a cytokine that may be particularly involved in implantation and *in vitro* LIF production by decidual T-cells from unexplained recurrent miscarriage women is reportedly lower than that produced by decidual T cells from normal fertile women (Piccinni *et al.*, 1998).

Having determined the expression of LIF, IL-6, IL-1 α and IL-1 β in the endometrium of normal fertile women throughout the menstrual cycle, this study was performed to determine if endometrial expression of these cytokines during the peri-implantation period is altered in women who suffer unexplained recurrent miscarriage. The expression of LIF, IL-6, IL-1 α and IL-1 β in endometrium of normal fertile, at the

time of implantation, was therefore compared to that in normal fertile women at the same time in the menstrual cycle.

4.2 Materials and Methods

Expression of LIF, IL-6, IL-1 α and IL-1 β protein was analysed in biopsies obtained from recurrent miscarriage women at the time of implantation. The staining intensities were analysed quantitatively and compared to staining seen at the same times in the menstrual cycle of normal fertile women (data shown in chapter 3).

4.2.1 Endometrial biopsies

Biopsies from women suffering unexplained recurrent miscarriage were timed from the LH surge and obtained between days LH+6 and LH+10. Each biopsy was then assessed according to Noyes *et al.* (1950) to determine the morphology of the biopsy. This was required to ensure that any biopsies that had retarded development were accounted for. Expression of LIF was investigated in sections from 24 biopsies. IL-6 expression was assessed in sections from 15 biopsies. Expression of IL-1 α was investigated in sections from 12 biopsies and IL-1 β expression was investigated in sections from 11 biopsies.

4.2.2 Immunocytochemistry

Immunocytochemical staining was carried out according to the protocol described in chapter 2. The staining procedure and antibodies used for all four cytokines were the same as those in chapter 3. All staining was repeated on at least three sections from each biopsy. A negative control was run parallel to each section where the next serial section was incubated with primary antibody, which had been neutralised for 24 h prior to the experiment. For each experiment, sections obtained from biopsies from normal fertile women at the same time in the cycle, were run parallel to the recurrent miscarriage sections.

4.2.3 Semi-quantitative analysis

All staining was assessed semi-quantitatively according to the method described previously.

4.3 Results

The results for the morphological analysis of the biopsies used in staining for each of the cytokines is shown in table 4.1. The results show that although all samples were obtained between days LH+6 and LH+10, 5 biopsies stained for LIF and 2 biopsies stained for IL-6 had the morphology of day LH+3 or LH+4, suggesting that these women had retarded endometrium.

4.3.1 Leukaemia Inhibitory Factor

Staining for LIF in sections from biopsies obtained from women suffering recurrent miscarriage showed that LIF protein was present in all endometrial biopsies, and as in normal fertile women, staining was predominantly seen in epithelial cells.

However, the intensity of staining for LIF was highly variable between different individuals. Semi-quantitative analysis of the staining intensity for each biopsy is shown in table 4.2. The results show that 13 of the biopsies had similar staining to that seen in normal fertile women at the same time in the menstrual cycle, but staining was decreased, particularly in the epithelial cells, in sections from biopsies from 11 different women with recurrent miscarriage (biopsies 7, 11, 12, 13, 14, 16, 17, 19, 22, 23 and 24). Figure 4.1 shows examples of the staining obtained for LIF, where staining was similar to that seen in normal fertile women (figure 4.1 a and b) and weaker than that seen in normal fertile women (figure 4.1 c and d).

4.3.2 Interleukin-6

Immunocytochemical staining for IL-6 was seen in all 15 biopsies from recurrent miscarriage women. The staining again varied greatly between individuals. The semi-quantitative analysis in all recurrent miscarriage biopsies stained for IL-6 is shown in table 4.3. The results showed that very little, if any IL-6 was present in the stromal compartment in any of the biopsies, agreeing with that seen in normal fertile women. This semi-quantitative assessment showed that epithelial staining was weaker in 9 of the biopsies (biopsies 1, 4, 5, 7, 9, 10, 11, 12 and 13) from recurrent miscarriage women, when compared to staining seen in normal fertile women at the same time in the cycle. Figure 4.2 shows the intensity of staining seen in four biopsies from women who suffer unexplained recurrent miscarriage, two of which

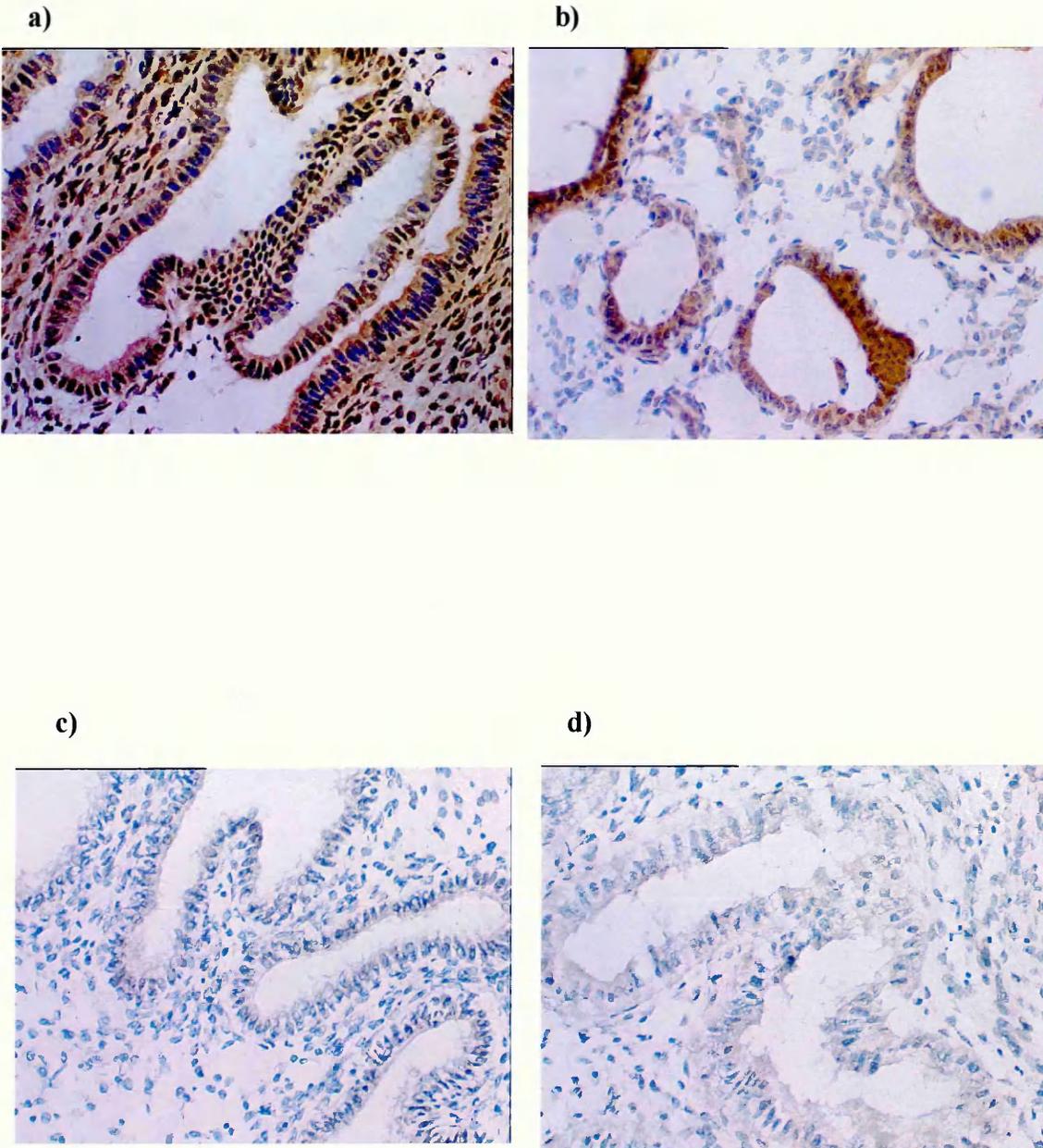
Table 4.1: Morphological dating of the recurrent miscarriage biopsies stained for LIF, IL-6, IL-1 α and IL-1 β

LH Day	Cytokine			
	LIF	IL-6	IL-1 α	IL-1 β
3	3	1	0	0
4	2	1	0	0
6	2	1	1	1
7	3	1	2	2
8	7	9	4	4
9	4	2	2	2
10	3	0	3	2

Table 4.2: Semi quantitative analysis of staining intensity for LIF in normal fertile women and in 24 endometrial biopsies from women with recurrent miscarriage. All biopsies were obtained during the implantation period. The LH days shown are those calculated from the morphological appearance of the tissue.

Biopsy	Day of cycle	Epithelial staining	Stromal staining
N	20	+++	++
O	20	++/+++	+
R	21	+++	++
M	21	+++	+
A	21	+++ /++++	+/++
L	22	++++	+/++
S	23	++++	+
	24	++++	+
1	LH+3	+++ /++++	+/++
2	LH+3	+++	+
3	LH+3	+++	+
4	LH+4	++++	+
5	LH+4	+++	+
6	LH+6	+++	+
7	LH+6	++	+/++
8	LH+7	+++	+
9	LH+7	++++	+/++
10	LH+7	+++	+
11	LH+8	++	+/++
12	LH+8	+	+
13	LH+8	++ /+++	+
14	LH+8	+/++	+
15	LH+8	+++	+
16	LH+8	+	-/+
17	LH+8	++	+/++
18	LH+9	+++	+
19	LH+9	++	+/++
20	LH+9	++++	++
21	LH+9	++++	+/++
22	LH+10	++ /+++	+
23	LH+10	++	++
24	LH+10	+	+

Figure 4.1: Staining for LIF in endometrial biopsies from women who suffer unexplained recurrent miscarriages. Examples of (a and b) normal and (c and d) weak staining.



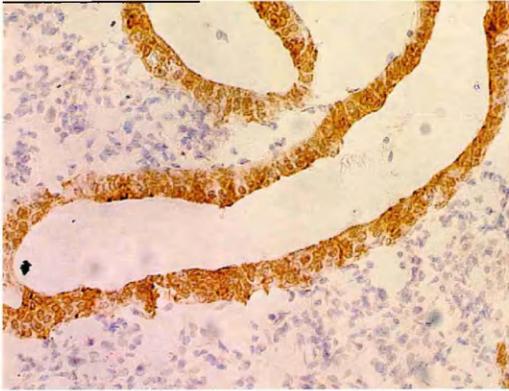
Magnification = X400

Table 4.3: Semi quantitative analysis of staining intensity for IL-6 in normal fertile women and in 15 endometrial biopsies from women with recurrent miscarriage. All biopsies were obtained during the implantation period. The LH days shown are those calculated from the morphological appearance of the tissue.

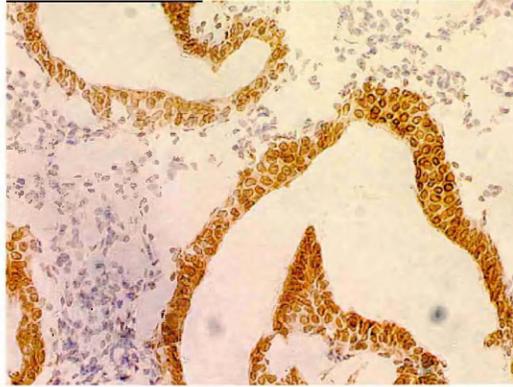
Biopsy	Day of cycle	Epithelial staining	Stromal staining
	20	+++ /++++	-
N	21	+++ /++++	-
O	22	+++ /++++	-
R	23	+++ /++++	-
M	23	++++	-
A	23	++++	-
L	24	++++	-
S	24	++++	-
	24	++++	-
1	LH+3	++	-
2	LH+4	++++	-
3	LH+6	+++ /++++	-
4	LH+7	++	-
5	LH+8	++	-
6	LH+8	++++	-
7	LH+8	+	-
8	LH+8	++++	-
9	LH+8	++	-
10	LH+8	++	-
11	LH+8	+	-
12	LH+8	+	-
13	LH+8	++	-
14	LH+9	++++	-
15	LH+9	+++ /++++	-

Figure 4.2: Staining for IL-6 in endometrial biopsies from women who suffer unexplained recurrent miscarriages. Examples of (a and b) normal and (c and d) weak staining.

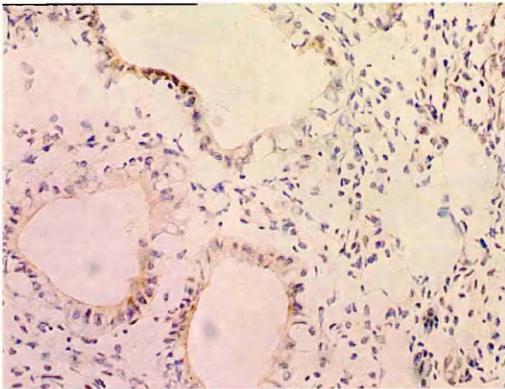
a)



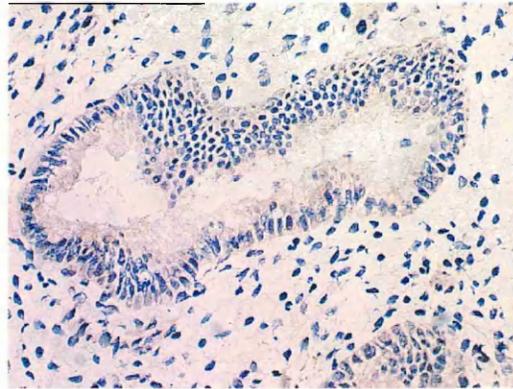
b)



c)



d)



Magnification = X400

show similar staining to that seen in normal fertile women (figure 4.2a and b), and two of which show weaker staining than that seen in normal fertile women (figure 4.2c and d).

4.3.3 Interleukin-1 α

Staining for IL-1 α was assessed in 12 biopsies from recurrent miscarriage women and compared to staining seen in 7 biopsies from normal fertile women obtained at the time of implantation. All recurrent miscarriage biopsies showed positive staining for IL-1 α although the intensity varied between individuals. Table 4.4 shows the semi-quantitative analysis of IL-1 α intensity in the recurrent miscarriage biopsies. Of the 12 biopsies assessed, 5 showed decreased stromal and epithelial staining when compared to normal fertile women (biopsies 1, 2, 5, 6 and 12) and another 2 biopsies showed a slight decrease in stromal staining only (biopsies 8 and 9). Examples of staining for IL-1 α in biopsies from recurrent miscarriage women are shown in figure 4.3, where (a) and (b) show similar staining and (c) and (d) show weaker staining than that seen in normal fertile women at the time of implantation.

4.3.4 Interleukin-1 β

Staining for IL-1 β was assessed in 11 biopsies from recurrent miscarriage women and compared to that seen in 10 biopsies from normal fertile women at the time of implantation. Staining for IL-1 β was apparent in all recurrent miscarriage biopsies, but again varied between individuals. The semi-quantitative analysis of staining intensity is shown in table 4.5. Staining for IL-1 β was weaker in both the epithelial and stromal cells of 2 of the recurrent miscarriage biopsies (biopsies 1 and 4), when compared to staining in normal fertile women. However, epithelial staining in some biopsies was very variable throughout the section. In some biopsies only parts of glands stained, whereas in others some glands showed positive staining and others showed no staining. Even when glandular epithelial staining appeared variable throughout the sections staining in the luminal epithelium was always strong. This pattern of staining was not seen in normal fertile women, where epithelial staining

Table 4.4: Semi quantitative analysis of staining intensity for IL-1 α in normal fertile women and in 12 endometrial biopsies from women with recurrent miscarriage. All biopsies were obtained during the implantation period. The LH days shown are those calculated from the morphological appearance of the tissue.

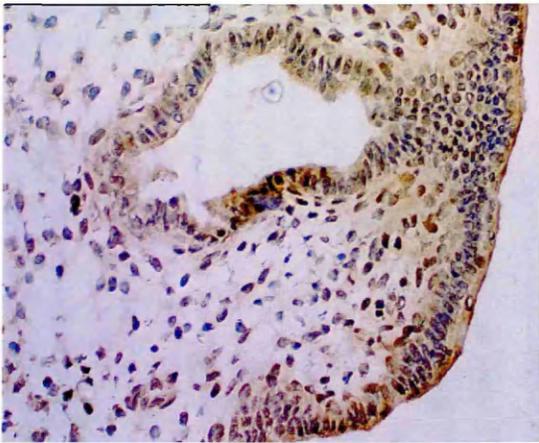
Biopsy	Day of cycle	Epithelial staining	Stromal staining
N	20	+++	++
O	20	+++	+ / ++
R	20	+++	++
M	21	+++	+ / ++
A	21	+++	++
L	23	++++	++ / +++
S	24	+++ / +++++	++ / +++
1	LH+6	++	+
2	LH+7	++	- / +
3	LH+7	+++ / +++++	+ / ++
4	LH+8	+++	++
5	LH+8	+ / ++	- / +
6	LH+8	+	-
7	LH+8	++++	++
8	LH+9	+++ / +++++	+
9	LH+9	+++	+
10	LH+10	+++	+ / ++
11	LH+10	+++	+
12	LH+10	- / +	-

Figure 4.3: Staining for IL-1 α in endometrial biopsies from women who suffer unexplained recurrent miscarriages. Examples of (a and b) normal and (c and d) weak staining.

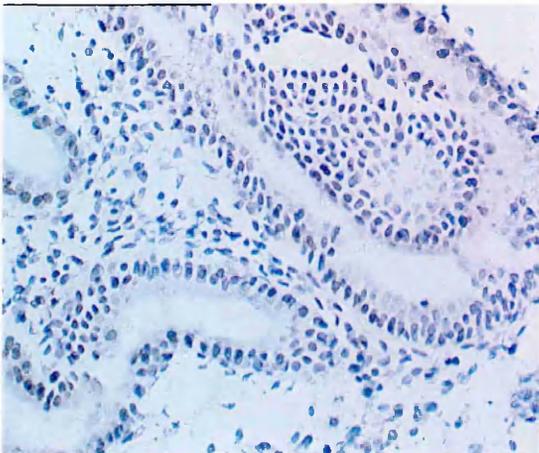
a)



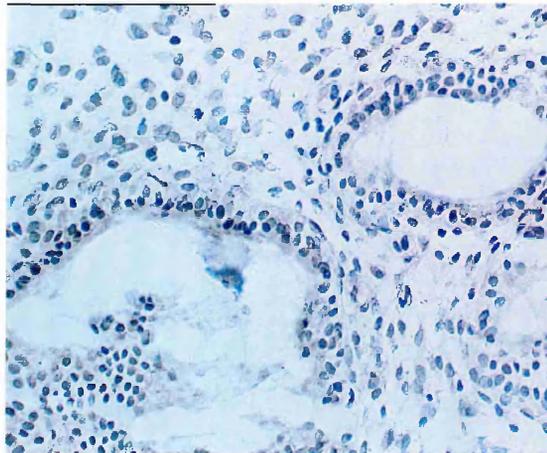
b)



c)



d)



Magnification = X400

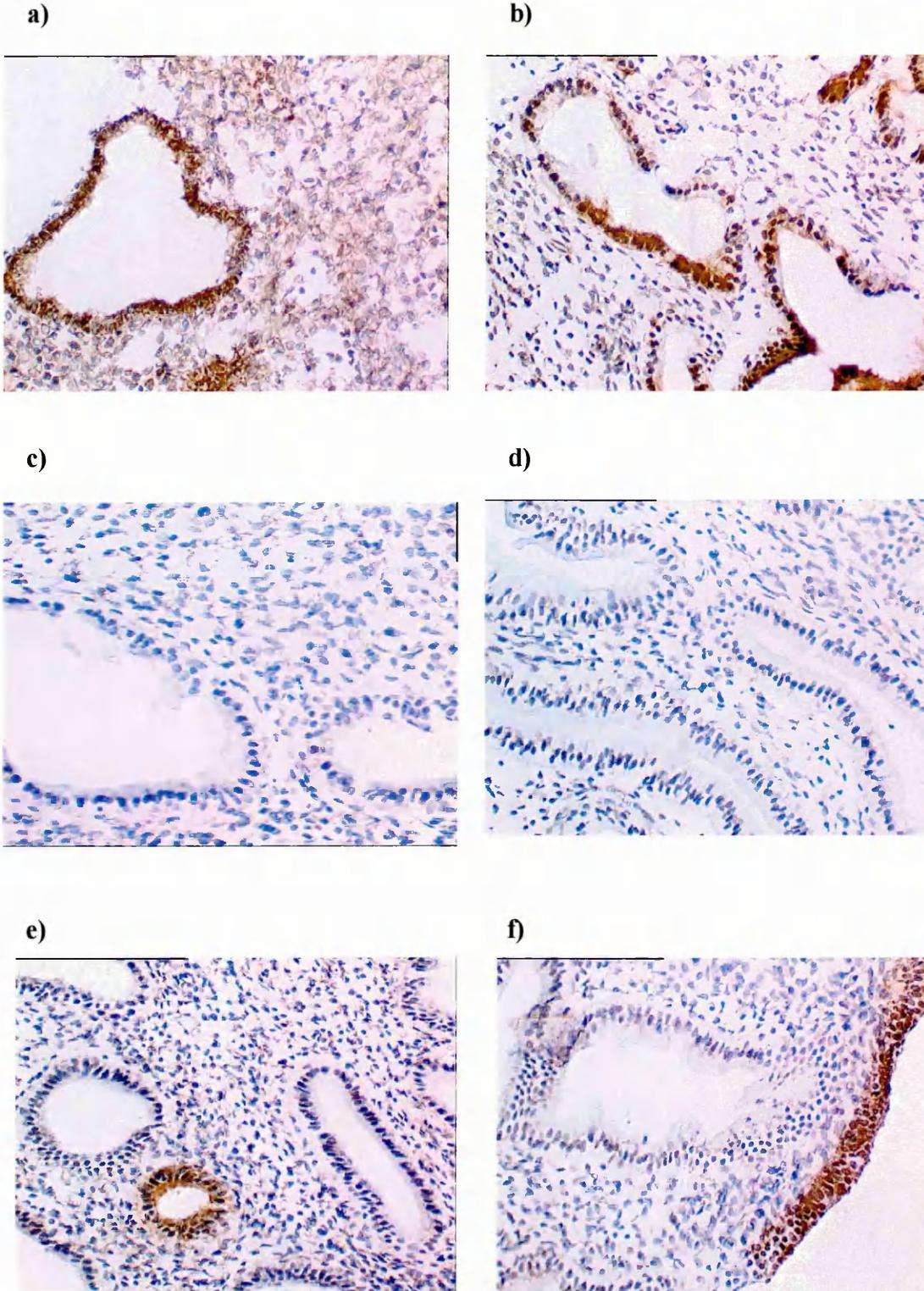
Table 4.5: Semi quantitative analysis of staining intensity for IL-1 β in normal fertile women and in 11 endometrial biopsies from women with recurrent miscarriage. All biopsies were obtained during the implantation period. The LH days shown are those calculated from the morphological appearance of the tissue.

Biopsy	Day of cycle	Epithelial staining	Stromal staining
	20	+++	+/**
N	20	++++	+
O	20	+++/**	+
R	21	++++	**
M	21	++++	+/**
A	21	++++	+/**
L	21	++++	+
S	22	++++	+
	23	+++	**
	24	+++	**
1	LH+6	+	-/**
2	LH+7	++++	+/**
3	LH+7	+++	+/**
4	LH+8	+	-/**
5	LH+8	++++	+
6	LH+8	++++	+
7	LH+8	+++	+
8	LH+9	+++	+
9	LH+9	++++	+
10	LH+10	++++	**
11	LH+10	++++	+

always appeared uniform throughout the glandular and luminal epithelium. Figure 4.4 shows examples of staining seen in recurrent miscarriage biopsies. Figure 4.4 (a) and (b) shows staining similar to that seen in normal fertile women, figure 4.4 (c) and 4.4 (d) shows staining weaker than that in normal fertile women and figure 4.4 (e) and (f) shows examples of variable epithelial staining seen in biopsies from recurrent miscarriage women. This variable pattern of staining was seen on each occasion that the immunocytochemistry was carried out.

Not all of the recurrent miscarriage biopsies were tested for all of the four cytokines. However, some of the biopsies were stained for more than one cytokine. Table 4.6 shows all of the biopsies tested, which of the cytokines each biopsy was tested for and if the expression was similar or weaker than that in normal fertile women at the time of implantation. There are some biopsies which show normal staining intensities for all the cytokines tested and some which show low staining intensities for all cytokines tested. However, for the majority there are too many gaps in staining to assess whether when the expression of one cytokine is low, all the others are also decreased.

Figure 4.4: Staining for IL-1 β in endometrial biopsies from women who suffer unexplained recurrent miscarriages. Examples of (a and b) normal, (c and d) weak and (e and f) variable staining



Magnification = X400

**Table 4.6: Table to show which biopsies were tested for which cytokine.
 NT= not tested, += normal staining and - = weak staining when
 compared to normal fertile women at the time of implantation**

Biopsy	Day	LIF	IL-6	IL-1 α	IL-1 β
1	LH+3	+	-	NT	NT
2	LH+3	+	NT	NT	NT
3	LH+3	+	NT	NT	NT
4	LH+4	+	+	NT	NT
5	LH+4	+	NT	NT	NT
6	LH+6	+	+	NT	NT
7	LH+6	-	NT	-	-
8	LH+7	+	NT	NT	NT
9	LH+7	+	NT	NT	NT
10	LH+7	+	-	NT	NT
11	LH+7	NT	NT	-	+
12	LH+7	NT	NT	+	+
13	LH+8	-	-	NT	NT
14	LH+8	-	-	NT	NT
15	LH+8	-	NT	NT	NT
16	LH+8	-	NT	NT	NT
17	LH+8	NT	+	NT	NT
18	LH+8	NT	-	NT	NT
19	LH+8	+	+	NT	NT
20	LH+8	NT	-	NT	NT
21	LH+8	NT	-	NT	NT
22	LH+8	NT	-	NT	NT
23	LH+8	-	NT	+	-
24	LH+8	-	-	NT	NT
25	LH+8	NT	NT	-	+
26	LH+8	NT	NT	-	+
27	LH+8	NT	NT	+	NT
28	LH+8	NT	NT	NT	+
29	LH+9	NT	+	NT	NT
30	LH+9	+	+	NT	NT
31	LH+9	-	NT	e=+ s=-	+
32	LH+9	+	NT	NT	NT
33	LH+9	+	NT	e=+ s=-	+
34	LH+10	-	NT	NT	NT
35	LH+10	NT	NT	+	+
36	LH+10	-	NT	+	+
37	LH+10	-	NT	NT	NT
38	LH+10	NT	NT	-	NT

4.4 Discussion

The results from this study suggest that expression of LIF, IL-6, IL-1 α and IL-1 β protein is decreased in the endometrium of some recurrent miscarriage women at the time of implantation. This agrees with previous work that has shown a decreased expression of IL-6 mRNA and protein and IL-1 β mRNA (von Wolff *et al.*, 2000; Lim *et al.*, 2000) in recurrent miscarriage women compared to normal fertile women.

Protein expression was measured using immunocytochemical staining. Intensity was assessed using semi-quantitative analysis. In all cases the staining was carried out at least three times and a negative control run parallel to the sections whereby the antibody had been neutralised for 24h prior to incubation. Each time a set of slides with sections from recurrent miscarriage women was analysed, a section from a biopsy obtained at the same time in the cycle from a normal fertile women was included, to aid in comparison between the staining.

4.4.1 Leukaemia Inhibitory Factor and Interleukin-6

LIF expression was significantly weaker in 11 of the 16 (69%) recurrent miscarriage biopsies included in the experiment, mainly in epithelial cells. The results also showed a similar proportion of biopsies from recurrent miscarriage women had reduced epithelial IL-6 expression (9 of the 15 biopsies (60%)). These results therefore suggest that both endometrial LIF and IL-6 may be contributing factors to preventing recurrent miscarriage.

The results of the previous chapter show that changes in LIF and IL-6 occur through the menstrual cycle with maximum expression in the late secretory phase. A number of the endometrial biopsy samples from recurrent miscarriage women had retarded development, a phenomenon that is known to be associated with recurrent miscarriage. It is therefore possible that the reduced staining for LIF and IL-6 seen in some of the recurrent miscarriage women may be due to delayed endometrial development. However, the results in table 4.2 and 4.3 show that there is no relationship between LH day and intensity of staining and therefore this observed

decrease in LIF and IL-6 staining appears independent of whether the endometrium is retarded or not.

4.4.2 Interleukin-1 α and Interleukin-1 β

In contrast to IL-6 and LIF a smaller proportion of recurrent miscarriage women showed lower epithelial expression of IL-1 α (4 out of 12; 25%) and IL-1 β (2 out of 11; 18%). However, for IL-1 β a very variable pattern of staining intensity was seen. The glandular epithelial staining in biopsies from normal fertile women was uniform throughout the sections from all biopsies tested. However, in some biopsies from recurrent miscarriage women the staining was very patchy in some glands and in some cases was entirely absent. In other cases it was seen in the luminal epithelium, but not in the glandular epithelium. The reason for this variation in staining is not known.

These results show that in some women who suffer recurrent miscarriage there is a decreased expression of more than one cytokine. Cytokines are known to be very redundant and it is therefore likely that the decreased expression of just one cytokine could not affect implantation. However, if more than one cytokine is affected it suggests a greater chance of implantation being unsuccessful and in these women may be a cause of pregnancy loss. Because of this redundancy, and the fact that recurrent miscarriage women are a very heterogeneous population, it would have been sensible to try and analyse all 4 cytokines in each biopsy sample. However, this was not possible, partly due to lack of planning, but also because there was a limited number of sections, which could be obtained from a single biopsy.

Chapter 5

Effect of cytokines on endometrial MMP production

5.1 Introduction

One of the autocrine/paracrine actions of cytokines may be to control the expression of MMPs. MMPs are enzymes that are produced by the endometrium and are known to be important in the degradation process associated with menstruation. It is also thought that because MMPs are capable of degrading the extracellular matrix (ECM), they could play a role in the invasion of trophoblast cells into the maternal decidua (Bischof *et al.*, 1995b; Hulboj *et al.*, 1997). Although the pattern of expression of MMPs in normal cycling endometrium is understood, the precise regulation of these enzymes has yet to be fully elucidated.

The endometrial cytokines, IL-1, TNF α , IL-6 and LIF have been postulated to be involved in the control of implantation (Stewart *et al.* 1992; Hunt *et al.* 1992; Sharkey, 1998; Simon *et al.*, 1998) and thus may affect the production of MMPs during this process. Studies have shown that IL-1 and TNF α affect MMP-2 and MMP-9 production by endometrial stromal cells (Rawdanowicz *et al.* 1994; Singer *et al.*, 1999) and that IL-1, TNF α , LIF and IL-6 affect gelatinase production by cytotrophoblast cells (Bischof *et al.*, 1995a; Meisser *et al.*, 1999a; Meisser *et al.*, 1999b). To date there are no reports of effects of LIF and IL-6 on MMP-2 and MMP-9 production by endometrial cells and studies of the effects of any of these cytokines on MMP production by epithelial cells are limited.

Therefore the aims of this study were to compare the production of MMP-2, MMP-9 and MMP-7 by cultured human endometrial epithelial and stromal cells and to determine if the cytokines LIF, IL-6, IL-1 α or TNF α have any effect on the production of these MMPs *in vitro*. In particular, if LIF and IL-6 affect MMP-2 and MMP-9 production as they do in cytotrophoblast cells.

5.2 Materials and Methods

5.2.1 Tissue culture

Endometrial biopsies were obtained from 12 normal fertile women as described in chapter 2. Primary epithelial and primary stromal cell cultures were prepared from each biopsy as previously described. Once the cells reached confluency, they were incubated for a further 48 hours, with either no additions, 0.1ng/ml, 1ng/ml or 10ng/ml of either IL-1 α , TNF α , IL-6 or LIF. Epithelial and stromal cell cultures from three biopsies obtained on days 7, 17 and 25 of the menstrual cycle were incubated with LIF. Epithelial and stromal cell cultures from three biopsies obtained on days 9, 14 and 17 were incubated with IL-6. Epithelial and stromal cell cultures from three biopsies obtained on days 9, 14 and 17 were incubated with IL-1 α and from biopsies obtained on days 14, 22 and 24 were incubated with TNF α . After incubation the supernatants were removed from the cells and stored at -20°C until analysed using zymography or ELISA.

5.2.2 Zymography

Zymography was used to analyse MMP-2 and MMP-9 activity in the supernatants of cultured endometrial cells, as described in chapter 2. Supernatants from each cell culture were run on gels on at least three separate occasions. In each gel, supernatants from cells cultured with 0, 0.1 ng/ml, 1 ng/ml and 10 ng/ml of each cytokine were run parallel to a marker and supernatants from the D3X cell line, which was used as a positive control. The bands representing MMPs were analysed visually and also using densitometric analysis, as described in chapter 2.

5.2.3 ELISA

The amounts of MMP-2, MMP-9 and MMP-7 produced from cultured endometrial epithelial and stromal cells was measured quantitatively using commercially available BIOTRA ELISA kits (Amersham Life Sciences), as described in chapter 2.

5.2.4 Statistical analysis

Data was expressed as group means \pm SEM (n = 4, number of replicate wells from a single experiment). Results shown are those obtained from cells prepared from a single biopsy and are typical of those obtained on each of the 3 occasions that the

experiment was repeated. Differences in the amounts of MMP produced in the presence of different cytokines were analysed using ANOVA.

5.3 Results

5.3.1 Basal production of MMP-2, MMP-9 and MMP-7

Analysis of supernatants from endometrial epithelial (figure 5.1a) and stromal (figure 5.1b) cells, cultured without cytokines, by gelatin zymography showed between 2 and 6 bands of gelatinase activity, with molecular weights of 234, 116, 90, 84, 70, and 66kDa (Figure 5.1). A summary of the results obtained from cells prepared from all twelve biopsies is shown in Table 5.1. Bands of molecular weights 90kDa, 84kDa, 70kDa and 66kDa showed the greatest activity. It is likely that these bands represent the pro and active (cleaved) forms of MMP-9 (90kDa and 84kDa) and MMP-2 (70kDa and 66kDa) and this is supported by results from the D3X cell supernatants, which also show bands at 90kDa, 70kDa and 66kDa. The band at 84kDa is not present in D3X cell supernatants, but its position in the gel and molecular weight, suggest that it is active MMP-9. The identity of the higher molecular weight bands is not known, but may represent MMP-2 and MMP-9 bound to TIMPs. Bands corresponding to proMMP-2 and proMMP-9 were seen in all cell supernatants while the band corresponding to active MMP-9 was much weaker than that for pro MMP-9 and was only seen in some cell supernatants. Bands for active MMP-2 were seen in all supernatants from all cultured epithelial cells, but only in some supernatants from cultured stromal cells. Although similar band sizes for MMP-2 were seen in supernatants from stromal and epithelial cells, the bands for MMP-9 produced from stromal cell cultures were smaller than that seen in epithelial cell supernatants.

Basal production of MMP-2, MMP-9 and MMP-7 was also measured using ELISA (Table 5.2). This analysis showed that total MMP-2 (i.e. both pro and active forms were detected by the antibody), pro-MMP-9 and pro-MMP-7 were present in epithelial cell supernatants. However, under basal conditions only MMP-2 was detected in stromal cell supernatants and the amounts present were higher than those present in epithelial cell supernatants. None of the MMPs were detected in unconditioned CDMEM. These results confirm the zymography results showing that basal stromal cell production of MMP-9 is very low, and that epithelial cells produce both MMP-2 and MMP-9.

Figure 5.1: Zymography gels showing gelatinase activity in supernatants from unsupplemented (a) epithelial and (b) stromal cells. Lane 1 contains the molecular weight marker; and lanes 2-6 contain supernatants from different cell cultures. In (a) lanes 2 and 5 contain supernatants from cells prepared from proliferative endometrium, while lanes 3, 4 and 6 contain supernatants from cells prepared from secretory endometrium. In (b) lanes 2, 5 and 6 contain supernatants from cells prepared from proliferative endometrium, while lanes 3 and 4 contain supernatants from cells prepared from secretory endometrium. Arrows indicate the 6 bands of gelatinase activity seen at 234, 116, 90, 84, 70 and 66kDa.

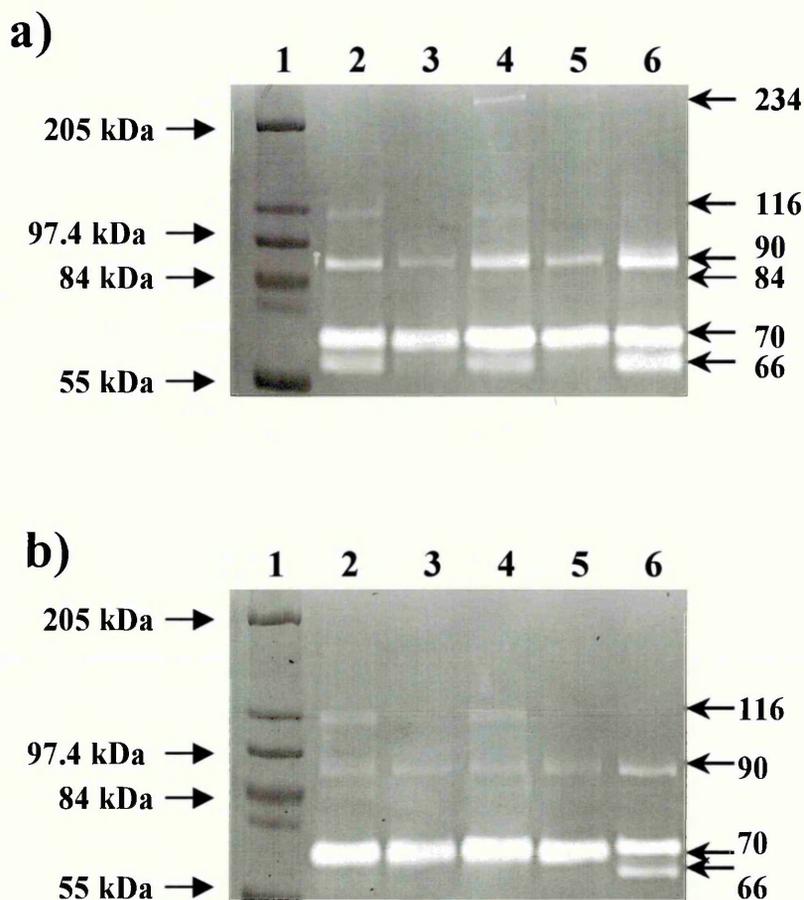


Table 5.1: Semi-quantitative analysis of bands of gelatinase activity in supernatants from unsupplemented cultured (a) epithelial and (b) stromal cells, +++ = high gelatinase activity, ++ = moderate gelatinase activity, + = low gelatinase activity and - = no gelatinase activity.

a)

Biopsy	Time in Cycle	Band MW	1 234	2 116	3 90	4 84	5 70	6 66
1	P		-	-	++	-/+	+	+
2	P		-	-	-/+	-	+	-/+
3	P		-	-/+	++	-	++	+/>++
4	P		-/+	-	+	-	++	-/+
5	P		-/+	-	+/>++	-/+	++	-/+
6	P		-	-	-/+	+	-/+	+
7	S		-/+	-	++	-	+++	++
8	S		-	-	+	-	++	-/+
9	S		-	-	+	-	++	++
10	S		-	-	++	-	++	+
11	S		-	-	-/+	-	+/>+++	-/+
12	S		+	-/+	++	-	+++	+/>++

b)

Biopsy	Time in cycle	Band MW	1 234	2 116	3 90	4 84	5 70	6 66
1	P		-	-	-/+	-	+	-/+
2	P		-/+	-	-/+	-	+/>++	-/+
3	P		-	-/+	-/+	-/+	+++	-
4	P		-	-	-/+	-	+/>+++	-
5	P		-	-	-/+	-	++	-
6	P		-	-	+	-	++	+/>++
7	S		-	-/+	-/+	-	+/>++	-/+
8	S		++	-/+	++	+/>++	+	++
9	S		-/+	-	+	-	++	+
10	S		-	-	-/+	-	+/>++	-
11	S		-	-	-/+	-	+/>+++	-
12	S		-	-/+	-/+	-/+	+++	-

Table 5.2: Basal production of MMP-2, MMP-9 and MMP-7 by endometrial epithelial and stromal cells. Values shown are ranges from cells prepared from 12 different biopsies.

	Epithelial (ng/ml/24h)	Stromal (ng/ml/24h)
Total MMP-2	24.8-503.2	59.2-742.2
Pro MMP-9	0.86-100.1	<0.6
Pro MMP-7	2.9-693.2	<0.16

ELISA measurements showed that basal levels of pro-MMP-9 in epithelial cell supernatants and total MMP-2 in stromal cell supernatants were similar in cells prepared from both proliferative and secretory endometrium. However, differences were seen in total MMP-2 and pro-MMP-7 in epithelial cell supernatants between cells prepared from proliferative and secretory endometrium (Figure 5.2). Epithelial total MMP-2 levels were significantly higher ($p < 0.01$) in supernatants from cells prepared from the secretory phase compared to cells from the proliferative phase.

Zymography also suggested that pro-MMP-2 was higher in supernatants from epithelial cells prepared from secretory endometrium (band 5, Table 5.1a), compared to that from cells prepared from proliferative endometrium. In contrast pro-MMP-7 levels in supernatants from epithelial cells were significantly lower ($p < 0.01$) in cells prepared from secretory phase endometrium compared to proliferative phase endometrium.

5.3.2 Effects of cytokines on pro-MMP-9 production

Results from zymography (Figure 5.3) and densitometric analysis (Table 5.3) showed that levels of proMMP-9 in supernatants from epithelial cells were increased in the presence of $\text{TNF}\alpha$ but not $\text{IL-1}\alpha$, whereas both $\text{IL-1}\alpha$ and $\text{TNF}\alpha$ caused an increase in proMMP-9 levels in supernatants from stromal cells. ELISA measurements showed that $\text{IL-1}\alpha$ caused a concentration dependent increase in stromal proMMP-9 production ($p < 0.001$) (figure 5.4b) and $\text{TNF}\alpha$ caused a concentration dependent increase in both epithelial ($p < 0.01$) (figure 5.4c) and stromal ($p < 0.001$) (figure 5.4d) proMMP-9 production. No significant difference in epithelial proMMP-9 production was seen following incubation with $\text{IL-1}\alpha$ (figure 5.4a).

Visual analysis of the zymographs (figure 5.5) suggested that LIF and IL-6 had no significant effect on proMMP-9 levels in supernatants from epithelial and stromal cells and this was confirmed by densitometric analysis (table 5.3). In addition, no significant effects of LIF or IL-6 on proMMP-9 production were detected when the supernatants from epithelial and stromal cells were analysed using ELISA (figure 5.6).

Figure 5.2: Production of total MMP-2 and pro-MMP-7 by unsupplemented endometrial epithelial cells, prepared from secretory and proliferative endometrium. Values are \pm SEM (n=6 proliferative, n=6 secretory). ** = significantly different to cells from proliferative endometrium at $p < 0.01$

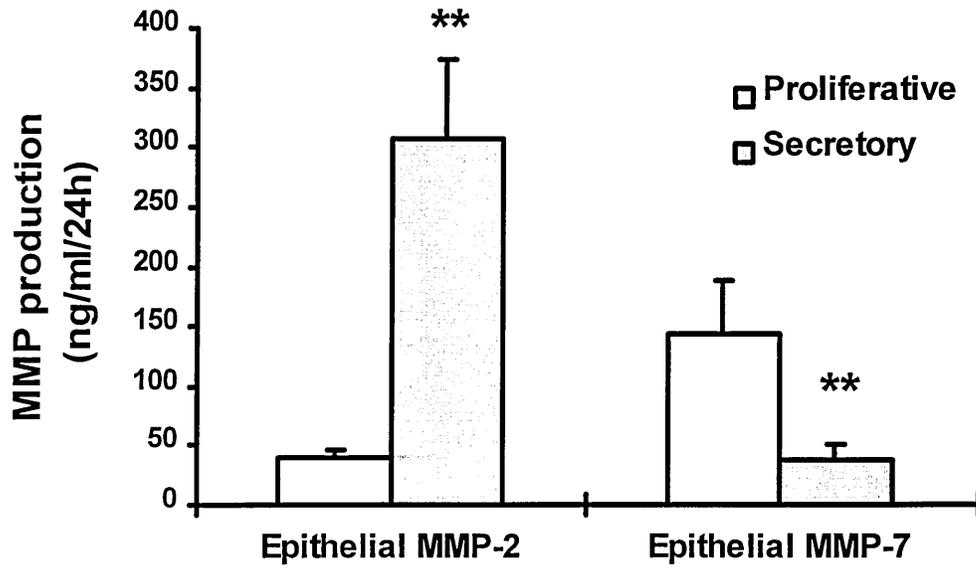


Figure 5.3: Zymography gels showing the effect of (a and b) IL-1 α and (c and d) TNF α on gelatinase secretion by cultured (a and c) epithelial and (b and d) stromal cells. Lane 1 contains the supernatant from D3X cells and lanes 2-5 supernatants from cells incubated with 0, 0.1, 1 and 10ng/ml of each cytokine. Examples shown are from cells prepared from secretory endometrium

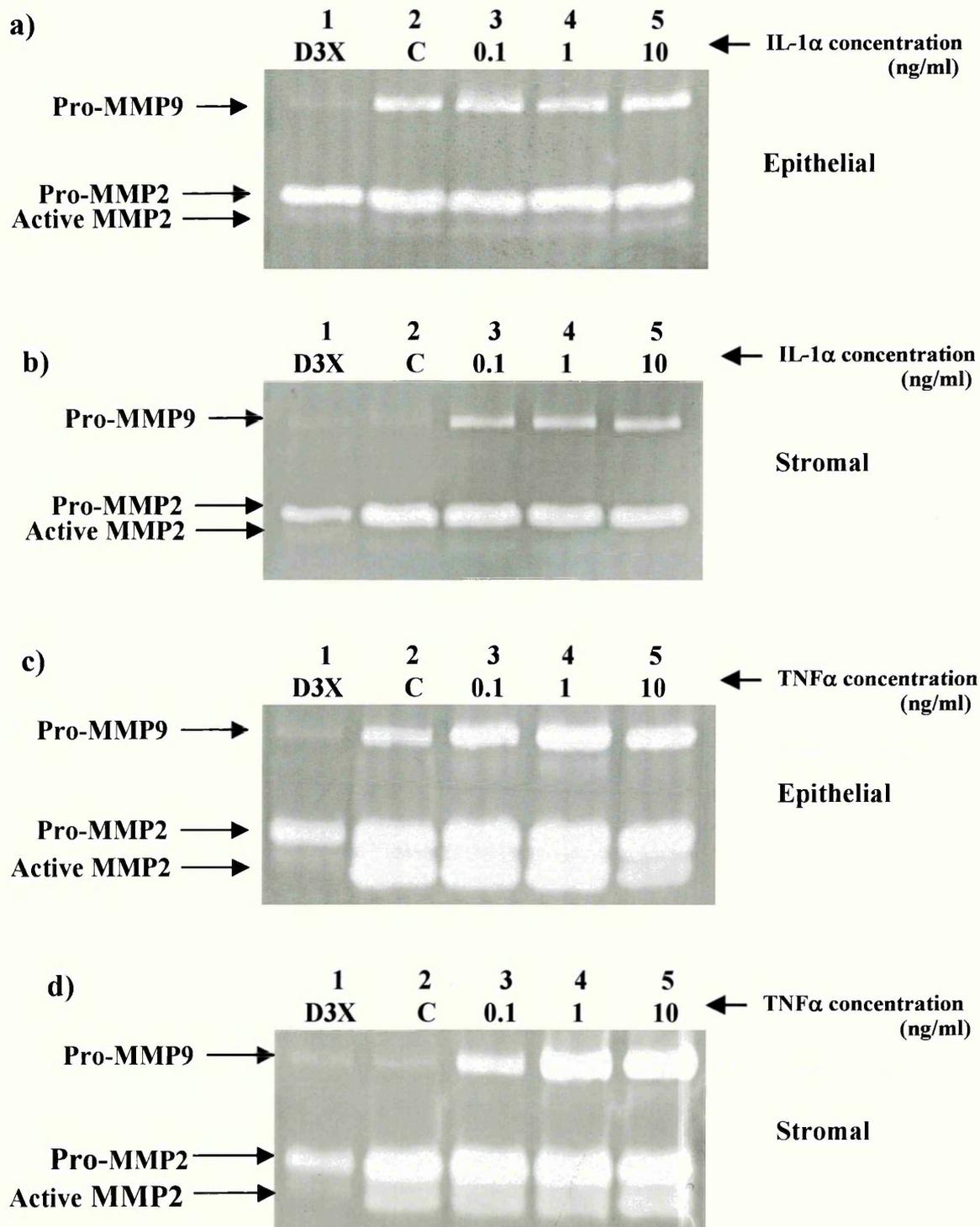


Table 5.3: Densitometric analysis of MMP-2 and MMP-9 activity bands following stimulation of cells with cytokines. Values shown are percentage change in net intensity when compared to control bands from the same gel

		Epithelial ProMMP2	Stromal ProMMP2	Epithelial ProMMP9	Stromal ProMMP9
IL-1 ng/ml	0.1	-1.1	-0.06	9.2	520.1
	1	5.2	-4.6	3	523.9
	10	12.3	-7.8	0.7	512.7
TNF ng/ml	0.1	1.7	-8.4	38.1	643.8
	1	11	3.2	86.5	1159.3
	10	-21.8	19	63.9	1385.5
LIF ng/ml	0.1	-5.4	-11.3	-25.1	8.5
	1	8.5	-1.3	12.3	12.5
	10	0.5	0.53	9.4	2.6
IL-6 ng/ml	0.1	4.5	-2.5	-7.1	-6.3
	1	-24.3	6.9	-12.2	-4.8
	10	-15.3	15.6	-14.5	-2.1

following stimulation with (a and b) IL-1 α and (c and d) TNF α . Values are mean \pm SEM (n = 4). ** = significantly different to controls at p<0.01, *** = significantly different to controls at p<0.001. Results shown are from cells prepared from a single biopsy and are typical of that seen in 3 different biopsies. All graphs show results from biopsies obtained during the secretory phase of the menstrual cycle

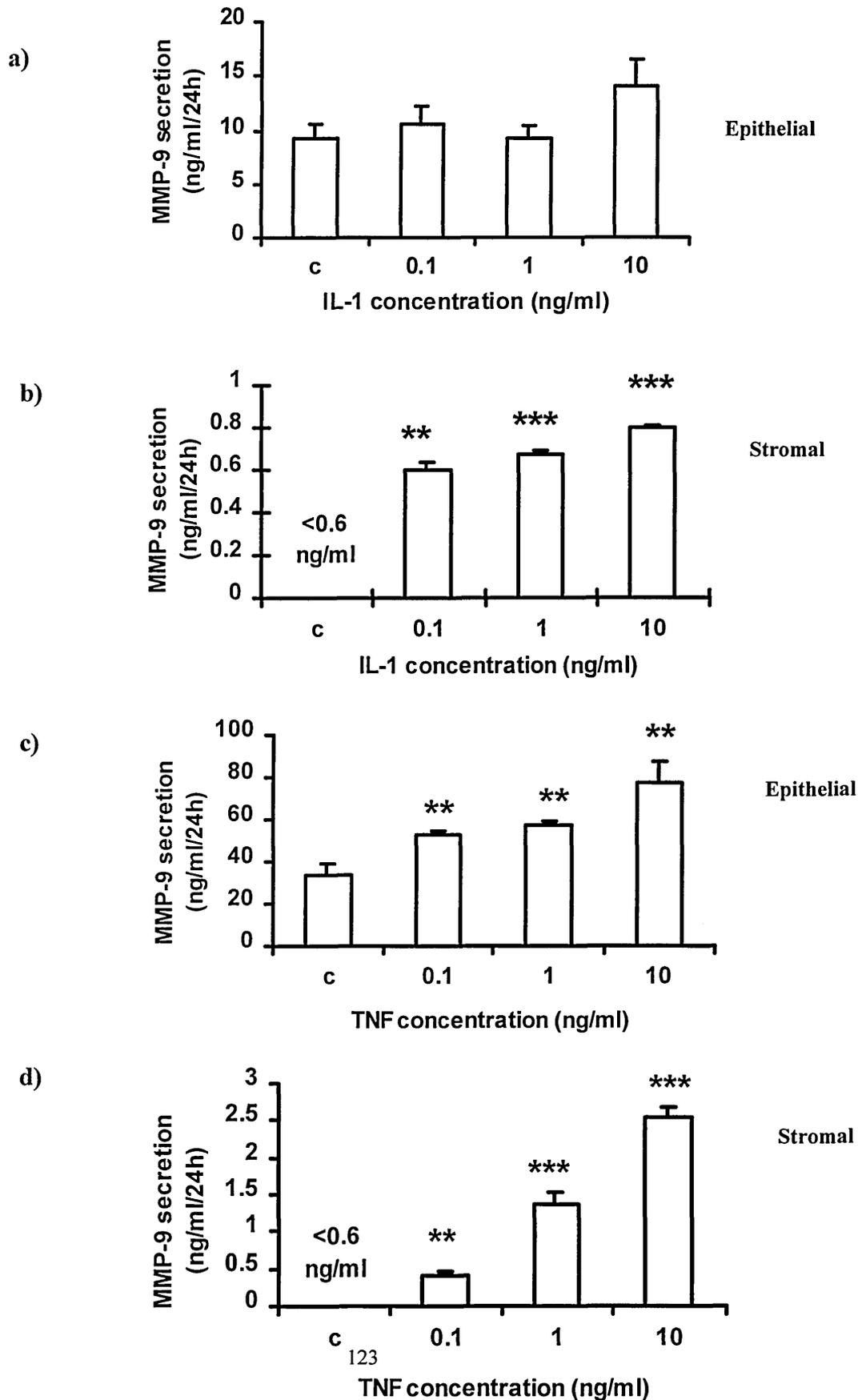


Figure 5.5: Zymography gels showing the effect of (a and b) LIF and (c and d) IL-6 on gelatinase secretion by cultured (a and c) epithelial and (b and d) stromal cells. Lane 1 contains the supernatant from D3X cells and lanes 2-5 supernatants from cells incubated with 0, 0.1, 1 and 10ng/ml of each cytokine. Examples shown are from cells prepared from secretory endometrium

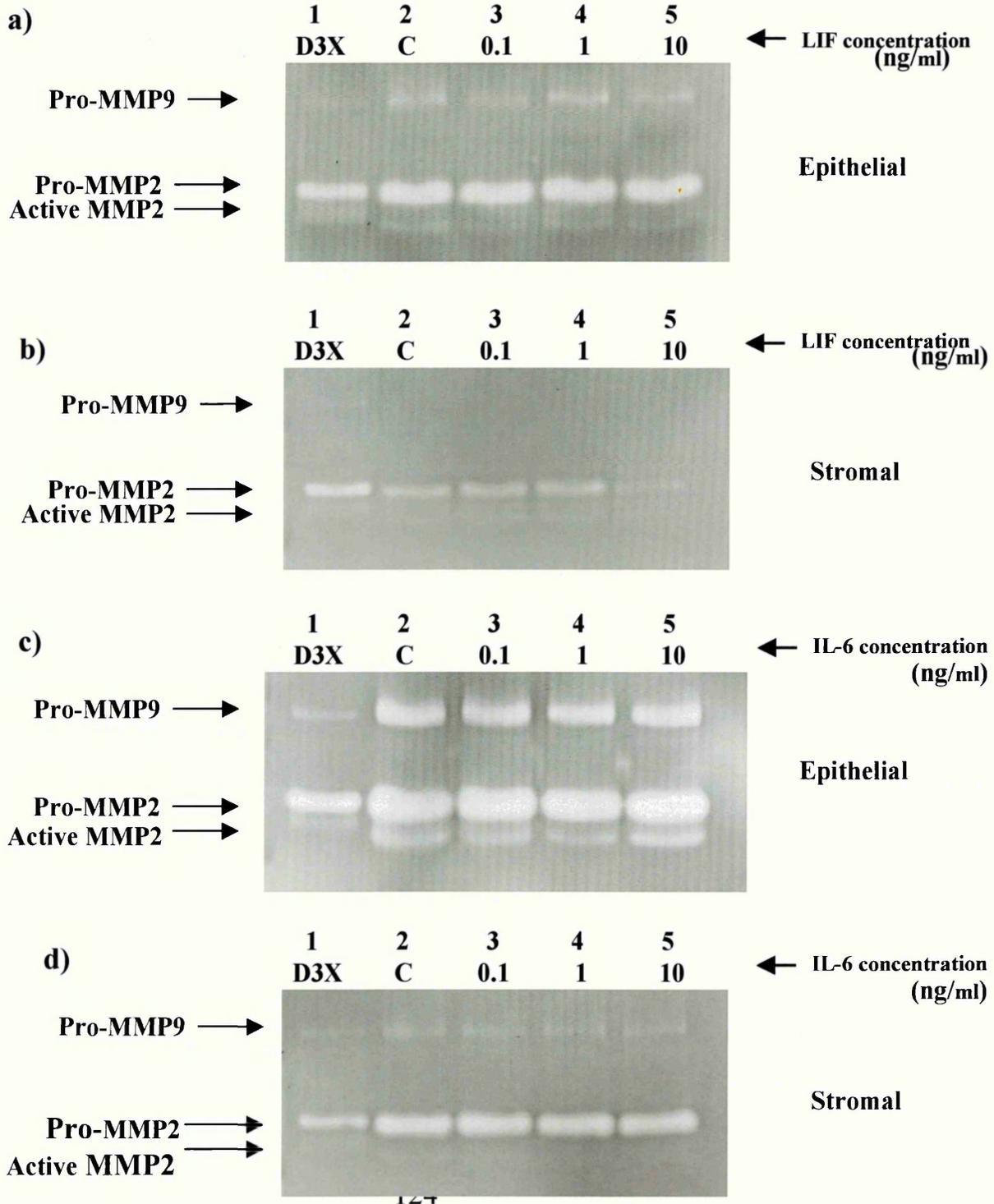
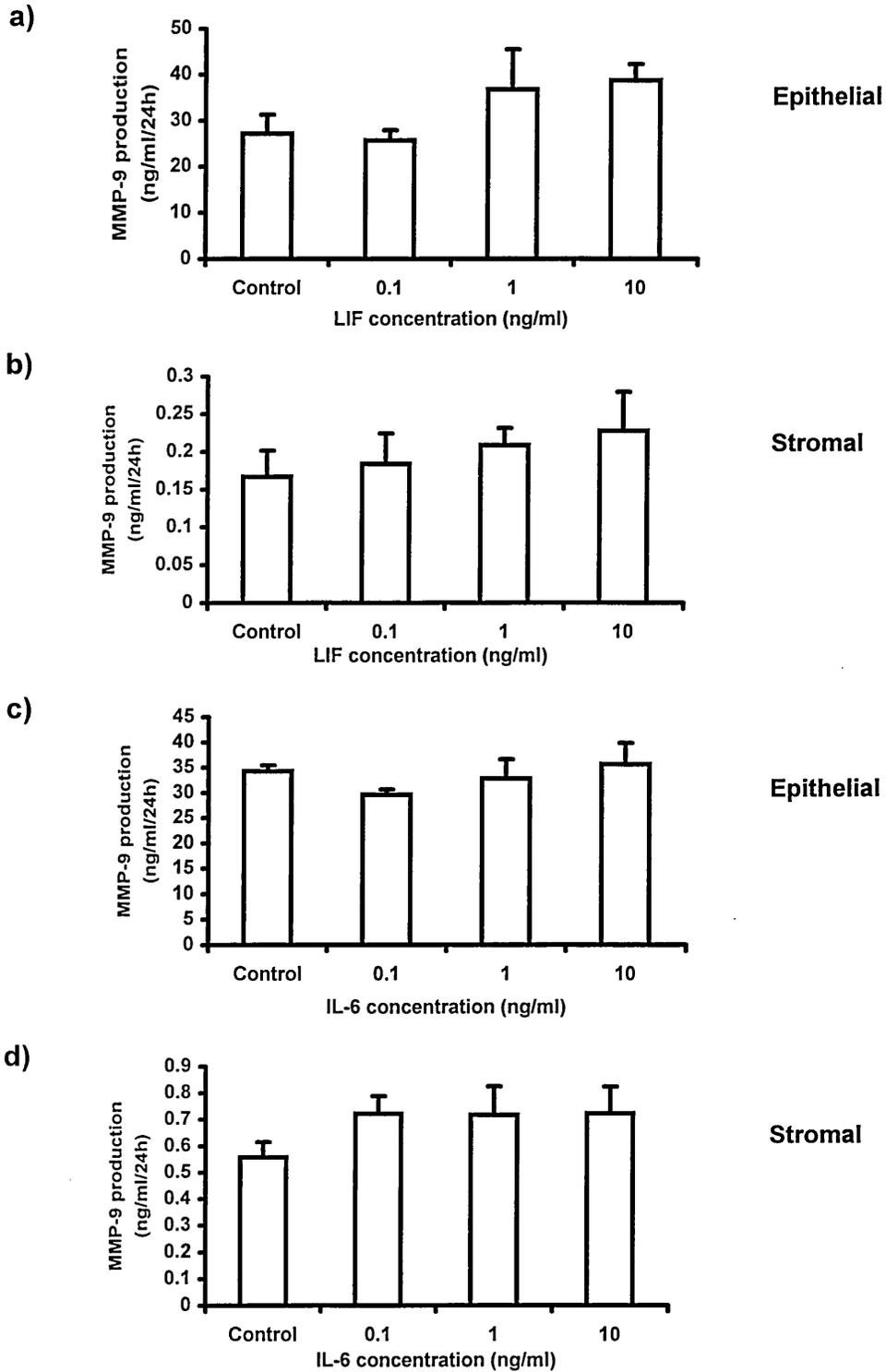


Figure 5.6: Pro MMP-9 production by (a and c) epithelial and (b and d) stromal cells following incubation with (a and b) LIF and (c and d) IL-6. Values are mean + SEM (n = 4). Results shown are from cells prepared from a single biopsy and are typical of that seen in 3 different biopsies. All graphs show results from biopsies obtained during the secretory phase of the menstrual cycle



5.3.3 Effects of cytokines on MMP-2 production

The effects of cytokines on MMP-2 production were less dramatic than that seen for MMP-9. No effect of IL-1 α on either stromal or epithelial pro-MMP-2 levels was seen using zymography (Figure 5.3) even after densitometric analysis (Table 5.3). However, a small significant increase ($P<0.05$) in epithelial total MMP-2 levels was seen at higher concentrations of IL-1 α , using ELISA (Figure 5.7a). ELISA also suggested a significant decrease ($P<0.05$) in stromal total MMP-2 production at 1ng/ml IL-1 α (figure 5.7b). Again the effect of TNF α on MMP-2 production was small. However, a significant decrease in total MMP-2 levels in epithelial cell supernatants ($P<0.01$) was seen using ELISA (Figure 5.7c) and zymography showed a decrease in both the pro and active MMP-2 bands in supernatants from epithelial cells cultured with TNF α (Figure 5.3). No significant effect of TNF α on stromal cell MMP-2 production was seen. The effects of both TNF α and IL-1 α on MMP-2 production as assessed by both zymography and ELISA, although significant, are small and therefore the physiological significance of this response is questionable.

Zymographic (Figure 5.4) and densitometric analysis (Table 5.3) suggested that LIF and IL-6 had no effect on pro or active MMP-2 levels in the supernatants of either epithelial or stromal cells. This was confirmed using ELISA (Figure 5.8), which showed that neither LIF nor IL-6 had a significant effect on epithelial or stromal total MMP-2 production.

5.3.4 Effects of cytokines on pro-MMP-7 production

Levels of pro-MMP-7 in supernatants from epithelial cells following incubation with IL-1 α , TNF α , LIF and IL-6 were measured using ELISA only. TNF α caused a significant increase in pro-MMP-7 production by epithelial cells, but only at 10ng/ml ($p<0.01$). IL-1 α , LIF and IL-6 had no significant effect on pro-MMP-7 production (Figure 5.9).

Figure 5.7: Total MMP-2 production by (a and c) epithelial and (b and d) stromal cells following incubation with (a and b) IL-1 α and (c and d) TNF α . Values are mean + SEM (n = 4). * = significantly different from controls at p<0.05, **=significantly different from control at p<0.01. Results shown are from cells prepared from a biopsy and are typical of that seen in 3 different biopsies. All graphs show results from biopsies obtained during the secretory phase of the menstrual cycle

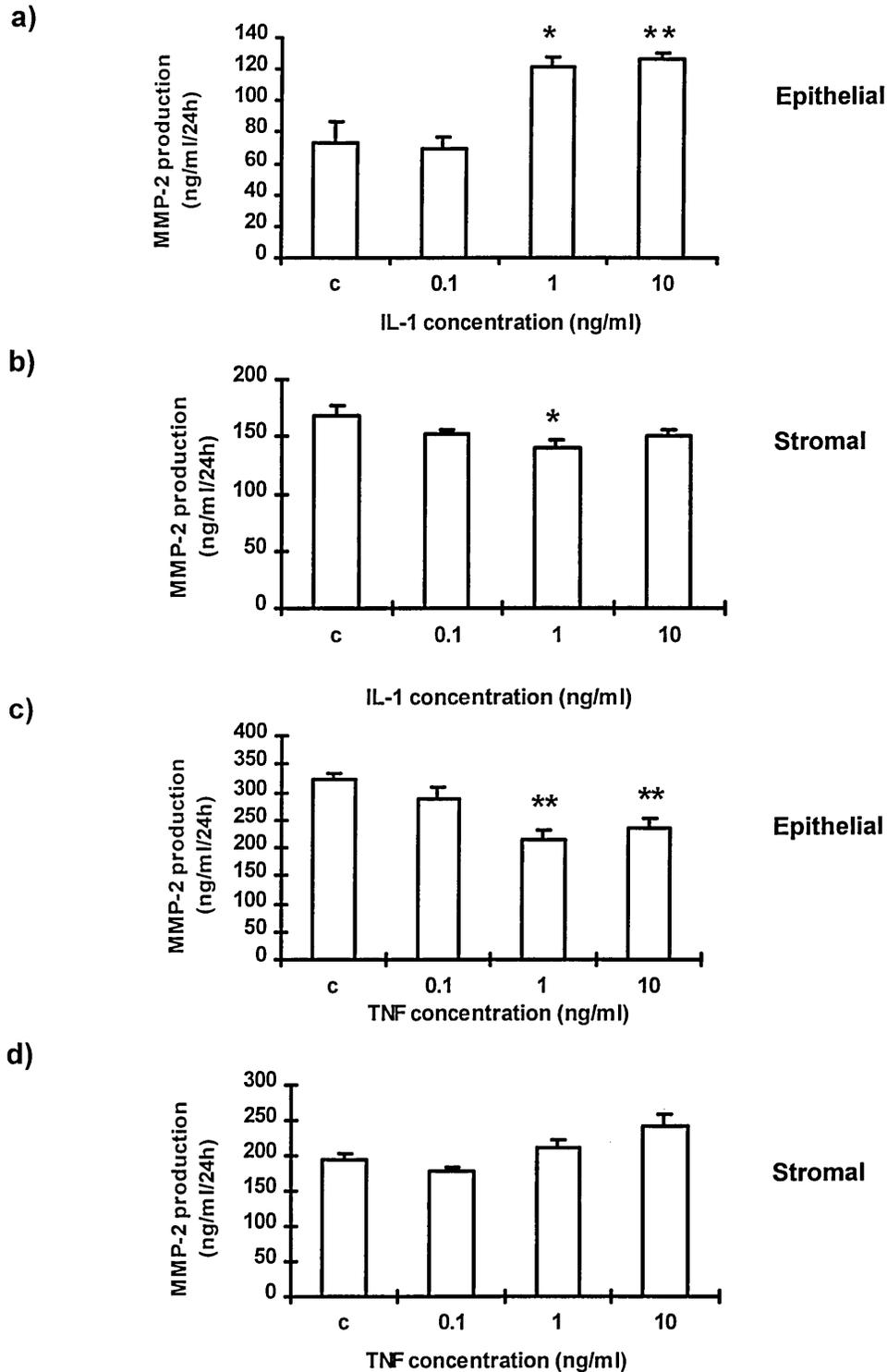


figure 5.8: Total MMP-2 production by (a and c) epithelial and (b and d) stromal cells following incubation with (a and b) LIF and (c and d) IL-6. Values are mean + SEM (n = 4). Results shown are from cells prepared from a single biopsy and are typical of that seen in 3 different biopsies. All graphs show results from biopsies obtained during the secretory phase of the menstrual cycle

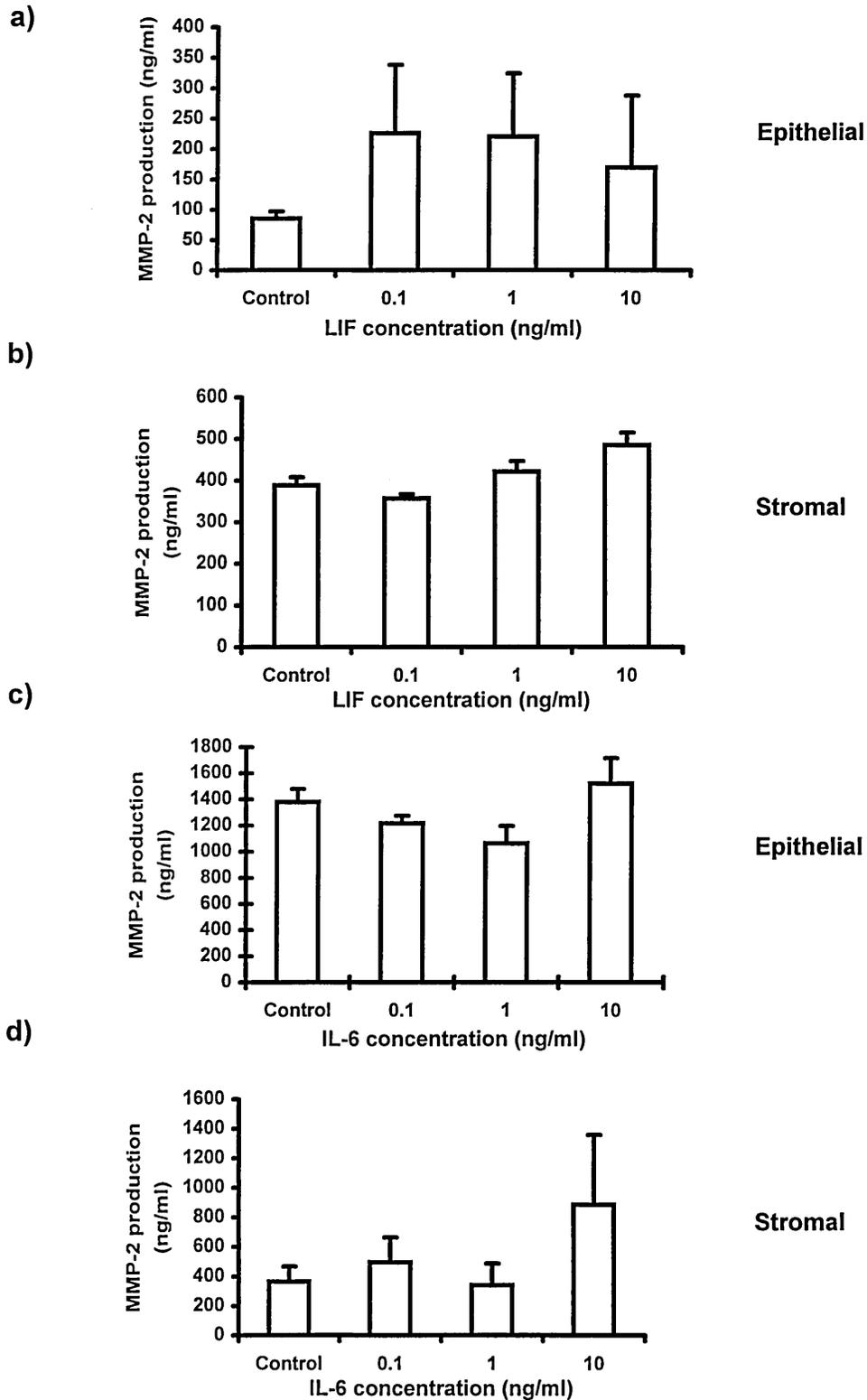
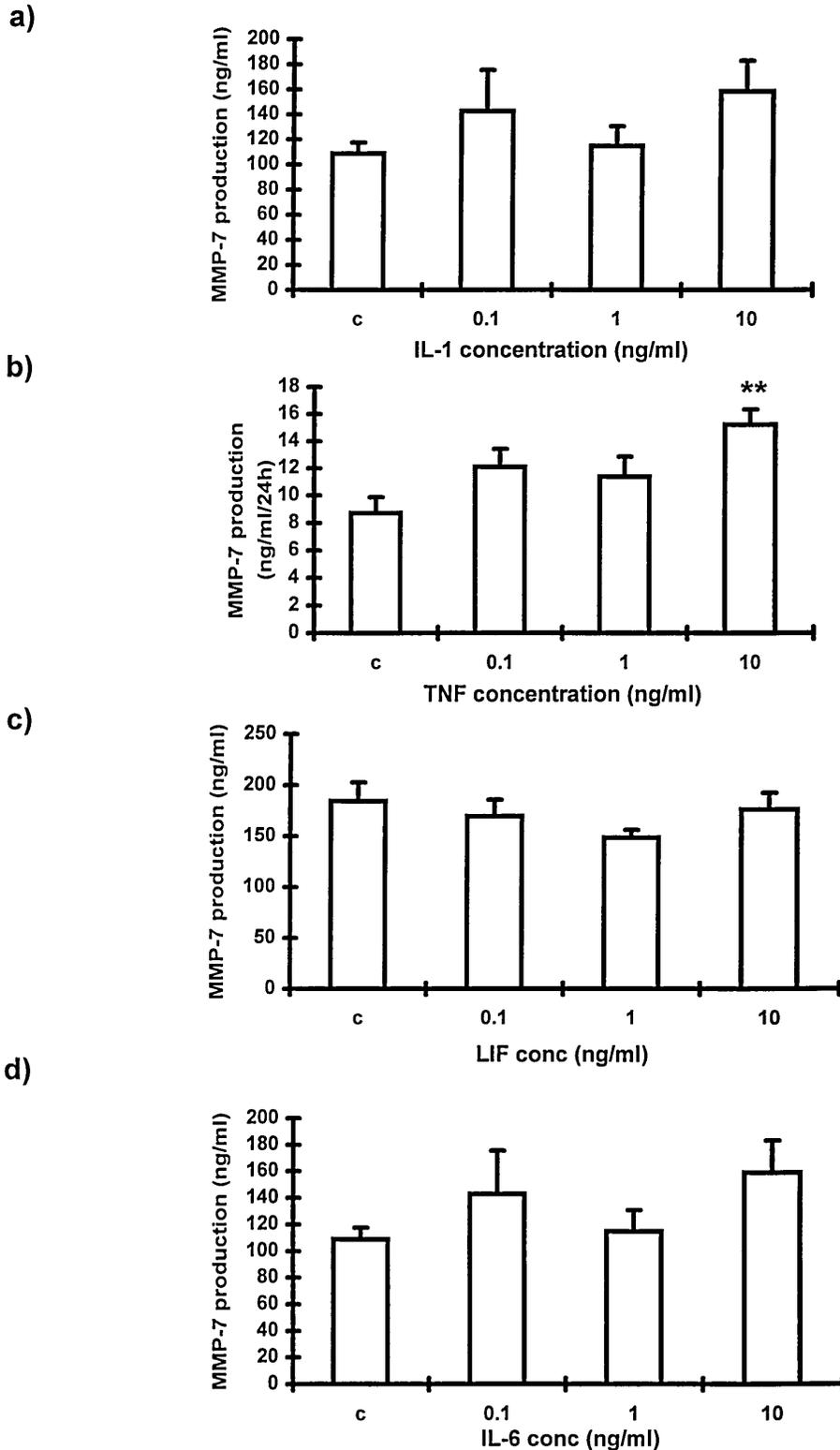


Figure 5.9: Pro MMP-7 production by epithelial cells following incubation with (a)IL-1 α , (b)TNF α , (c)LIF and (d)IL-6. Values are mean +SEM (n=4). **= significantly different from controls at p<0.01. Results shown are from cells prepared from a single biopsy and are typical of that seen in 3 different biopsies. All graphs show results from biopsies obtained during the secretory phase of the menstrual cycle



5.4 Discussion

In this study zymography and ELISA were used to detect MMP secretion by human endometrial cells *in vitro*. It was shown that pro and active MMP-2 and MMP-9 were secreted by both epithelial and stromal cells, although basal stromal MMP-9 production was very low, while pro-MMP-7 was secreted from epithelial cells only. Supplementation with various cytokines caused a concentration dependent modulation of proMMP secretion. In particular, TNF α and IL-1 α caused a large increase in pro-MMP-9 production, while having smaller stimulatory or inhibitory effects on MMP-2 production. In contrast to previous studies using cytotrophoblast cells (Bischof *et al.*, 1995a; Meisser *et al.*, 1999a; Meisser *et al.*, 1999b) addition of LIF and IL-6 had no effect on MMP production.

5.4.1 Basal production of endometrial MMPs

Gelatin zymography showed the presence of gelatinase activity at 6 different molecular masses in the endometrial cell culture supernatants. The most intense bands seen were the pro form of MMP-9 and the pro and active forms of MMP-2. This is supported by the presence of bands at the same molecular weight in D3X cell supernatants, which are known to produce both MMP-2 and MMP-9 (Laird *et al.*, 1999). A less intense band was also seen in epithelial cell culture supernatants at 84 kDa and although this was not seen in the D3X cell supernatants, its molecular weight suggests that it is active MMP-9. Both zymography and ELISA analysis showed that MMP-2 was produced by both stromal and epithelial cell cultures under basal conditions. The production of MMP-2 by epithelial cells is controversial. *In vivo* studies have suggested its presence only in stromal cells (Freitas *et al.*, 1999; Rodgers *et al.*, 1994), while other *in vivo* and *in vitro* studies have shown its presence and production by both stromal and epithelial cells (Martelli *et al.*, 1993; Zhang *et al.*, 2000).

The secretion of MMP-2 by epithelial cells seen in this study could possibly be due to contamination with stromal cells or leukocytes. However, epithelial cells prepared in this way are essentially pure and free of stromal and leukocyte contamination (Laird *et al.*, 1993). The finding that epithelial cells are the major source of MMP-9 agrees with that reported in a number of other studies (Freitas *et al.*, 1999; Skinner *et*

al., 1999; Jeziorska *et al.*, 1996). In particular it has been shown that basal production of MMP-9 by cultured stromal cells is very low (Huang *et al.*, 1998).

The results also suggest that MMP-2 production by epithelial cells, but not stromal cells, is significantly higher in cells obtained from secretory endometrium compared to cells from proliferative endometrium. These *in vitro* findings agree with previous *in vivo* studies which have shown a higher concentration of MMP-2 in endometrial flushings taken during the secretory phase compared to those obtained during the proliferative phase (Laird *et al.*, 1999) and more MMP-2 mRNA in secretory endometrium compared to proliferative endometrium (Irwin *et al.*, 1996). However, other studies have suggested that endometrial stromal cell MMP-2 production does not change significantly throughout the cycle (Hulboy *et al.*, 1997). Also in contrast to others, who have shown an increase in MMP-9 secretion (Jeziorska *et al.*, 1996) and immunostaining (Skinner *et al.*, 1999) during the peri-implantation period, our results showed that MMP-9 secretion by epithelial cells *in vitro* was similar in cells prepared from proliferative and secretory phase endometrium. This may be due to the fact that in this study only 2 biopsies were obtained during the peri-implantation period and thus differences at this time in the cycle were not observed.

The presence of MMP-7 in supernatants of endometrial epithelial cells, but not stromal cells, found in this study agrees with other *in vivo* and *in vitro* studies, where MMP-7 mRNA and protein were found predominantly in the epithelial compartment (Rogers *et al.*, 1993; Rogers *et al.*, 1994; Osteen *et al.*, 1994). The results from this study suggest that more MMP-7 is produced from cells prepared from proliferative endometrium compared to cells prepared from secretory endometrium. Other studies have suggested that endometrial MMP production is suppressed during the secretory phase of the menstrual cycle due to the action of steroid hormones which may influence cytokine secretion (Marbaix *et al.*, 1992; Marbaix *et al.*, 1995). The results from this study suggest that MMP-7 production may also be decreased in the secretory phase of the cycle.

5.4.2 Effects of cytokines on endometrial MMP production

Both zymography and ELISA results show that of the four cytokines studied, TNF α has the most dramatic effect on MMP production. The stimulatory effect of TNF α on MMP-9 production by stromal cells agrees with previous reports (Rawdanowicz *et al.*, 1994; Singer *et al.*, 1999) and is similar to the effect of TNF α on cytotrophoblastic cells (Meisser *et al.*, 1999a). In this study IL-1 α also increased MMP-9. A similar effect of IL-1 α on MMP-9 production by endometrial stromal cells (Rawdanowicz *et al.*, 1994; Huang *et al.*, 1998) and cytotrophoblast cells has been reported (Meisser *et al.*, 1999a). The importance of IL-1 α in MMP production has also been suggested by other studies, where the stimulatory effect of IL-1 α on MMP-1 expression by endometrial fibroblasts was blocked by anti-IL-1 antibodies (Singer *et al.*, 1997). In this study, the results show that TNF α stimulates MMP-9 production by epithelial cells and that IL-1 α stimulates MMP-2 production by epithelial cells, suggesting that cytokines may also be important in the control of MMP production in epithelial cells.

In contrast to IL-1 α and TNF α , IL-6 and LIF had little effect on MMP production by endometrial cells. This is surprising as both LIF and IL-6 have been shown to affect gelatinolytic production by trophoblast cells (Bischof *et al.*, 1995a; Meisser *et al.*, 1999b) and increased MMP-11 expression was seen in response to IL-6 in endometrial stromal cells (Singer *et al.*, 1999). LIFR, IL-6R and gp130 are expressed by both stromal and epithelial endometrial cells (Tabibzadeh *et al.*, 1995a; Senturk and Arici, 1998), suggesting that the differences in response to these cytokines by cytotrophoblast and endometrial cells are not due to differences in the expression of their receptors, but may be due to differences in intracellular signalling pathways.

In this study the cells were incubated in media containing fetal calf serum which was not further supplemented with steroids, both prior to and during incubation with cytokines. Previous studies have shown the importance of combined oestradiol and progesterone in the control of MMP production. Steroid withdrawal stimulates MMP production both *in vitro* and *in vivo* (Schatz *et al.*, 1999; Salamonsen *et al.*, 1997; Keller *et al.*, 2000) and progesterone can prevent IL-1 stimulation of MMP

production by endometrial stromal cells (Singer *et al.*, 1997; Keller *et al.*, 2000). In addition, the fact that differences in MMP-2 and MMP-7 secretion were seen in this study in cells prepared from secretory and proliferative endometrium, also suggest an influence of steroids. It may therefore be possible that the increases in MMP production seen on addition of cytokines may be due to the effects of steroid withdrawal. The experiments should therefore be repeated in the presence of oestradiol and progesterone. The fact that stimulatory effects on MMP production were specific for IL-1 α and TNF α and not seen for LIF and IL6, and that differences were seen in the effects of TNF α and IL-1 α on MMP-2 and MMP-9 production, suggest that the effect is not just a general effect of steroid withdrawal. It is also possible that steroid exposure *in vivo* prior to surgery and cell preparation may also affect the response of cells to cytokines. However in this series of experiments each cytokine was added to cells prepared from biopsies obtained both during the secretory and proliferative phases of the cycle with no differences in response seen.

These results therefore show that both stromal and epithelial cells are capable of producing MMP-2 and MMP-9, although basal production of MMP-9 by stromal cells is very low. In contrast, MMP-7 is only produced by epithelial cells. The results also suggest that TNF α and IL-1 α , but not IL-6 and LIF, play an important role in the regulation of MMP-2 and MMP-9 production by both endometrial stromal and epithelial cells. This pattern of MMP stimulation is different to that seen for cytotrophoblast cells. As the interaction between cytotrophoblast and endometrial cells is important in the control of embryo implantation, an understanding of the different control mechanisms may result in an increased understanding of this process and thus to treatments for conditions, where implantation is defective such as infertility and recurrent miscarriage.

Chapter 6

Interleukin-11 in the human endometrium

6.1 Introduction

Recent studies in mice have suggested the importance of IL-11 and IL-11R α in embryo implantation (Robb *et al.*, 1998; Bilinski *et al.*, 1998). Both of these studies have shown that IL-11R α expression is essential for normal decidual development. Female mice with either an inactive or null mutation for the IL-11R α chain are fertile and their blastocysts implant and elicit an initial decidual response. However, only small decidua form due to reduced cell proliferation, resulting in progressive degeneration of the decidua. Consequently, embryo-derived trophoblast cells generate a network of trophoblast cells but fail to form a chorio-allantoic placenta, showing that the decidua is essential for normal fetoplacental development (Bilinski *et al.*, 1998). The presence of IL-11 and IL-11R α has been shown in mice endometrium, with increased expression around the time of decidualisation (Davidson *et al.*, 1997).

Previous studies have shown that IL-11 production by other cell types is controlled by other cytokines. IL-1 α and TNF α synergistically stimulate rheumatoid synovial fibroblasts to produce IL-11 at the mRNA and protein levels (Mino *et al.*, 1998) and IL-1 α has also been shown to stimulate IL-11 production from lung epithelial cells (Elias *et al.*, 1994a). Transforming growth factor beta (TGF β) has also been shown to stimulate IL-11 production *in vitro* from a number of other different cell types including lung epithelial cells (Elias *et al.*, 1994a), fibroblasts (Elias *et al.*, 1994b), osteoblasts (Elias *et al.*, 1995; Morinaga *et al.*, 1997), chondrocytes (Maier *et al.*, 1993) and breast cancer cells (Lacroix *et al.*, 1998).

These previous results have suggested the importance of endometrial IL-11 in the murine decidual response; and it is possible that IL-11 may also play an important role in implantation and decidualisation in humans. The expression of IL-11 in the human endometrium throughout the menstrual cycle was therefore studied. The

effects of IL-1 α , TNF α and TGF β on IL-11 production by cultured human epithelial and stromal endometrial cells in culture was also investigated.

6.2 Materials and Methods

Immunocytochemistry was used to determine the expression of IL-11 in sections taken from endometrial biopsies obtained throughout the menstrual cycle. Staining was then evaluated by semi-quantitative analysis. Endometrial epithelial and stromal cultures were grown and the effects of IL-1 α , TNF α and TGF β on IL-11 production measured using ELISA.

6.2.1 Endometrial sections

Serial cryostat sections of 15 μ m were taken from endometrial biopsies obtained from normal fertile women, as described in the materials and methods section. For this study one biopsy was obtained from the early proliferative phase (days 0-5), two during the mid-proliferative phase (days 5-9), four during the late proliferative phase (days 10-14), five during the early secretory phase (days 15-19), five during the mid secretory phase (days 20-24) and two from the late secretory phase (days 25+). The sections were fixed as described in chapter 2. Dating of the biopsies was calculated from the time of the last menstrual period.

6.2.2 Immunocytochemistry

All sections were stained according to the protocol previously described in chapter 2. The antibodies used in this experiment were as follows: two primary antibodies for IL-11 were used, a goat anti-human IL-11 polyclonal antibody (Santa Cruz Technology Inc, U.K.) and a mouse anti-human IL-11 human monoclonal antibody (R+D Systems, Abingdon, U.K.), both at a concentration of 10 μ g/ml and incubated for 24h at +4 $^{\circ}$ C. The secondary antibodies were a rabbit anti-goat immunoglobulin at a dilution of 1:100 (18 μ g/ml) in 10% normal rabbit serum for the polyclonal primary antibody, and a rabbit anti-mouse immunoglobulin at a dilution of 1:25 (140 μ g/ml) in 10% normal rabbit serum for the monoclonal antibody. Both were obtained from DAKO, Ely, U.K. and incubated for 30min at room temperature. Binding was visualised using PAP antibodies and DAB colour reagent as previously described.

6.2.3 Quantification of staining

All staining was assessed using the semi-quantification method.

6.2.4 Cell culture

All cell cultures were prepared as previously described. For this experiment biopsies were used from nine different women and both epithelial and stromal cell cultures were prepared from each biopsy. IL-1 α (0.1-10ng/ml) was added to replicate wells of epithelial and stromal cells prepared from three biopsies, which had been obtained on days 9, 16 and 17 of the menstrual cycle. TNF α (0.1-10ng/ml) was added to cells prepared from three biopsies obtained on days 16, 22 and 24 of the menstrual cycle and TGF β (0.1-10ng/ml) was added to cells prepared from biopsies obtained on days 7, 17 and 21 of the menstrual cycle. Again the timing of the biopsies was calculated from the last menstrual period. Control wells containing no additions were included in each experiment. After incubation for 48h the supernatants were removed and stored at -20°C until analysed for IL-11 by ELISA (see chapter 2 for details).

6.3 Results

6.3.1 Human endometrial expression of IL-11 *in vivo*

Immunocytochemical staining showed that IL-11 was present in the human endometrium throughout the menstrual cycle in both epithelial and stromal cells. Results obtained were similar when using both the goat anti-human IL-11 and mouse anti-human IL-11 antibodies. Figures 6.1 and 6.2 show staining obtained in sections from four biopsies taken during the proliferative, early secretory, mid secretory and late secretory phases of the cycle, with either the goat anti-human IL-11 (figure 6.1) or the mouse anti-human IL-11 (figure 6.2) antibodies. No true negative control was used for these experiments, however a section was run parallel to each section, whereby the primary antibody was omitted from the protocol, and instead the sections incubated with just normal rabbit serum.

6.3.2 Expression throughout the menstrual cycle

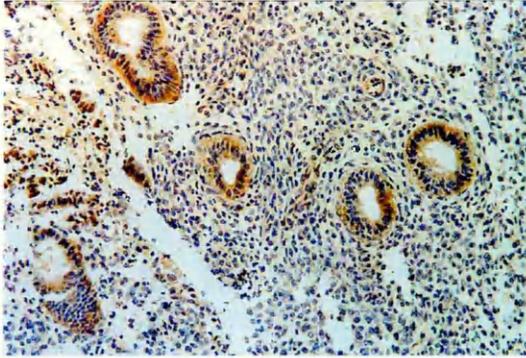
Glandular epithelial IL-11 staining was strong throughout the cycle, with a small increase in expression during the late proliferative – early secretory phase. Although luminal epithelium was not observed in sections from all biopsies, when it was observed, it also stained intensely and in some cases showed slightly stronger staining than that seen in the glandular epithelium of the same section (see Figure 6.2d). In both luminal and glandular epithelial cells staining was particularly intense at the luminal side of the cell, particularly during the secretory phase of the cycle (see Figure 6.2a), suggesting that the IL-11 produced is secreted. During the proliferative and early to mid secretory phases of the cycle, stromal IL-11 expression was weak and it remained relatively constant throughout the cycle, with only a small increase during the late proliferative phase. However stromal cell staining increased during the late secretory phase of the cycle (Figure 6.1d, 6.2c and d) and its expression at this time was similar to that seen in the epithelium.

6.3.3 Semi-quantitative analysis of IL-11 staining

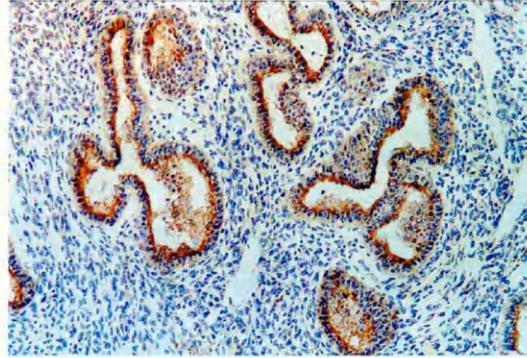
The semi-quantitative analysis of staining intensity in sections obtained from 19 biopsies is summarised in Table 6.1. These results were obtained using the mouse

Figure 6.1: Immunocytochemical staining for IL-11 in sections obtained from endometrial biopsies during the (a) proliferative, (b) early secretory, (c) mid secretory and (d) late secretory phases of the menstrual cycle. A negative control is also shown (e). Results shown were obtained using the goat anti-human IL-11 antibody.

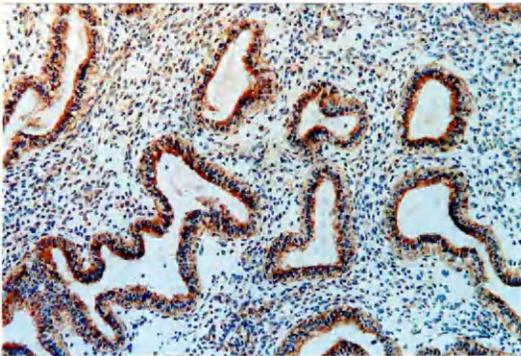
a)



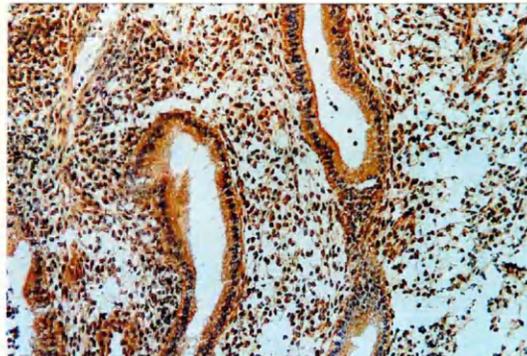
b)



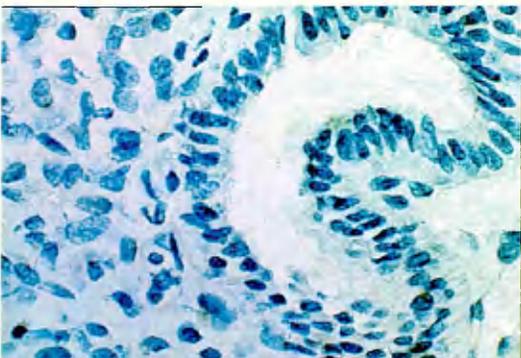
c)



d)



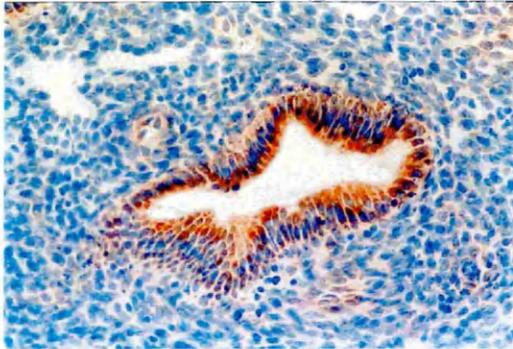
e)



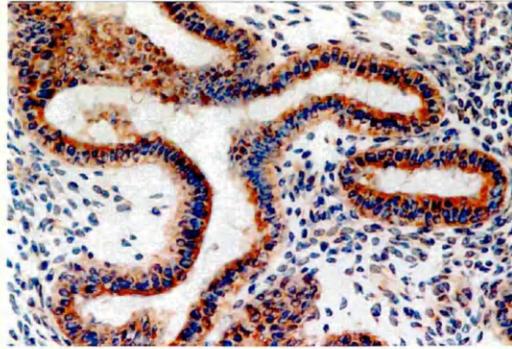
Magnification = X200, on all figures except (e) where the magnification = X400

Figure 6.2: Immunocytochemical staining for IL-11 in sections obtained from endometrial biopsies during the proliferative (a), early secretory (b), mid-secretory (c) and late secretory (d) phases of the menstrual cycle. A negative control is also shown (e). Results shown were obtained using the mouse anti-human IL-11 antibody.

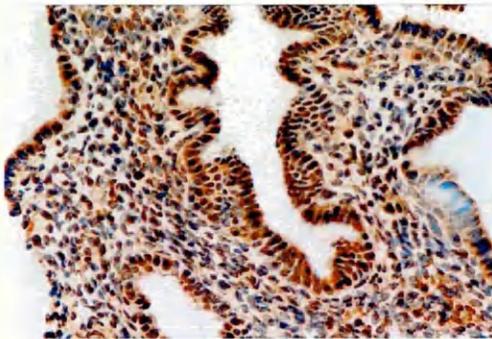
a)



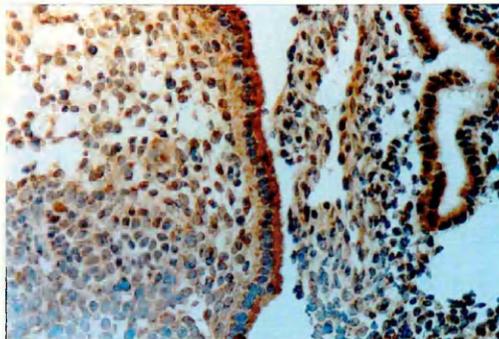
b)



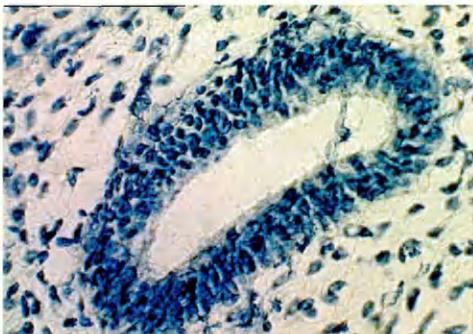
c)



d)



e)



Magnification = X400

Table 6.1: Semi-quantitative analysis of staining intensity for IL-11 in epithelial and stromal cells of 19 biopsies from normal fertile women obtained at different times throughout the menstrual cycle

Biopsy	Day of cycle	Epithelial staining	Stromal staining
1	3	++/+++	+
2	5	+++	+
3	7	+++	+
4	10	+++	+
5	11	+++/>++++	+
6	12	+++/>++++	+
7	14	++++	+
8	15	++++	+
9	15	++++	-/+
10	16	+++/>++++	-/+
11	17	+++	+
12	19	+++	+
13	20	+++	++
14	20	+++	++
15	21	+++	++
16	22	+++	++
17	23	+++	++
18	26	+++/>++++	++/>+++
19	33	+++	++

anti-human IL-11 antibody, however a similar pattern of staining was seen when using the goat anti-human IL-11 antibody.

6.3.4 Basal production of IL-11 from endometrial cell cultures

Basal production of IL-11 was measured in supernatants from cultured epithelial and stromal cells. The results agreed with the immunocytochemical staining and showed that IL-11 production was significantly lower in supernatants from cultured stromal cells when compared to that from epithelial cells (Figure 6.3). No differences were seen in IL-11 production by cells prepared from proliferative and secretory endometrium. However, only two of the nine biopsies used for cell culture in these experiments were obtained from proliferative phase endometrium and therefore absolute comparisons are difficult. The results shown here are a culmination of the controls used in the stimulatory experiments. Also the same biopsies were used for each of the stromal and epithelial cell cultures.

6.3.5 Effects of cytokines on IL-11 production from endometrial cell cultures

TNF α , IL-1 α and TGF β all caused a concentration-dependent increase in IL-11 production by both stromal and epithelial cells (Figure 6.4).

Significant increases in IL-11 production by epithelial cells were seen at concentrations of 1 (p<0.05) and 10ng/ml (p<0.001) TNF α (figure 6.4a). While all concentrations of TNF α used caused a highly significant increase (p<0.001) in IL-11 production by stromal cells (figure 6.4b). Epithelial cell IL-11 production was significantly increased following incubation with 1ng/ml (p<0.01) and 10ng/ml (p<0.001) IL-1 α (Figure 6.4c), and IL-11 in supernatants from stromal cells was also significantly higher (p<0.001) when cells were incubated with all concentrations of IL-1 α (figure 6.4d). Like IL-1 α and TNF α , TGF β caused a highly significant increase (p<0.001) in stromal (figure 6.4f) and also in epithelial (figure 6.4e) cell IL-11 production, at all concentrations of cytokine used.

All three cytokines caused a greater stimulation of IL-11 by stromal cells than epithelial cells. The stimulated:basal ratio at 10ng/ml of TNF α was 10:1 and 2:1 for stromal and epithelial cells respectively. Similarly the stimulated:basal ratio at 10ng/ml of cytokine was 4:1 and 2:1 for stromal and epithelial cells in the presence of both IL-1 α and TGF β .

Figure 6.3 : Production of IL-11 in supernatants from unsupplemented epithelial and stromal cell cultures. Values are mean \pm SEM (n=9 epithelial, n=9 stromal). *** = significantly different to epithelial production at $p < 0.001$.

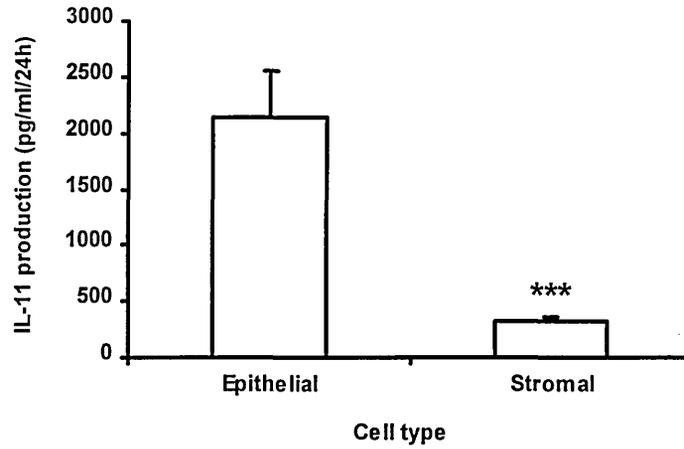
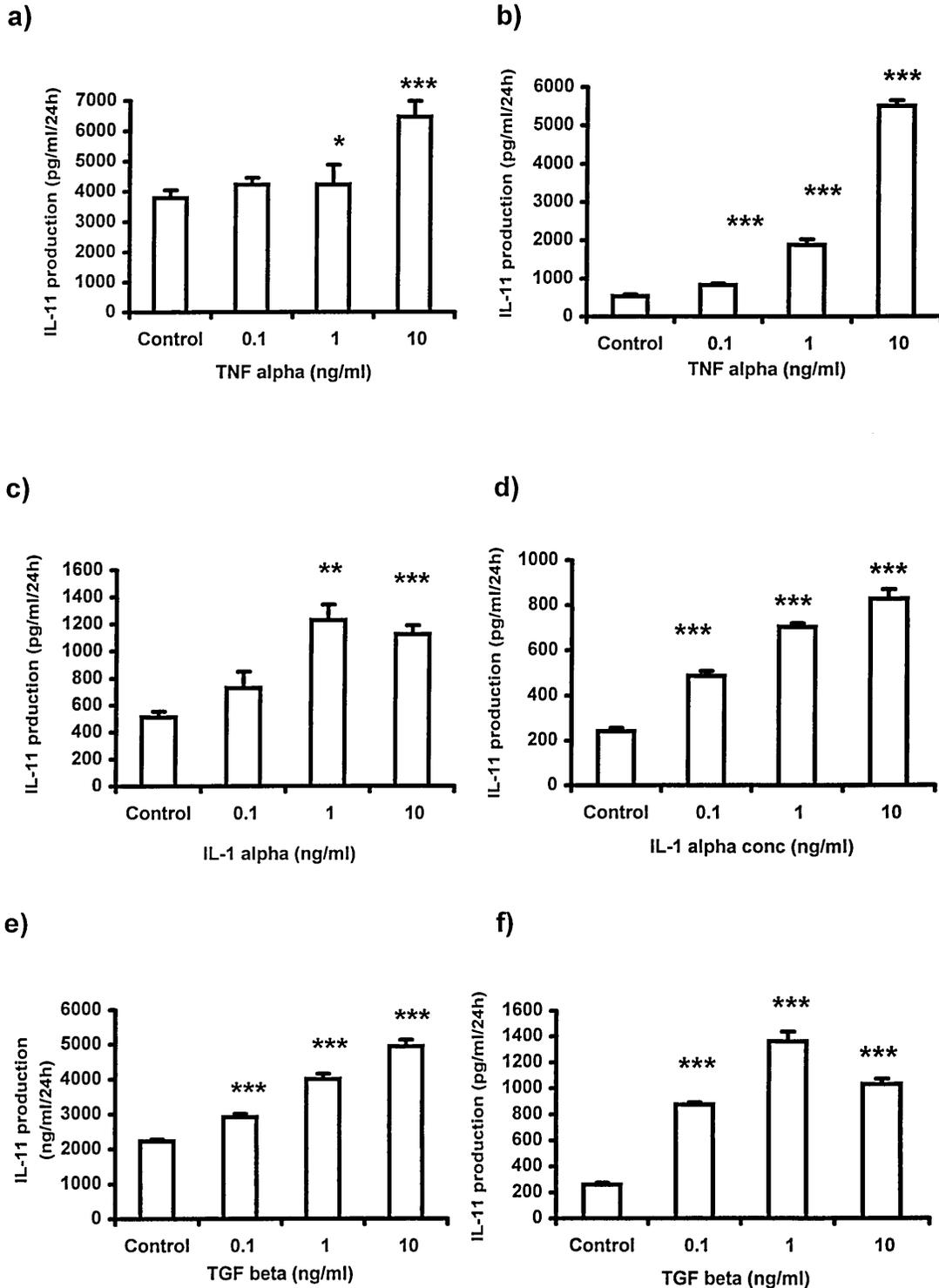


Figure 6.4: IL-11 production by (a, c and e) epithelial and (b,d and f) stromal cells following stimulation with (a and b) TNF α , (c and d) IL-1 α and (e and f) TGF β . Values are mean + SEM (n=4). Results shown are from cells from a single biopsy and are typical of that seen in 3 different biopsies for each cytokine. * = significantly different from controls at p<0.05, ** = significantly different from controls at p<0.01 and *** = significantly different from controls at p<0.001



6.4 Discussion

6.4.1 Human endometrial IL-11 expression *in vivo*

This is one of the first studies that has investigated the expression of IL-11 protein in the human endometrium, although a recent report (Dimitriadis *et al.*, 2000) has shown that IL-11 mRNA is expressed in the human endometrium. Our results clearly show that IL-11 is present in human endometrium throughout the menstrual cycle and is expressed by both stromal and epithelial cells, agreeing with the results from Dimitriadis *et al.* (2000). The semi-quantitative analysis of intensity of staining suggests that until the late stages in the cycle, expression is greater in epithelial cells than stromal cells, but during the late secretory phase increased levels of stromal IL-11 are produced. No attempt was made to establish whether specific populations of stromal cells, such as the leukocytes, were staining particularly for IL-11. However, particularly in the late stages of the cycle, staining in the stromal compartment appeared fairly uniform, whereas leukocytes are present in discrete populations. There is a slight possibility that the staining may be due to IL-11 binding to its receptor. However the positioning in the cytoplasm of the cell suggests that it is produced by the cells themselves. In addition, the fact that there is more intense staining on the luminal side of the cytoplasm suggests that it is secreted.

Previous studies in mice have suggested that IL-11 is only expressed by decidualised cells in the pregnant endometrium and not during the non-pregnant cycle (Bilinski *et al.*, 1998; Robb *et al.*, 1998; Davidson *et al.*, 1997). Our results, and those of Dimitriadis *et al.* (2000) suggest that in human endometrium it is also expressed in the non-pregnant cycle.

6.4.2 Human endometrial IL-11 production *in vitro*

In this study we also used our cell culture system to investigate the effects of other cytokines on endometrial IL-11 production. Immunocytochemical analysis has previously suggested that both the epithelial and stromal cell cultures are free from leukocyte contamination (Laird *et al.*, 1993) and therefore the IL-11 in the cell culture supernatants is likely to originate from either stromal or epithelial cells. Basal

production of IL-11 by epithelial cells was greater than basal stromal cell IL-11 production and although care should be made in extrapolating between *in vivo* and *in vitro*, taken together these results suggest that epithelial cells produce more IL-11 than stromal cells. The number of cells present at the end of the incubation was not calculated, so it is possible that the differences seen between epithelial and stromal IL-11 production could be due to a smaller number of cells being present in the stromal cell cultures. However, both the epithelial and stromal cell cultures were grown to confluency before stimulation and therefore it is unlikely that the 5 fold difference is due entirely to a difference in cell numbers. None of the nine biopsies used in the cell culture part of this study were obtained during the late secretory phase of the cycle, therefore the suggestion of an increase in production by stromal cells at this time, suggested by the immunocytochemistry results can not be confirmed by the cell culture results.

6.4.3 Effects of cytokines on endometrial IL-11 production

Our results suggest that IL-11 production by both stromal and epithelial endometrial cells is increased by IL-1 α , TNF α and TGF β , as has been shown for many other cell types. IL-1 α and TNF α are known to be present in the human endometrium (Stewart *et al.*, 1992; Simon *et al.*, 1995b) and are postulated to have a role in implantation, possibly by affecting the production of other endometrial cytokines such as IL-6 and LIF (Laird *et al.*, 1994). The results from this study suggest that IL-11 is also stimulated by IL-1 α and TNF α . TGF β is also expressed by the endometrium in a menstrual cycle dependent manner (Arici *et al.*, 1996; Casslen *et al.*, 1998) and has been shown to affect proteinase and proteinase inhibitor production by endometrial stromal cells (Godkin *et al.*, 1998).

IL-11 stromal cell production appeared to be more sensitive to stimulation by cytokines, as evidenced by higher stimulated:basal values. This greater effect of cytokines on stromal cell IL-11 production compared to epithelial cell production seen in this study is similar to that reported by others on the effects of IL-1 α and TNF α on IL-6 and LIF production (Arici *et al.*, 1995).

The work in transgenic mice provides strong evidence for a possible role for IL-11 in the decidual reaction, which is an essential response of the maternal endometrium for implantation and feto-placental development (Bilinski *et al.*, 1998; Robb *et al.*, 1998). Decidualisation results in a change in morphology and functional activity of the stromal cells characterised by an increased secretion of prolactin (Schatz *et al.*, 1997). In addition stromal cell extracellular matrix (ECM) production is altered resulting in significant remodelling of the ECM (Church *et al.*, 1996).

Decidualisation is initiated by the effects of progesterone on the oestradiol-primed endometrium and in humans is also seen in late secretory endometrium, even in the absence of an implanting embryo (Lockwood *et al.*, 1997). The increased expression of IL-11 in the endometrial stromal compartment seen in this study may suggest that decidual cells also produce more IL-11 than non-decidual cells. An alternative explanation for the increased stromal staining at this time may be due to increased binding to the IL-11 receptor. Whichever explanation is correct the results suggest that IL-11 may also play a role in decidualisation in the human endometrium.

Chapter 7

Expression of Interleukin 11 Receptor in human endometrium and effects of IL-11 on endometrial cell function

7.1 Introduction

The work described in the previous chapter shows that IL-11 is produced by the human endometrium. However, the effects of IL-11 on endometrial function have not previously been reported in humans. In order for IL-11 to have an effect on endometrial cells *in vivo* the endometrium must express IL-11R α and gp130. Previous reports (Tabibzadeh *et al.*, 1995a; Cullinan *et al.*, 1996) have shown that gp130 is expressed by the human endometrium. These studies showed that expression of gp130 mRNA and protein is greater in the glandular and luminal epithelium than the stromal cell compartment and suggested that levels may increase during the secretory phase of the cycle. We therefore wanted to determine if IL-11R α protein is expressed in the human endometrium, and if the expression varies throughout the menstrual cycle. Any expression of IL-11R α would suggest that IL-11 is capable of functioning within the human endometrium by signalling through the IL-11R α /gp130 complex.

Studies have shown that IL-11 is essential for normal decidualisation in mice (Robb *et al.*, 1998; Bilinski *et al.*, 1998), and IL-11 may also be important in human implantation and decidualisation. These processes involve remodelling of the endometrium and may be controlled by cytokines. The postulated role of MMPs in endometrial remodelling associated with embryo implantation has previously been discussed (Bischof *et al.*, 1995b) and our results have shown that endometrial MMP production *in vitro* can be influenced by the presence of other cytokines, particularly IL-1 α and TNF α . We therefore wanted to determine if IL-11 has any effect on endometrial MMP production.

Several studies have shown that IL-11 can affect cytokine production by other cells. Work on psoriasis, a chronic inflammatory skin disease has also shown that

treatment with recombinant human IL-11 (rhIL-11) results in a decreased expression of disease-related genes including the cytokines IFN γ , IL-8, IL-12, TNF α and IL-1 β and also an increased expression of endogenous IL-11. This resulted in amelioration of the disease as shown by reduced keratinocyte proliferation and chronic inflammation (Trepicchio *et al.*, 1999). This indicates that pro-inflammatory cytokines can be selectively suppressed, in this disease, by rhIL-11 and suggests that IL-11 may play a role in the regulation of these cytokines in other tissues. Therefore, we wanted to determine if IL-11 would effect the production of pro-inflammatory cytokines from cultured endometrial cells.

The aims of this study were therefore to determine the expression of IL-11R α in human endometrium throughout the menstrual cycle using immunocytochemistry and to study the effects of IL-11 on production of MMP-2, MMP-9, MMP-7, TNF α and IL-1 β by cultured human endometrial epithelial and stromal cells. We initially investigated whether cultured endometrial stromal and epithelial cells *in vitro* expressed the IL-11R and therefore had the potential to respond to IL-11.

7.2 Materials and Methods

Immunocytochemistry was used to determine the expression of IL-11R α in sections taken from endometrial biopsies obtained throughout the menstrual cycle.

Immunostaining was also used to show expression of IL-11R α in cultured stromal and epithelial cells. Staining in the endometrial sections was then evaluated by semi-quantitative analysis.

Endometrial epithelial and stromal cells were cultured and the effects of IL-11 on MMP-2, MMP-9, MMP-7, TNF α and IL-1 β production measured using ELISA.

7.2.1 Endometrial sections

Cryostat sections of 15 μ m were taken from nineteen endometrial biopsies obtained from normal fertile women. For this study, one biopsy was obtained from the early proliferative phase (days 0-5), one during the mid-proliferative phase (days 5-9), four during the late proliferative phase (days 10-14), four during the early secretory phase (days 15-19), seven during the mid secretory phase (days 20-24) and two from the late secretory phase (days 25+). The sections were fixed as previously described in chapter 2. Dating of the biopsies was calculated from the time of the last menstrual period.

7.2.2 Cell culture

Endometrial stromal and epithelial cells were grown in chamber slides, until confluent. The cells were then fixed and stained as described for endometrial sections.

7.2.3 Immunocytochemistry

All sections and cells were stained according to the protocol previously described. The primary antibody used in this experiment was a goat anti-human IL-11R α polyclonal antibody (Insight Biotechnologies, Wembley, U.K.) at a concentration of 10 μ g/ml and an incubation time of 24h at +4 $^{\circ}$ C. A parallel section was included, where the section was incubated with 10% normal rabbit serum instead of the

primary antibody, but no negative control (i.e. goat IgG) was included in these experiments. The secondary antibody was a rabbit anti-goat immunoglobulin at a dilution of 1:100 (18 µg/ml) in 10% normal rabbit serum (DAKO, Ely, U.K.) and an incubation time of 30min at room temperature. The binding was visualised using PAP antibodies and DAB substrate.

7.2.4 Quantification of staining

Staining in the endometrial sections was assessed using the semi-quantification method.

7.2.5 Cell culture

All cell cultures were prepared as described in chapter 2. For these experiments, endometrial biopsies were used from five different women and both epithelial and stromal cell cultures were prepared and grown from each biopsy. The timing of each biopsy was calculated from the time of the last menstrual period. Cells were prepared from biopsies obtained on days 7, 10, 12, 17 and 21 of the menstrual cycle and each biopsy was taken from a different woman. At confluency, the cells were either stimulated with IL-11 at concentrations of 0.1ng/ml, 1ng/ml and 10ng/ml, or incubated with no additions for a further 48h. The supernatants were then removed and stored at -20°C until analysed for MMP production by ELISA. Epithelial and stromal MMP-2 production was measured in cell cultures prepared from the biopsies obtained on days 7, 12 and 17 of the menstrual cycle. Epithelial and stromal MMP-9 production was measured in cell supernatants prepared from the three biopsies obtained on days 7, 10 and 17 of the menstrual cycle and epithelial MMP-7 and TNF α production was measured in supernatants of cell cultures from all five biopsies.

7.3 Results

7.3.1 Human endometrial expression of IL-11R α

Immunocytochemical staining showed that IL-11R α was present in the human endometrium obtained throughout the menstrual cycle in both epithelial and stromal cells. Figure 7.1 shows staining obtained from four biopsies taken during the proliferative, early secretory, mid secretory and late secretory phases of the cycle. A negative control has been included where the primary antibody was replaced with normal rabbit serum. Figure 7.2 shows the expression of IL-11R α in cultured epithelial (figure 7.2a) and stromal (figure 7.2b) cells.

7.3.2 Expression throughout the menstrual cycle

Expression of IL-11R α in the glandular epithelium was strong, with little variation between the different phases of the menstrual cycle. However, slightly more intense staining for IL-11R α was seen during the late proliferative and secretory phases compared to the early-mid proliferative phases of the menstrual cycle. Although luminal epithelium was not observed in sections from all biopsies, when it was observed, it also stained intensely and showed similar staining to that seen in the glandular epithelium of the same section (Figure 7.1d). Staining of the stromal compartment also remained relatively constant throughout the cycle, with only a small increase during the late proliferative and early secretory phases. However stromal cell staining was less intense than that seen in the epithelium at all times.

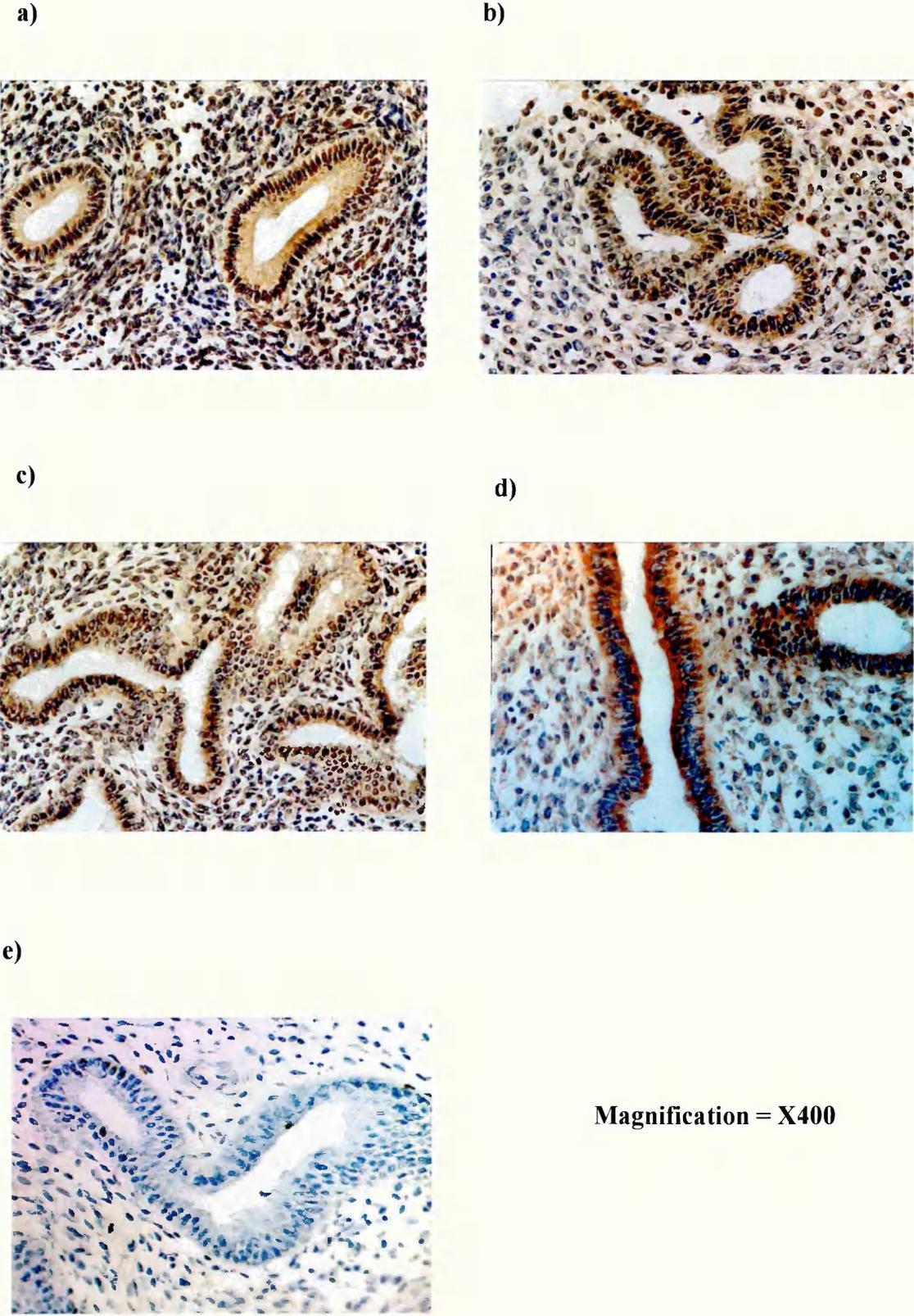
7.3.3 Semi-quantitative analysis of IL-11 staining

The semi-quantitative analysis of staining intensity in sections obtained from 19 biopsies is summarised in Table 7.1.

7.3.4 Effects of IL-11 on endometrial MMP production

The basal production of MMP-2, MMP-9 and MMP-7 in these experiments was similar to that described in chapter 5 and showed that epithelial MMP-2 was

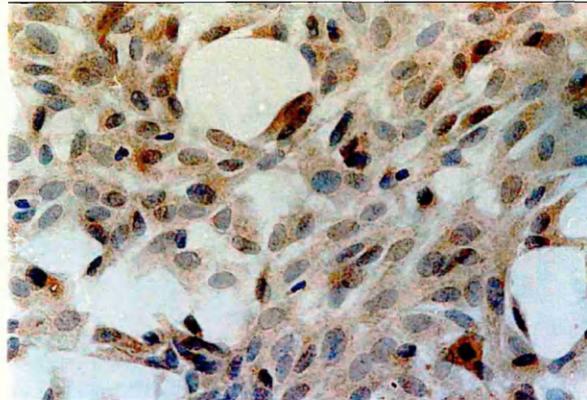
Figure 7.1: Immunocytochemical staining for IL-11R α in sections obtained from endometrial biopsies during the (a) proliferative, (b) early secretory, (c) mid secretory and (d) late secretory phases of the menstrual cycle. A negative control is also shown (e).



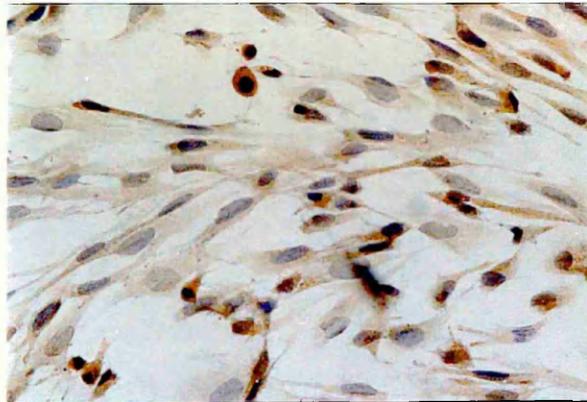
Magnification = X400

Figure 7.2: Immunocytochemical staining for IL-11R α in cultured (a) epithelial and (b) stromal cells. A negative control is also shown (c).

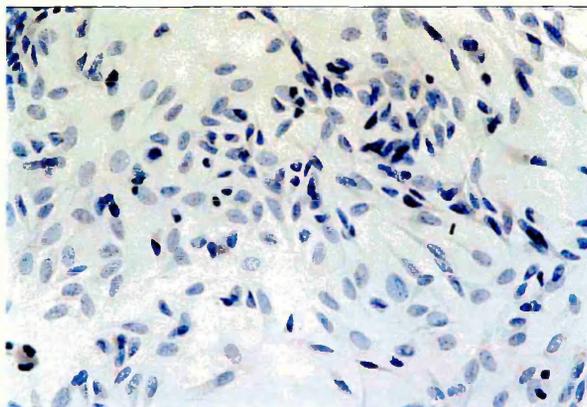
a)



b)



c)



Magnification = X400

Table 7.1: Semi-quantitative analysis of staining intensity for IL-11R α in epithelial and stromal cells of 19 biopsies obtained at different times throughout the menstrual cycle

Biopsy	Day of cycle	Epithelial staining	Stromal staining
1	3	++	+
2	5	++	+/++
3	10	++	+/++
4	11	++	+/++
5	12	+++	++
6	12	+++	++
7	15	+++	++
8	16	++++	++
9	17	++/+++	++
10	19	++/+++	++
11	20	++/+++	++
12	20	+++	++
13	20	+++	++
14	21	+++	++
15	22	+++	+/++
16	23	+++	+/++
17	23	++++	+/++
18	25	+++	+/++
19	27	+++	++

produced from both epithelial and stromal cells, but that stromal MMP-2 production was greater than epithelial production. MMP-9 production was predominantly seen in epithelial cells and MMP-7 production was only seen in epithelial cell cultures.

7.3.5 MMP-2 production

The effects of IL-11 on epithelial (figure 7.3a,c and e) and stromal (figure 7.3b, d and f) MMP-2 production by cells prepared from biopsies obtained on days 7 (figure 7.3a and b), 12 (figure 7.3c and d) and 17 (figure 7.3e and f) of the menstrual cycle are shown in figure 7.3. IL-11 significantly increased MMP-2 production in supernatants from epithelial cell cultures prepared from two of the three biopsies used (figure 7.3a and e). Stromal MMP-2 production was significantly increased in supernatants from cells prepared from only one of these experiments (figure 7.3f), with IL-11 having no significant effect on MMP-2 production by stromal cells prepared from the other two experiments (figure 7.3b and d).

7.3.6 MMP-9 production

Figure 7.4 shows the effects of IL-11 on MMP-9 production from epithelial cells *in vitro*. In this experiment cells were prepared from biopsies obtained on days 7 (figure 7.4a), 10 (figure 7.4b) and 17 (figure 7.4c) of the menstrual cycle. IL-11 caused a significant decrease ($p < 0.05$ at 10ng/ml) in epithelial MMP-9 production in cells prepared from two of the three biopsies (figure 7.4b and c). However, the decreases seen were only very small. In the stromal cell culture supernatants levels of MMP-9 were below the level of detection of the ELISA kit, both in the presence and absence of IL-11, showing that IL-11 did not cause a large increase in MMP-9 production by these cells (data not shown).

7.2.7 MMP-7 production

The effect of IL-11 on epithelial MMP-7 production in cells cultured from five different biopsies is shown in figure 7.5. The biopsies were obtained on days 7 (figure 7.5a), 10 (figure 7.5b), 12 (figure 7.5c), 17 (figure 7.5d) and 21 (figure 7.5e)

Figure 7.3: Effect of IL-11 on MMP-2 production from (a, c and e) epithelial and (b, d and f) stromal cells *in vitro*. Values are mean + SEM (n=4). *= significantly different from controls at p<0.05, **= significantly different from controls at p<0.01. Each graph represents the results obtained from cells prepared from a single biopsy

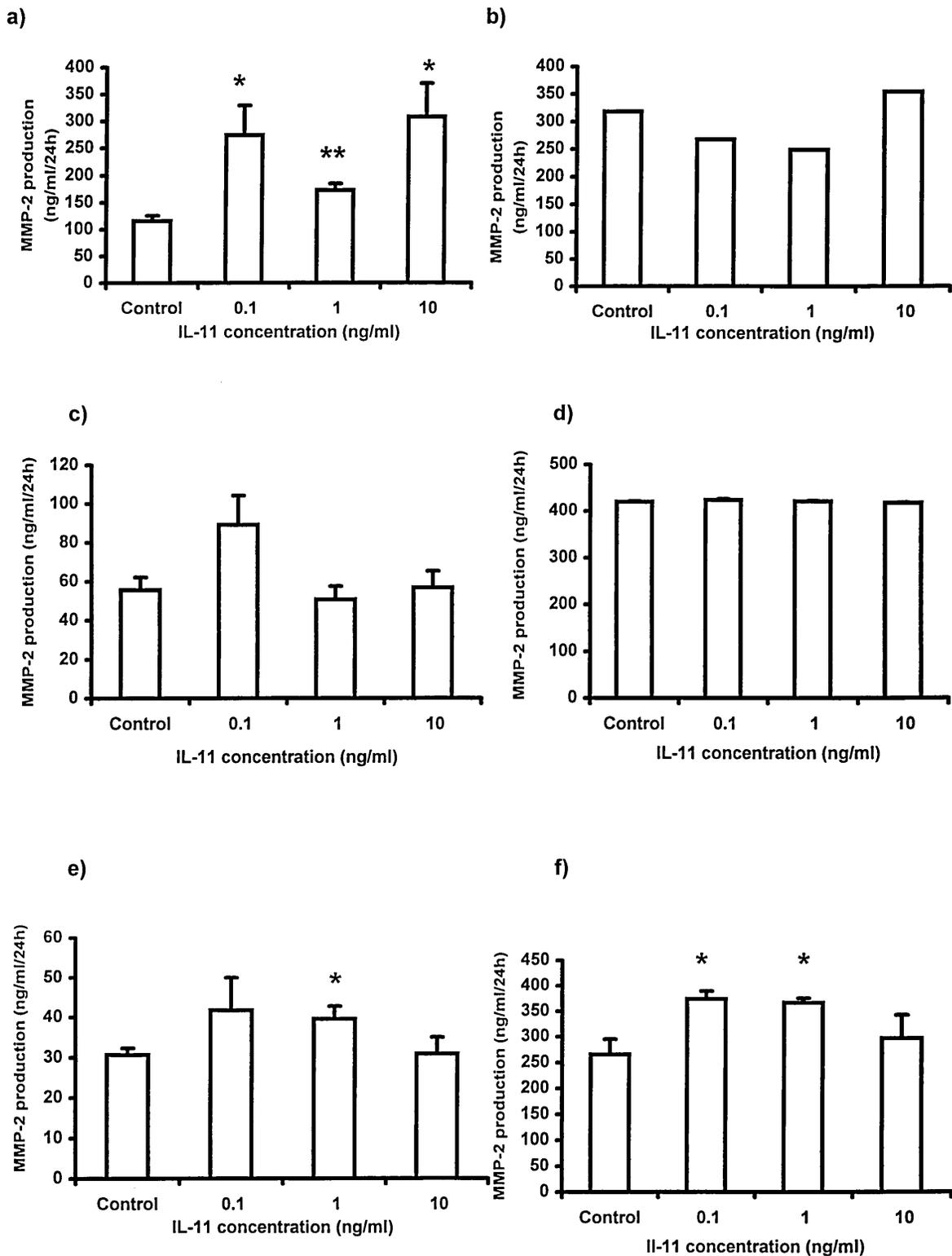
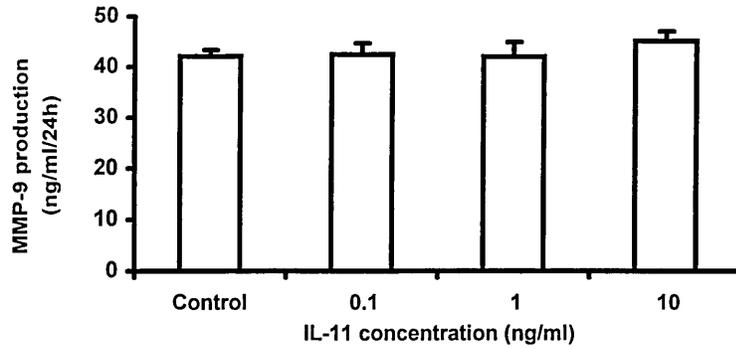
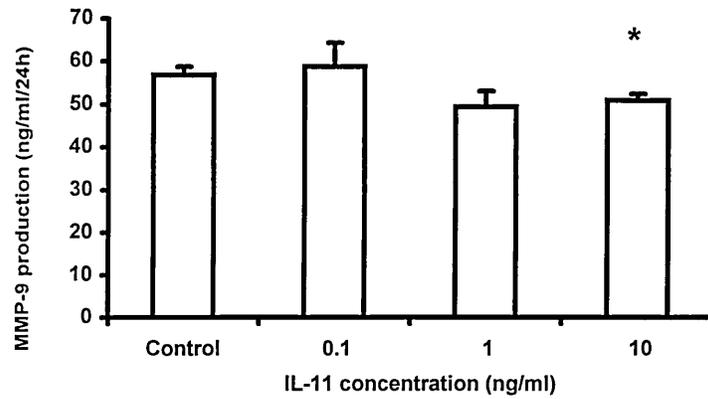


Figure 7.4: Effect of IL-11 on MMP-9 production from epithelial cells *in vitro*. Values are mean + SEM (n=4). *= significantly different from controls at p<0.05. Each graph represents results obtained from cells prepared from a single biopsy

a)



b)



c)

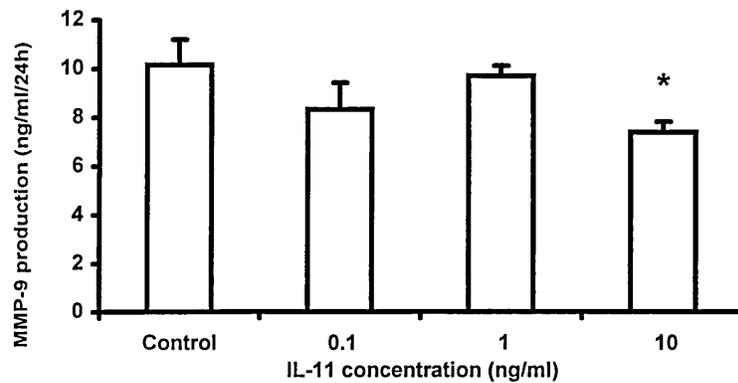
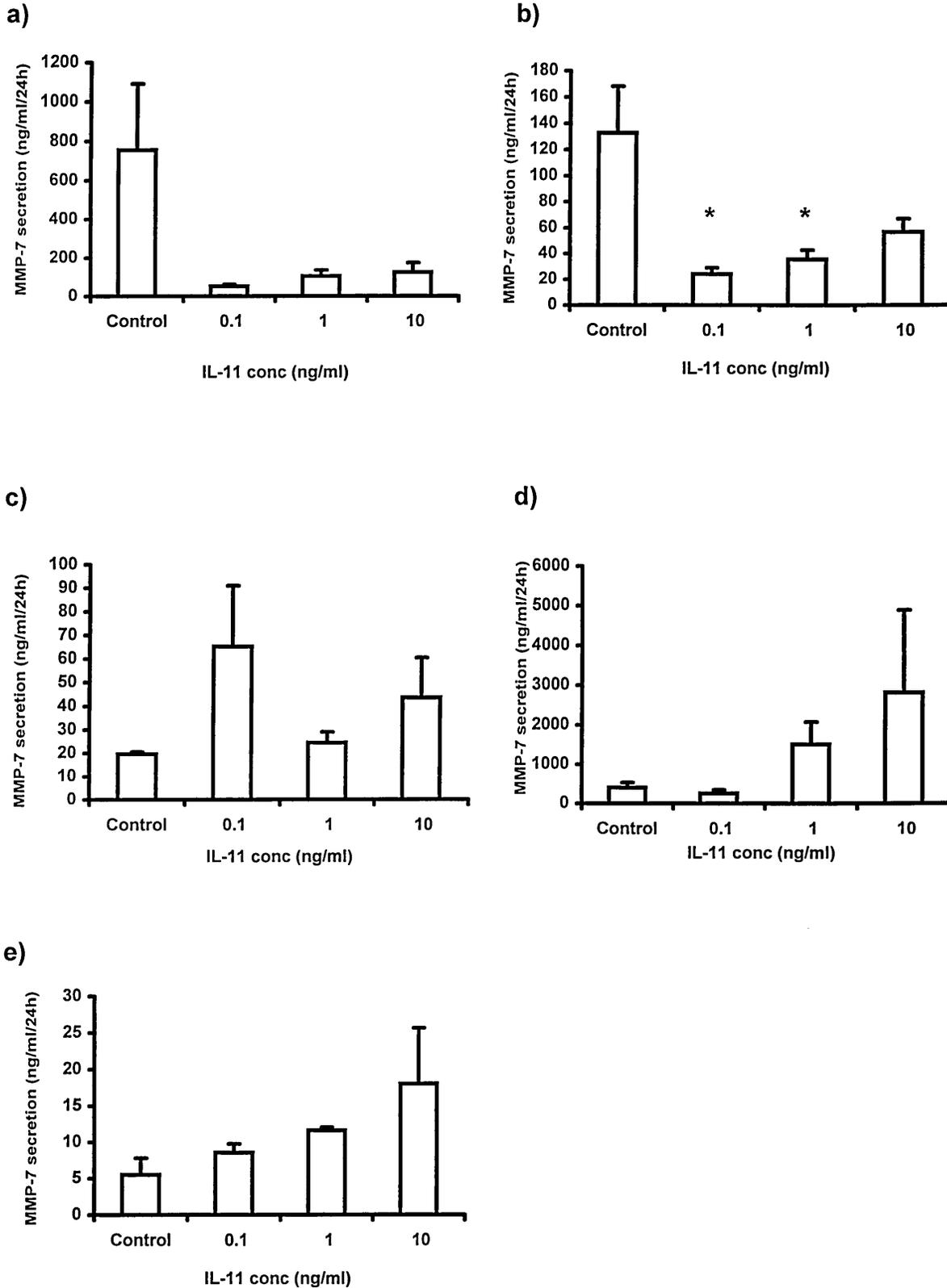


Figure 7.5: Effect of IL-11 on epithelial MMP-7 production in supernatants from cell cultures of biopsies obtained on (a) day 7, (b) day 10, (c) day 12, (d) day 17 and (e) day 21 of the menstrual cycle. Values are mean + SEM (n=4). *=significantly different from controls at $p < 0.05$. Each graph represents results from cells prepared from a single biopsy.



of the menstrual cycle. The results suggest that IL-11 had no significant effect on MMP-7 production by endometrial epithelial cells prepared from four of the five biopsies, and although a significant decrease ($p < 0.05$) in MMP-7 production was seen in cells prepared from the other biopsy (figure 7.5b), the effect was not dose-dependent.

7.3.8 Effects of IL-11 on endometrial cytokine production

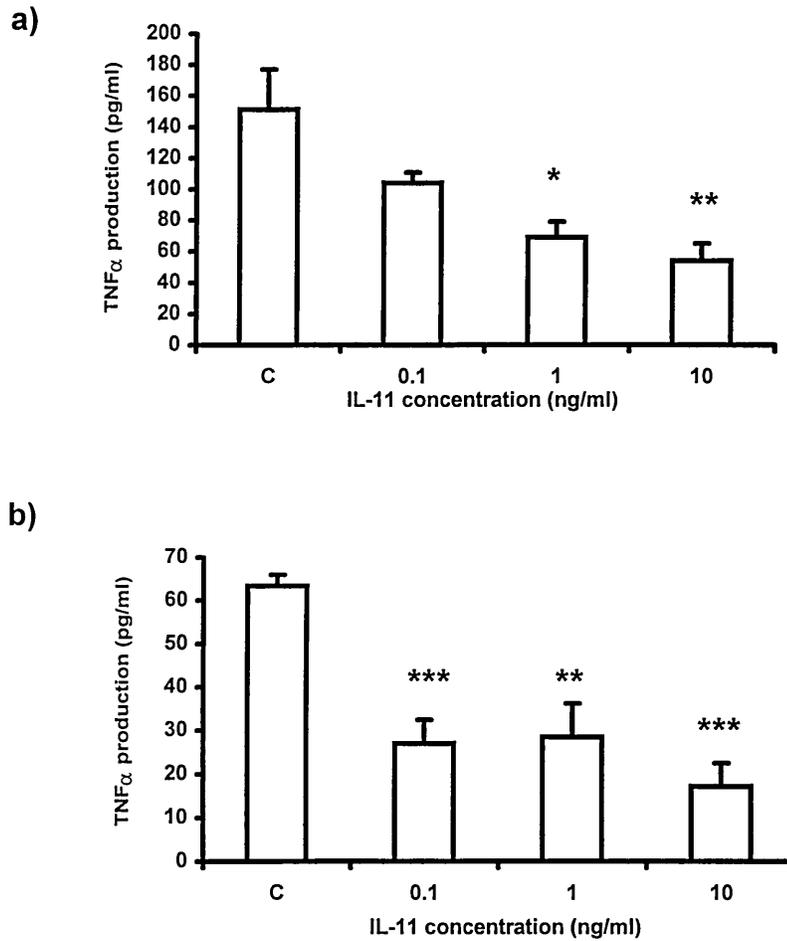
7.3.8.1 Tumour Necrosis Factor α

Production of TNF α from epithelial cells was seen to decrease following incubation with IL-11. Figure 7.6 (a) shows the results from cells prepared from a single biopsy, when levels of TNF α in cell culture supernatants were measured using ELISA. IL-11 caused a dose-dependent decrease in TNF α production with significant decreases seen at 1 and 10ng/ml. A similar decrease was seen in 3 of the other 4 experiments carried out. In the other experiment, IL-11 had no effect on TNF α production. Changes in levels of TNF α measured by bioassay showed strong correlation with that seen when TNF α was measured by ELISA. Figure 7.6 (b) shows the dose-dependent decrease in TNF α production by epithelial cells as is seen in figure 7.6 (a). Figures 7.6 (a) and (b) show the results from the same cell culture experiment measured using the two methods, and similar patterns of TNF α levels were seen in all experiments when analysed by bioassay and ELISA. Differences in the amount of TNF α measured using the two different methods is due to the bioassay only measuring unbound/active TNF α , whereas ELISA will also measure any TNF α that is bound to the soluble form of the receptor, therefore producing higher measurements of TNF α present. No differences were seen in responses of cells prepared from the proliferative and secretory endometrium.

7.3.8.2 Interleukin-1 β

The effect of IL-11 on IL-1 β production from cultured epithelial and stromal cells was also investigated. However, in both epithelial and stromal cell cultures the concentration of IL-1 β in the supernatants was below the level of detection of the

Figure 7.6: Effect of IL-11 on endometrial TNF α production by cultured epithelial cells. TNF α was measured by (a) ELISA and (b) bioassay. Values are mean + SEM (n=4). *= significantly different from controls at p<0.05, ** = significantly different from controls at p<0.01 and * = significantly different from controls at p<0.001. The results shown are from a single biopsy, but similar results were seen in the other four experiments.**



ELISA kit. The levels of IL-1 β remained below the level of detection following incubation of the cell cultures with IL-11.

7.4 Discussion

This study is one of the first to investigate the presence of IL-11R α protein in the human endometrium. The results clearly show that IL-11R α is expressed in the endometrium throughout the menstrual cycle. This agrees with a previous recent study which showed, using RT-PCR, that IL-11R α mRNA was expressed in the human endometrium throughout the menstrual cycle with minimal variation (Dimitriadis *et al.*, 2000). The results suggest that IL-11R α expression in epithelial and stromal cells remains relatively constant throughout the menstrual cycle, with epithelial expression being greater than stromal expression at all times in the menstrual cycle. The intensity of staining in the epithelial cells appeared to be slightly greater during the early secretory phase of the menstrual cycle. The presence of IL-11R α in the human endometrium taken together with results from other studies, which have shown the presence of gp130 in the human endometrium (Dimitriadis *et al.*, 2000; Tabibzadeh *et al.*, 1995a), suggests that IL-11 should be capable of functioning within the human endometrium. It has been shown that mice with either a null or inactivating mutation in the IL-11R α gene were infertile, as they had an impaired decidual response, which resulted in pregnancy loss (Robb *et al.*, 1998; Bilinski *et al.*, 1998). Therefore, IL-11R α may also play an important role in the decidualisation process in humans. The results also show that cultured epithelial and stromal cells express IL-11R α . Thus the cells in culture should be able to respond to IL-11.

MMPs are thought to be involved with the endometrial remodelling associated with implantation of the embryo in humans. It has previously been shown that MMP-2, MMP-9 and MMP-7 are produced by epithelial cells and that other cytokines, in particular, IL-1 α , TNF α can affect endometrial MMP production *in vitro* (data shown in chapter 5). We therefore wanted to determine if IL-11 had any effect on endometrial MMP production. The results show varying effects of IL-11 on endometrial MMP-2 production *in vitro*. In some cases significant increases were seen, while in others no effects were seen. Even when significant increases were seen the effects were not dose-dependent and therefore, there is little convincing evidence for IL-11 affecting stromal or epithelial MMP-2 production. The results suggest that

IL-11 may have a slight inhibitory effect on MMP-9 production at 10ng/ml. It is possible that higher doses of IL-11 may cause a further decrease. This was not tested as we have previously found that cytokines have their effects at the concentrations used in these experiments (0.1-10ng/ml). Levels of stromal MMP-9 production were below the level of detection of the ELISA kit. This is expected for the basal values of MMP-9, but because the levels were still below the level of detection following incubation with IL-11 it suggests that IL-11 has no significant stimulatory effect on stromal MMP-9 production.

The results show considerable variation in basal MMP-7 production from cells cultured from biopsies obtained at different times in the menstrual cycle. Overall, basal MMP-7 concentrations were highest in supernatants from cells cultured from biopsies obtained in the proliferative phase of the menstrual cycle, which agrees with that seen in previous studies (Rodgers *et al.*, 1993; Rodgers *et al.*, 1994) and results obtained in chapter 5. The results showing the effect of IL-11 on epithelial MMP-7 production were also very varied and the standard error bars were very large. The reason for the high level of variation for MMP-7 in this series of experiments compared to those described in chapter 5 is not known. As in chapter 5 these experiments were carried out without the addition of steroids to the cell culture media and in media containing fetal calf serum. This may have accounted for the lack of effect of IL-11 on the cells and for the variation seen. However, as discussed in chapter 5, consistent responses to IL-1 α and TNF α were seen when cells were cultured in the same conditions. In these experiments the levels of MMPs were not assessed by zymography. The results described in chapter 5 show that similar effects are seen by zymographic analysis and ELISA analysis, and as ELISA is more quantitative than zymography, only this type of analysis was chosen.

The results suggest that IL-11 significantly decreases TNF α production from most endometrial epithelial cells. No attempt was made to measure TNF α levels in stromal cell supernatants as they do not produce TNF α (Laird *et al.*, 1996). An attempt to measure any effect of IL-11 on epithelial and stromal IL-1 β production was made, but the amounts of IL-1 β measured were below the level of detection of the ELISA kit both before and after addition of IL-11. It would be difficult to

hypothesise a possible reason for this effect of IL-11 on TNF α production, however, it may be that it acts as a feedback mechanism to prevent over-production of TNF α .

Chapter 8

General Discussion

8.1 Introduction

This study has concentrated on the expression of cytokines in the human endometrium and the possible role that they may play in normal endometrial function. The role of these cytokines in recurrent miscarriage has also been investigated. Although much previous work has concentrated on T_H1 and T_H2 cytokines in successful pregnancy outcome, this study has concentrated on the pro-inflammatory cytokines, IL-1, TNF α , LIF and IL-6, as previous studies have suggested that these are important in endometrial function. During this period of study, the work with IL-11R knockout mice was published (Bilinski *et al.*, 1998; Robb *et al.*, 1998), suggesting the importance of IL-11 in successful pregnancy outcome. As IL-11 belongs to the same family of cytokines as IL-6 and LIF this was also studied.

The main techniques used were immunocytochemical analysis of cytokines in endometrial biopsies and cell cultures, and some attempt was made to measure cytokine mRNA by RT-PCR analysis. Immunocytochemistry has the advantage of enabling detection of cytokine production in the *in vivo* situation, and in particular, in determining which cell populations are involved. However, quantification using this technique is difficult and it is not possible to study mechanisms of actions of cytokines in human endometrial function in this way. Cell culture has the advantage of enabling control mechanisms to be investigated, but care should be taken in extrapolating the results obtained to the *in vivo* situation, as influences of other cells, either structurally or functionally have been removed. If it had been successful, the RT-PCR results would have shown expression of mRNA, which confirms synthesis, rather than presence of cytokines in the tissue. Showing the presence of both mRNA and protein would also have strengthened this study. The reason for the lack of success of the RT-PCR is probably due to the way the biopsies were collected and due to the impurity of the RNA samples following the RNA extraction procedure. It is however, unlikely that the lack of success is a result of inadequate optimisation of the PCR reactions as the reactions were optimised, as discussed previously in chapter 3.

The results from these studies clearly provide more evidence for the presence of cytokines, in particular LIF, IL-6, IL-1, TNF α and IL-11 in the human endometrium. With the exception of TNF α all of these cytokines have been localised in human endometrium using immunocytochemistry, and in this work, cultured endometrial cells were shown to express TNF α and IL-11. LIF and IL-6 have previously been shown to be produced from endometrial cells cultured using the same cell culture technique (Laird *et al.*, 1994; Laird *et al.*, 1997).

8.2 Human endometrial cytokine expression

8.2.1 *In vivo* expression of endometrial LIF, IL-6, IL-1 and IL-11

The results from this work agrees with that from others (Charnock Jones *et al.*, 1994; Tabibzadeh *et al.*, 1995a; Tabibzadeh and Sun, 1992; Simon *et al.*, 1993a; Dimitriadis *et al.*, 2000), in demonstrating that LIF, IL-6, IL-1 α , IL-1 β and IL-11 are expressed by the human endometrium throughout the menstrual cycle. The most significant findings are that LIF and IL-6 expression, in the epithelial glands, is maximal during the secretory phase of the cycle, when implantation occurs. Previous studies have also reported a similar increase in epithelial LIF expression (Charnock Jones *et al.*, 1994; Vogiagis *et al.*, 1996; Arici *et al.*, 1995) and epithelial IL-6 expression (Tabibzadeh *et al.*, 1995a) in the secretory phase of the menstrual cycle. This increased expression suggests that LIF and IL-6 may therefore be important in the implantation process in humans, particularly as LIF has previously been shown to be essential (Stewart *et al.*, 1992), and it has been suggested that IL-6 may also play an important role (Tabibzadeh *et al.*, 1995a), in implantation in mice.

IL-11 is a member of the IL-6 family of cytokines, and also signals through the signal transducing receptor gp130. Although the expression of IL-11 in the human endometrium was not seen to increase as drastically as LIF and IL-6, at the time of implantation, epithelial expression was slightly increased during the secretory phase of the cycle. As previous studies have shown that mice with a null or inactivating mutation for IL-11R α are infertile as a result of inadequate decidualisation (Bilinski

et al., 1998; Robb *et al.*, 1998), it is possible that IL-11 may be important in the decidualisation process in humans. This provides a possible explanation as to why stromal IL-11 expression is increased during the mid-late secretory phase of the menstrual cycle. Further evidence to support this possibility are the results showing that IL-11R α is expressed in the epithelial and stromal compartment of the human endometrium, although there appears to be little variation throughout the menstrual cycle.

Expression of IL-1 α and IL-1 β was seen to remain relatively constant throughout the menstrual cycle in both stromal and epithelial cells. This agrees with a previous report of human endometrial IL-1 expression (Tabibzadeh and Sun, 1992). However, other studies have suggested that IL-1 β expression is only seen during the mid-late secretory phase of the menstrual cycle (Kauma *et al.*, 1990; Simon *et al.*, 1993a). Although the results from this study showed that expression of IL-1 β in epithelial cells was maximal at the time of implantation, it was also present at all other times in the menstrual cycle. The differences in staining seen between these studies is not known, but may be due to different antibodies being used in each of the experiments.

8.2.2 Differences in cytokine expression of normal fertile women and women who suffer recurrent miscarriage

The results from this study showed that LIF expression was decreased in 11 out of 24 women who suffer unexplained recurrent miscarriage when compared to normal fertile women at the time of implantation. Expression of IL-6 was also decreased in 9 out of 15 biopsies obtained from recurrent miscarriage women and IL-1 α was decreased in at least 4 out of 12 biopsies obtained from recurrent miscarriage women when compared to staining seen in normal fertile women at the time of implantation. IL-1 β expression was only decreased in 2 out of 11 biopsies obtained from women who suffer recurrent miscarriage, however of the remaining biopsies that were used in this experiment, there was some variable staining. In some biopsies, epithelial glands stained the same intensity in recurrent miscarriage biopsies when compared to those obtained from normal fertile women, but the staining was not uniform throughout the sections, some glands showed a complete absence of IL-1 β . In other biopsies the

luminal epithelium stained normally but there was no staining present in any of the glands.

These differences seen between recurrent miscarriage women and normal fertile women were greater for LIF and IL-6 than for IL-1 α and IL-1 β . Previous studies have reported a similar decreased endometrial expression of both IL-1 β (von Wolff *et al.*, 2000) and IL-6 (von Wolff *et al.*, 2000; Lim *et al.*, 2000) mRNA transcripts in women with habitual abortion when compared to normal fertile women during the mid secretory phase of the cycle.

Having determined the pattern of expression of IL-11 in the endometrium throughout the menstrual cycle, the expression of IL-11 needs to be investigated in women suffering recurrent miscarriages. This work would determine if there are any differences in endometrial IL-11 expression between these two groups of women, particularly at the time of implantation and provide a greater understanding of the possible role of IL-11 in human endometrial function.

The results have shown the limitations of using immunocytochemistry to determine expression of cytokines in the endometrium, particularly when comparing results between the two groups of women. It might prove useful to use a more quantitative method to investigate this further. The measurement of protein in endometrial flushings has previously been used to compare production of endometrial proteins in normal fertile women and recurrent miscarriage women (Dalton *et al.*, 1995; Hey *et al.*, 1995) and provides a more quantitative assessment of differences. However, cytokine concentrations are very low in flushings and although attempts have been made to compare endometrial LIF production in various groups of women in this way, the levels present were close to the detection limit for the assay (Laird *et al.*, 1997). However, more sensitive assays for IL-6, IL-1 and IL-11 are now available and therefore this approach may prove productive in the future.

8.2.3 *In vitro* production of cytokines by cultured endometrial cells

The work presented here shows that cultured epithelial and stromal cells express IL-11R α and produce IL-11. TNF α was also shown to be produced by epithelial cells,

but not stromal cells. Other experiments attempted to measure IL-1 β production by cultured endometrial cells, however, the levels of IL-1 β in supernatants from cultured epithelial and stromal cells were below the level of detection of the ELISA kit, suggesting that if IL-1 β is secreted by these cells, it is only in very small amounts. Previous studies in this laboratory have shown that cultured epithelial, but not stromal cells produce IL-6 and LIF (Laird *et al.*, 1994; Laird *et al.*, 1997). The results above suggest that the cell culture system used acts as a good *in vitro* model when studying epithelial and stromal cells, as cytokines that are expressed *in vivo* can be detected in the cell cultures. However, when studying the endometrium *in vitro*, it can not be assumed that the results obtained would be an exact reflection of what is happening in the *in vivo* environment. These studies showed differences between *in vivo* and *in vitro* IL-1 β production, agreeing with other work that has also shown that IL-1 β is not produced by cultured endometrial cells (Simon *et al.*, 1998). We know that cytokines act in an autocrine and paracrine manner and it may be that in cell cultures, the paracrine action of some cytokines is altered due to the absence of other cells that would be present, and therefore would be influential, in the *in vivo* environment.

When studying cultured endometrial cells it is essential to determine the purity of the cell cultures, as contamination with leukocytes, stromal or epithelial cells would produce inaccurate results. Immunocytochemical analysis of vimentin (stromal cell marker), cytokeratin (epithelial cell marker) and CD45 (leukocyte marker) expression by stromal and epithelial cells prepared and cultured in this way has shown that the cells are essentially pure (>90%) and free of leukocyte contamination (Laird *et al.*, 1993).

8.3 Investigations into endometrial cytokine function

Epithelial and stromal cell cultures were used to study the effects of cytokines on MMP-2, MMP-9, MMP-7, IL-11 and TNF α production. In these experiments cytokines were added to confluent cells and were further incubated for 48 h. Effects of cytokines on cell growth were not investigated and it is therefore possible that the changes seen may be due to effects on cell number. However, previous published (Laird *et al.*, 1994; Laird *et al.*, 2000) and unpublished work has shown that neither

IL-1, TNF α , IL-6 and LIF have any effect on ^3H -thymidine uptake into either stromal or epithelial cells, suggesting that the effects seen are independent of cell growth.

Initial studies in the effect of IL-11 on ^3H -thymidine uptake into stromal and epithelial cells, also suggest that the effects of this cytokine are independent of cell growth (personal communication, S.M.Laird).

8.3.1 Basal endometrial MMP production

The production of MMP-2, MMP-9 and MMP-7 from cultured epithelial and stromal cells was measured using ELISA. The results showed that MMP-2 was produced from both epithelial and stromal cells, MMP-9 was produced mainly from epithelial cells, with only small amounts present in supernatants from cultured stromal cells and MMP-7 was produced from epithelial cells only. Epithelial MMP-2 production was seen to be significantly increased in supernatants from cells cultured from secretory endometrium when compared to that in supernatants from proliferative endometrium. Whereas epithelial MMP-7 production was significantly decreased in supernatants from cells cultured from secretory endometrium compared to proliferative endometrium. These findings are not surprising because it has previously been shown that progesterone and oestrogen are capable of influencing endometrial MMP production (Salamonsen *et al.*, 1997; Singer *et al.*, 1997; Schatz *et al.*, 1999; Keller *et al.*, 2000). Therefore the cyclical changes in steroid production would be expected to influence MMP production.

8.3.2 Effects of cytokines on endometrial MMP production

The results from this study have shown that cytokines, in particular, IL-1 α and TNF α are capable of modulating endometrial MMP production. In this study, cells prepared from only three endometrial biopsies were incubated with each cytokine and therefore it cannot be determined if the effects of the cytokines were dependent on the time in the cycle that the biopsy was obtained. The fact that differences in basal MMP production was seen in cells prepared from biopsies obtained at different times in the cycle, suggests that differences may be seen. Subsequent studies investigating the effects of cytokines at particular times in the cycle, in particular at the time of

implantation, would prove useful in order to establish the relationship between cytokines and MMPs in the implantation process.

The most surprising aspect of this study was the fact that IL-6 and LIF had no effect on MMP production by either stromal or epithelial cells. Both IL-6 and LIF have been shown to affect gelatinase production by cytotrophoblast cells (Bischof *et al.*, 1995a; Nachtigall *et al.*, 1995; Meissier *et al.*, 1999b). IL-6 receptor, LIF receptor and gp130 signal transducing receptor are all expressed in epithelial and stromal cells *in vivo* (Tabibzadeh *et al.*, 1995a; Sharkey *et al.*, 1999). Although there are no reports of their expression by cultured epithelial cells, loss of cytokine receptor expression is not characteristic of cultured cells. Taken together these results suggest that MMP production from embryonic and maternal cells are controlled by different mechanisms.

In contrast to IL-6 and LIF, IL-1 α and TNF α seem to be capable of modulating MMP production by both embryonic and maternal cells (Meissier *et al.*, 1999a). IL-11, like LIF and IL-6, had little effect on endometrial MMP production by either stromal or epithelial cells. This is perhaps not surprising as all three cytokines signal through gp130 and thus would be expected to have similar effects.

8.3.3 Effects of cytokines on endometrial IL-11 production

Previous studies have shown that IL-1 and TNF α stimulate endometrial LIF (Arici *et al.*, 1995) and IL-6 (Laird *et al.*, 1994; Laird *et al.*, 2000) production and that IL-1, TNF α and TGF β are capable of stimulating IL-11 production in other cell types (Elias *et al.*, 1994a; Morinaga *et al.*, 1997; Mino *et al.*, 1998). Therefore, we have used cultured stromal and epithelial cells to investigate the effects of IL-1 β , TNF α and TGF β on endometrial IL-11 production *in vitro*. The results showed that all three cytokines significantly increased IL-11 production from both stromal and epithelial cell cultures.

These results show the production of IL-11 by cultured endometrial cells for the first time and also indicate differences between IL-11, IL-6 and LIF production, in that IL-

IL-11 is produced by both stromal and epithelial cells, whereas previous studies have shown that LIF and IL-6 are only produced by cultured epithelial cells (Laird *et al.*, 1994; Arici *et al.*, 1995).

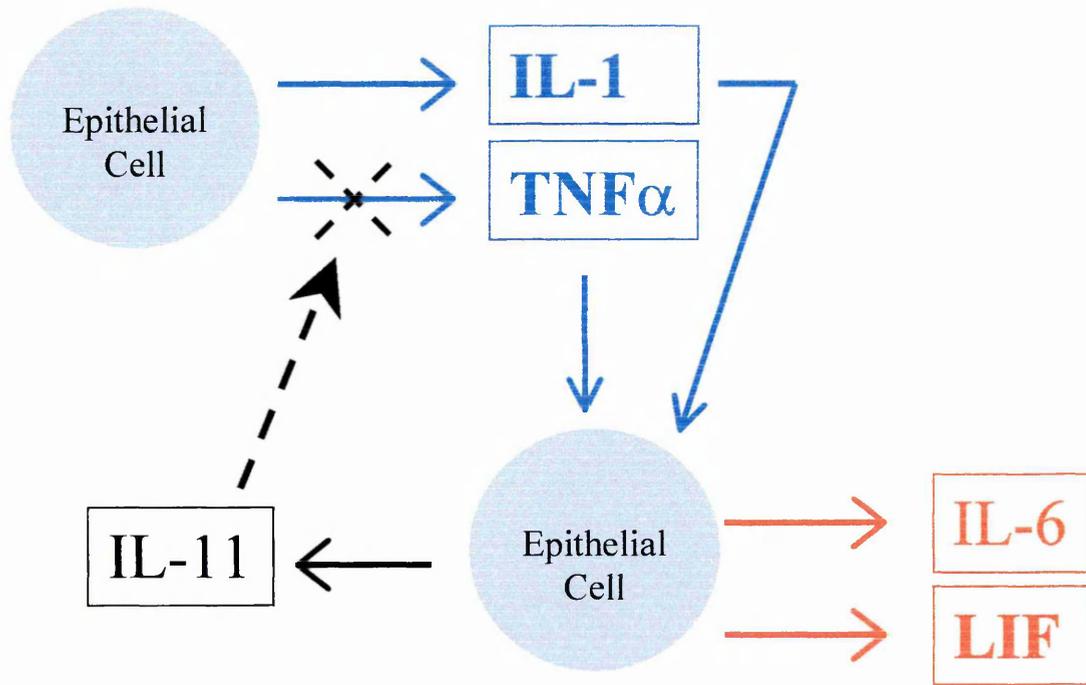
The effect of TGF β on cell growth has not been investigated and as a result, this may explain the increased expression of IL-11 in the presence of TGF β . However, the cells were only incubated with TGF β for 48h and the significant increase in IL-11 production seen in the cell cultures particularly stromal cells, is unlikely to be accounted for by an increase in cell number over 48h. Therefore the results suggest that the effects of all three cytokines on IL-11 production are independent of growth.

The results suggest that IL-11 production can be controlled by other cytokines in the endometrium, including IL-1, TNF α and TGF β . Effects of IL-1 and TNF α on endometrial LIF and IL-6 production have been reported (Laird *et al.*, 1994; Arici *et al.*, 1995). Effects of TGF β on endometrial cytokine production have not been reported and would warrant further study.

8.3.4 Effect of IL-11 on endometrial cytokine production

The endometrial expression of IL-11R α shown in this study and the fact that previous studies have shown expression of gp130 in the endometrium (Tabibzadeh *et al.*, 1995a; Cullinan *et al.*, 1996), suggests that IL-11 may have a role in endometrial function. In this study, the effect of IL-11 on cytokine production from cultured endometrial cells was also investigated. However, only TNF α production from epithelial cells could be measured because epithelial IL-1 β and stromal IL-1 β and TNF α levels were below the level of detection of the ELISA kits. TNF α production was significantly decreased following incubation of the cells with IL-11. Taken together with the findings that TNF α stimulates epithelial IL-11 production *in vitro*, this suggests that IL-11 may provide a possible negative feedback mechanism for the control of epithelial TNF α production in the human endometrium (figure 8.1). Thus IL-1 and TNF α stimulate epithelial production of not only IL-11 but also IL-6 and LIF; which becomes maximal at the time of implantation. Increased IL-11 production

Figure 8.1: Postulated function of IL-11 to provide a negative feedback mechanism for the control of endometrial epithelial TNF α production



appears to feedback onto the epithelial cells and inhibits TNF α production, thus turning off one of the major stimulants of its production. The effects of IL-6 and LIF on TNF α and IL-1 production have not been reported, but initial studies have suggested that IL-6 and LIF have no effect on TNF α production by epithelial cells and thus the feedback effect may be specific for IL-11.

8.4 Overall conclusion

In summary this study has shown the presence of LIF, IL-6, IL-1, TNF α and IL-11 in the human endometrium and provided evidence that they play a major role in human endometrial function. LIF and IL-6 in particular appear to be important in implantation, as expression is greatest at the time of implantation and is decreased in some women who suffer recurrent miscarriage. However, functional studies suggest that IL-1 and TNF α are also important, as it is these cytokines that produce more dramatic effects on cytokine and MMP production from endometrial cells in culture. More extensive research is required before the exact role of cytokines within the human endometrium can be determined. However, results from this study along with those previously reported by others show that cytokines are undoubtedly important mediators of endometrial function.

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