The Biological Control of Cervical Ripening

Cameron G Barclay

Submitted for the Degree of Doctor of Philosophy

University of Edinburgh

1995

THE UNIVERSITY *of* EDINBURGH

I hereby declare that the work contained in this thesis is my own and that this document was written by me.

Cameron G Barclay

I would like to thank Dr Rodney Kelly for his help and supervision of the work contained in this thesis. I would also like to thank him and Professor Andrew Calder for their editorial comments and assistance.

I am also grateful to Mrs Pam Warner, statistician with the Medical Research Council Reproductive Biology Unit, and to Dr Rob Elton, statistician with the University Department of Public health Sciences, for their help with the statistical analyses and instruction in using the computer software.

Human cervical tissue was obtained with the help of Dr Janet Brennand and the theatre staff.

Abstract

For many years the cervix was considered by clinicians to play a passive role in child birth. They assumed the expulsive forces applied to the fetus at term by the uterine musculature resulted in the forced dilatation of the cervix as the child was pushed through the birth canal. Few felt it necessary to investigate the subject any deeper. However, in recent years we have come to realise that there are major alterations in the structure of the cervix associated with pregnancy, especially during the latter stages. This is cervical ripening.

It has been shown that prostaglandins applied to the cervix can promote cervical ripening and they are now regarded as significant factors in the process. Hormonal alterations may also be important, with particular interest focusing on progesterone since the introduction of drugs with anti-progestogenic properties.

The consensus of opinion is currently that the activity taking place within the ripening cervix is very similar to an inflammatory reaction, involving white blood cells, hormonal control and very likely inflammatory mediators such as the cytokines.

A cell culture system was developed to study the production of prostaglandins by cervical cells from late pregnant guinea pigs. This permitted the examination of the influence of various substances on the synthesis of the prostaglandins, including progesterone and the anti-progesterone drug RU486 (Mifepristone/RU38486). RU486 has been found to promote cervical ripening in humans and guinea pigs. It is thought to increase the sensitivity of the uterus to prostaglandins and thereby promote muscle activity. It is licensed in the United Kingdom as an abortifacient. Since this antiprogestin can provoke

cervical ripening alone an accessible source of tissue from an animal with a similar physiology to the human seemed to be an appropriate starting point from which to investigate the effects of this steroidal derivative and how it may affect prostaglandin production.

The results show that prostaglandin output can be provoked *in vitro* by agents such as lipopolysaccharide and phorbol ester. The observed effects of RU486 were mixed. Giving the animals RU486 prior to sacrifice did not produce any measurable change in the response to most of the compounds examined, there were, however, some instances where a significant increase was detected, apparently associated with the calcium ionophore A23187, and others where there appeared to be a reduction. The inclusion of RU486 in the culture medium with other treatments did not produce any significant differences compared to the treatment on its own, and alone RU486 only produced a significant difference where the animal had been given the drug *in vivo*.

The cytokine/chemokine interleukin-8 was also examined. This peptide is a neutrophil chemoattractant and activator and thus is important in inflammatory reactions. The detection of interleukin-8 in the culture medium from the guinea pig cultures was not possible. However, maintaining cervical biopsy material from women using a tissue culture system it was possible to study this inflammatory mediator. Tissue was obtained from women hospitalised for surgical termination, hysterectomy or dilatation and curettage.

This work showed for the first time that human cervix was capable of producing interleukin-8 *in vitro* and that the level of production could be increased by the addition of various compounds to the culture medium. The tissue was obtained from different groups

of women and the results suggest that there may be a differential response related to the hormonal state of the patients, such as whether they were pre- or postmenopausal.

Basic immunohistochemical work appeared to show the presence of interleukin-8 in the connective tissue of the cervix which may be an indication that this peptide could attract cells to that area as a means of remodelling the connective tissue matrix.

Abbreviations

BSA	-	Bovine Serum Albumin
Cgem	-	Cervagem
DAB	-	Diaminobenzidine
DHA(S)	-	Dehydroepiandrosterone (sulphate)
DMF	-	Dimethylformamide
DNAse	-	Deoxyribonuclease
EDTA	-	Ethylenediaminetetraacetic Acid
Elisa	-	Enzyme Linked Immunosorbent Assay
FBS	-	Fetal Bovine Serum
GM-CSF	-	Granulocyte-Macrophage Colony-Stimulating Factor
HETE	-	Hydroxy-eicosatetraenoic-acid
HPETE	-	Hydroperoxy-eicosatetraenoic-acid
IL-1	-	Interleukin-1
IL-3		Interleukin-3
IL-7		Interleukin-7
IL-8	-	Interleukin-8
IPA	-	Isopropyl Alcohol
LPS	-	Lipopolysaccharide
MOX	-	Methyloximating solution
mRNA	-	messenger Ribonucleic Acid
NP	-	Nonpregnant
NSB	-	Non Specific Binding
NSS	-	Normal Swine Serum
OA	-	Okadaic Acid
Oe	-	Oestradiol
PAP	-	Peroxidase-Anti-Peroxidase
PBGS	-	Phosphate Buffered Gelatine Saline
PBS	-	Phosphate Buffered Saline
PG(s)	-	Prostaglandin(s)
PGE	-	Prostaglandin E
PGEM	-	Prostaglandin E Metabolite
PGF	-	Prostaglandin F
PGFM	-	Prostaglandin F Metabolite
PM	-	Postmenopausal
PMA	-	Phorbol Myristoyl/Myristate Acetate
Prog or P	-	Progesterone
RIA	-	Radioimmunoassay
RU486	-	11β-(4-N,N-dimethylaminophenyl)-17β-hydroxy-17α-propinyl-4,9(10)-oestradien-3-one
TBS	-	Tris Buffered Saline
TNF	-	Tumour Necrosis Factor-α
Tris	-	Tris(hydroxy)methylamine
TxB	-	Thromboxane

Chapter 1

Current Understanding of the Cervix and Cervical Ripening

Current Understanding of the Cervix and Cervical Ripening

Introduction

Throughout gestation the developing fetus must be protected from the harsh outside environment and as it grows it must be prevented from leaving the uterus. The cervix is a crucial element in accomplishing these requirements and therefore in maintaining the pregnancy. It does this by retaining its closed, rigid structure, acting in a sphincter-like manner.

When the fetus is mature the cervix experiences dramatic alterations in its function to allow the contents of the uterus to pass into the birth canal, culminating in the delivery of the child. In order for this to occur the cervix must dilate; however, it has become clear that the cervix is not a muscular sphincter and cannot merely relax. It is composed predominantly of connective tissue which must undergo fundamental changes to allow dilatation to take place. These changes are highly complex and are not fully understood. The process of change from a closed, rigid barrier to a soft, dilated ring of tissue are what we call cervical ripening.

Over one hundred years ago the cervix was shown to be an organ with a fundamentally fibrous structure (Hofmeier, 1886) but this idea was apparently overshadowed because the majority of workers regarded it as predominantly muscular in nature and therefore muscular in structure (Danforth, 1947). However, a few individuals pursued the notion that the cervix was constructed mainly of connective tissue (Stieve, 1927; Novak, 1944; Stander, 1945) and in 1947 Danforth (Danforth, 1947) published an article

which provided more widely accepted evidence of the fibrous character of the cervix. His work is often referred to and is seen by many as the real origin of the modern day view that the cervix is not a muscular sphincter and might be regarded as an organ in its own right.

For many years it was thought cervical ripening was a passive process that was dependent on the mechanical forces the cervix was subjected to by the action of the contracting uterus (Conrad and Ueland, 1983), despite the clinical observations that the cervix could ripen in the absence of uterine contractions or remain rigid and unyielding under their influence (Liggins, 1978). In sheep, cervical ripening was shown to occur even when the uterus and cervix were mechanically isolated from each other (Stys et al., 1978; Ledger et al., 1985).

The apparent lack of cervical smooth muscle meant that no major function for it was considered likely (Schwalm and Dubrauszky, 1966) and it was only relatively recently that a consensus was reached and the scientific community agreed that cervical ripening should be explained in terms of connective tissue biology (Danforth et al., 1980).

Although the cervix may be able to ripen without the contractions of the uterus it is obvious that the stresses they put on the cervix must in some way affect the connective tissue of the ripening cervix (Liggins, 1978; Calder, 1980; Hillier and Coad, 1982).

Recently, following Liggins' proposals, cervical ripening has been considered by most to be some form of inflammatory reaction (Liggins, 1981).

Since it is now clear that the cervix actively participates in parturition a great deal of work has been carried out in an effort to manipulate it and improve our understanding of its functioning.

As well as the intrinsic value of understanding the processes that take place during cervical ripening the knowledge gained has potentially useful clinical applications. As with all biological functions parturition and cervical ripening do not always adhere to anticipated patterns. In fact around 10% of pregnancies are complicated by a lack of cervical ripening at term and in a further 10% induction of labour and cervical ripening are required because of other indications (Ulmsten, 1988b; Ulmsten, 1988a). The need to promote cervical ripening is not the only clinical problem. In some women the cervix ripens and dilates relatively early in gestation and may be the cause of loss of the pregnancy through spontaneous abortion or premature delivery. This complication is known as cervical incompetence and current practice is to suture the cervix to keep it closed. Thus a fuller understanding of what processes underlie ripening may also permit pharmacological intervention in such cases.

The Cervix

The following pages detail cervical structure and the changes that are associated with cervical ripening.

Cervical Anatomy

The cervix is the lower most part of the uterus and in women is normally about 3cm long and approximately 2.5cm in outer diameter. It is anchored within the pelvic girdle by the pubocervical fascia, uterosacral and transverse cervical (or cardinal) ligaments (Uldbjerg and Ulmsten, 1990; Danforth, 1983).

Cervical Structure

Unlike the corpus of the uterus the uterine cervix is not a muscular organ but is predominantly composed of connective tissue with comparatively little smooth muscle (Danforth, 1947). It has been shown that, beginning at the uppermost reaches where it joins the myometrium, the amount of smooth muscle in the cervix decreases from around 29% to as little as 6% (Rorie and Newton, 1967) and generally it is accepted as being in the region of 10-15% (Danforth, 1983; Schwalm and Dubrauszky, 1966). The main cellular component of the cervix is the fibroblast (Junqueira et al., 1980) which is a cell type capable of the production of collagen, glycosaminoglycans and collagenase.

Around 75-80% of the cervix is water (Uldbjerg et al., 1983b; Danforth et al., 1974; Kleissl et al., 1978).

Most of the connective tissue of the cervix is collagen (Danforth, 1947; Junqueira et al., 1980) and around 70% of this is type I collagen, the remainder being type III (Danforth et al., 1974; Ito et al., 1979). However, Minamoto's group found type IV collagen associated with smooth muscle fibres and vascular basement membrane in human cervix (Minamoto et al., 1987). Elastin has been reported in small quantities (Danforth, 1947; Leppert et al., 1982) but there is disagreement about its significance (Danforth, 1983). The other main components of connective tissues are proteoglycans. They form a ground substance of protein and disaccharides in which the collagen is embedded (Uldbjerg et al., 1983c).

Collagen

Collagen is the most abundant protein in the body and there are at least seven genetically different forms (Uldbjerg et al., 1983c; Bornstein and Sage, 1980). Native collagen is found as fibrils (or fibres) and is built up from tropocollagen, each molecule of which consists of three collagen molecules twisted into a helical structure. The tropocollagen helices specifically congregate to produce collagen fibrils 60nm in diameter which are held together by cross links between hydroxyamino acids in the non helical end regions of the tropocollagen and hydroxyamino acids in the helix of adjacent tropocollagen molecules (Challis and Olson, 1988). Collagen is relatively rich in the amino acids hydroxyproline and hydroxylysine, and it is the quantification of hydroxyproline that is generally used to assess collagen concentration (Uldbjerg et al., 1983c; Uldbjerg and Ulmsten, 1990). As it matures the number of cross links within the fibrils increases (Uldbjerg et al., 1983c; Challis and Olson, 1988), as

does the stability of the protein. It is thought that more recently produced collagen is degraded more easily because of its simpler structure.

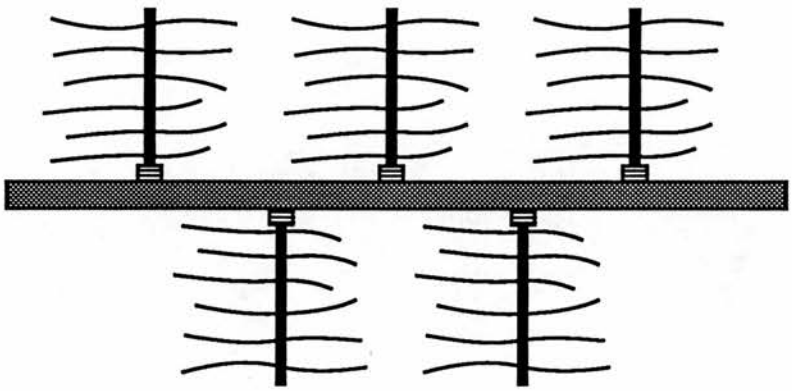
The collagen fibrils are embedded in a network of proteoglycans and the overall structure gives cervical connective tissue a high tensile strength (Conrad and Ueland, 1983) and it has been claimed that while smooth muscle does confer some contractile function on cervical tissue it could not contribute to its strength in any significant manner (Uldbjerg and Ulmsten, 1990; Schwalm and Dubrauszky, 1966; Danforth and Evanston, 1954). The histological appearance of the cervix is of an orderly, regimented structure where the collagen fibres are arranged in neat, tightly packed bundles (Danforth et al., 1960).

Proteoglycans

Proteoglycans are networks of protein covalently bound to glycosaminoglycans (GAGs). GAGs are large, unbranched chains of disaccharide units which have an amino sugar (hexosamine, glucosamine or galactosamine) and usually uronic acid, glucuronic acid or iduronic acid (Danforth et al., 1974; Uldbjerg and Ulmsten, 1990; Lindahl and Höök, 1978). They vary in the exact composition of the sugar residues and are often sulphated e.g. dermatan sulphate, chondroitin sulphate. Figure 1.1 shows a simplified representation of the proteoglycan structure.

As many as one hundred GAG chains bind to a glycoprotein core and the glycoproteins themselves associate with another large glycosaminoglycan, hyaluronic acid, probably through linking proteins. There may be between fifty and one hundred proteoglycans

non-covalently bound to each hyaluronic acid molecule (Uldbjerg and Ulmsten, 1990; Danforth et al., 1974; Uldbjerg et al., 1983c).



Hyaluronic Acid Core

Proteoglycan Protein Core

Linking Protein Between Hyaluronic Acid and Proteoglycan Core

Glycosaminoglycan

Figure 1.1 Representation of a section of proteoglycan complex

While the exact function of the proteoglycans is not clear it has been shown that the protein core of dermatan sulphate develops a relationship with collagen fibres and binds to them at specific points at 64nm intervals (Scott and Orford, 1981), corresponding to the banding of collagen fibres seen histologically. The GAG sidechains are also able to interact with the collagen or with other sidechains and appear to coat the collagen fibres binding them together to form large bundles of collagen (Uldbjerg and Danielsen, 1988). Thus the collagen is embedded in a network of protein and carbohydrate and it seems that the function of the proteoglycans may be to coordinate the

orientation of collagen fibres (Lindahl and Höök, 1978) and thus to increase the strength of the connective tissue matrix.

The proteoglycans are also able to interact with other macromolecules and the small amount of elastin that is present is bound into the ground substance in association with the collagen and GAGs (Lindahl and Höök, 1978).

Smooth Muscle

After Danforth published his findings in 1947 (Danforth, 1947) claiming to have observed a fibrous tissue with comparatively little smooth muscle, relative to the uterine corpus, Hughesdon (Hughesdon, 1952) claimed that the technique was at fault and that the muscular portion was lost when dissecting the tissue. Danforth then refuted these claims (Danforth and Evanston, 1954). While others have shown that the cervix does contain smooth muscle, in varying proportions between its proximal (vaginal) and distal (uterine) regions, and that it does confer some contractile properties on the cervix, its function is unclear (Rorie and Newton, 1967; Uldbjerg and Ulmsten, 1990; Schwalm and Dubrauszky, 1966; Hillier and Karim, 1970). It is considered insufficient to affect significantly the resistance of the cervix to dilatation. It may, however, have a role in returning the cervix to the nonpregnant state after delivery.

Changes in the Cervix Associated with Pregnancy

Physical Changes

During pregnancy there are changes in the cervix that are obvious to the clinician. Early on there may be some hypertrophy, it becomes vascularised and takes on a blue colour but remains relatively firm (Liggins, 1978; Calder, 1981; Danforth, 1983). Johnstone and colleagues (Johnstone et al., 1974) demonstrated an increase in the diameter of the cervical lumen in the first few weeks and this may be evidence that the cervix is enlarged. During the second half of pregnancy the cervix softens but retains its closed structure and remains resistant to forced dilatation (Liggins, 1978).

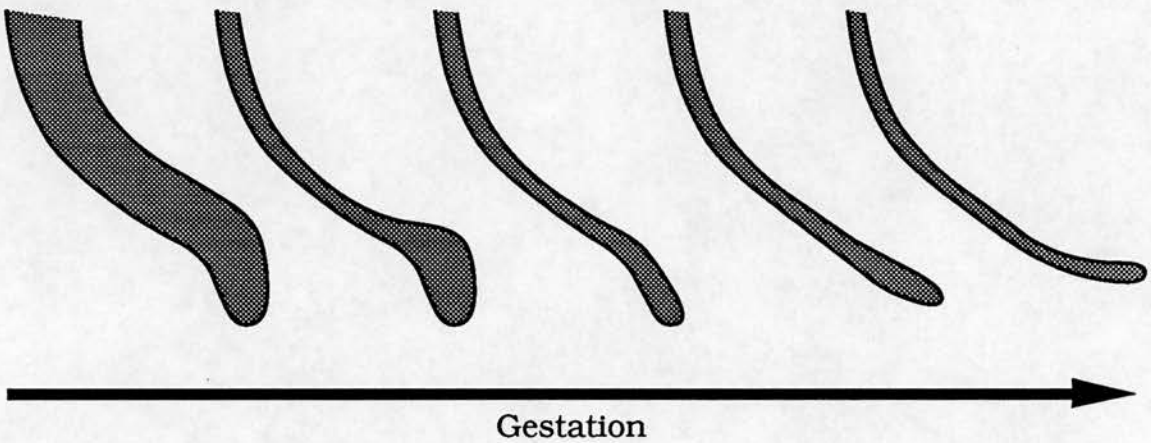


Figure 1.2 Representation of the changes in the cervix in relation to the uterus. Towards the end of the pregnancy the distinction between the uterine corpus and the uterine cervix becomes progressively less obvious as the cervix is "taken up."

There is then little outward change in appearance until the last few weeks of pregnancy when the early stages of ripening become evident. The tissue becomes softer and distensible and begins to efface, that is, it shortens and becomes less obvious as a separate entity as if the endocervical lumen opens to become part of the uterine cavity, following the contours of the uterus (Calder, 1981); this is represented diagrammatically in figure 1.2. When the cervix is ripe it is completely effaced and subsequently dilates to 10cm or more in order that the fetus can be expelled.

Histological Changes

Histologically, the changes in the cervix are every bit as radical as those required of it physically. This is hardly surprising in light of the fact that the cervix is not merely relaxing as a muscular sphincter would.

There are dramatic changes in the connective tissue the most obvious of which is the reorganisation, or more correctly the disorganisation, of the collagen fibres. In the nonpregnant and early pregnant cervix many collagen fibres with the same orientation are arranged in tightly packed bundles (Uldbjerg and Ulmsten, 1990; Uldbjerg et al., 1983b; Rath et al., 1988b; Junqueira et al., 1980; Parry and Ellwood, 1981). These associations between the collagen fibres are considered to give the cervix its great strength and rigidity. The dissolution of the regimented collagen bundles is central to the ripening of the cervix and the conversion to the resulting soft, dilatable organ.

Parry and Ellwood studied the cervical connective tissue of the sheep and showed that even at 130 days gestation the cervix still had a highly organised collagen fibre network (gestation in the sheep in their study was 137-145 days) and that in parturient animals the collagen became completely disorganised with large interfibrillar spaces. Their work also showed that the connective tissue quickly began to return to its previous regimented state with obvious associations between collagen fibres as early as five days postpartum (Parry and Ellwood, 1981).

In human cervix the situation is the same, spaces between the collagen fibres appear at term and the fibres become irregular and fragmented (Junqueira et al., 1980; Rath et al., 1988b; Minamoto et al., 1987; Kokenyesi and Woessner, 1990; Ulmsten, 1988b). Estimations of the collagen content of the cervix have been carried out by Junqueira using picrosirius red staining. The stain is said to specifically detect polymerised collagen when using a polarised light source (Junqueira et al., 1979). The results showed that there was a massive reduction in the level of similarly oriented collagen fibres, the intrapartum tissue possessing only 8.3% of the degree of organisation found in the nonpregnant specimens (Junqueira et al., 1980). A reduction in the diameter of the collagen fibres was also noted and the appearance of an amorphous material between them was suggested to be due to the products of degradation.

Other microscopically observed events include the activation of the cervical fibroblasts and the infiltration of the cervix by polymorphonuclear leucocytes at term. Basset found fibroblasts to be more active in sheep cervix (Basset, 1958) as did Parry (Parry and Ellwood, 1981) and Liggins suggested that cervical ripening depends

on changes in the functioning of fibroblasts (Liggins, 1981; Liggins, 1978). While there is some debate over the role the fibroblasts play in changing the state of the connective tissue, it is apparent that clinically used methods of promoting cervical ripening also promote fibroblast activity (Rath et al., 1988b; Uldbjerg et al., 1981).

Several reports of white blood cell infiltration of the cervix have been made. Junqueira's team observed that the fibroblast was the most abundant cell in the human cervix but that during labour there was an increase in cell numbers with the appearance of mast cells and macrophages but predominantly neutrophilic polymorphonuclear leucocytes (Junqueira et al., 1980). They found that once within the cervical tissue these neutrophils degranulated and the collagen fibres surrounding them disappeared. The function of the mast cells is unclear but they have been shown to stimulate collagenase secretion by fibroblasts (Dabbous et al., 1986). Two peptides of eosinophilic origin are said to modulate proteoglycan metabolism *in vitro*. These have been found in human, term cervix but not in nonpregnant or early pregnant tissue. It has been suggested that this indicates that eosinophils may also be involved in cervical ripening (Uldbjerg and Ulmsten, 1990) and they have been found in rat and guinea pig cervix at term (Luque and Montes, 1989; Liggins, 1981).

Minamoto et al. have shown that polymorphonuclear leucocytes invade the cervix at term. They observed them in clear spaces between the collagen fibres, possibly indicating degradation of the surrounding collagen (Minamoto et al., 1987). Another group has also seen leucocyte infiltration of the cervix during dilatation and parturition (Rath et al., 1988b) and found cells devoid of granules which they

suggest indicated that enzymes had been released into the cervical stroma.

A team from Japan found fat droplets in fibroblasts at term and less so earlier in pregnancy, but whether this signifies increased activity in these cells is not clear. They also claim to have observed leucocyte infiltration at term but only in the glandular tissue, at the same time showing general collagen dispersal in the cervix (Yoshida and Manabe, 1990).

Finally, a French group has extracted leucocytes from maternal blood, stimulated them and then, using a stereotactic device to accurately locate a syringe needle, they injected the woman's own cells into her cervix in an effort to bring about cervical ripening. They appear to have obtained a success rate of about 40% (Michelet et al., 1986).

Biochemical Changes

The biochemical alterations which accompany the histological changes are many and varied and some are still in dispute.

Collagen

Cervical collagen has been assessed by many different workers, not only in terms of the amount of collagen but also the ease with which it is degraded.

Various different parameters have been used to determine the changes that occur in collagen concentration in the cervix. It has been expressed in terms of the wet weight of the tissue, in terms of the dry weight and also in terms of the defatted, dry weight.

As a percentage of cervical protein collagen constitutes 82% in nonpregnant human tissue and only 52% of pregnant cervix (Danforth and Buckingham, 1973). Ito showed that the amount of collagen in defatted, dried cervical tissue was almost 50% lower in term cervix than in nonpregnant cervix (Ito et al., 1979). The level of collagen, as a fraction of the wet weight, has been shown to fall in pregnancy (Ulmsten, 1988b), the same author showing that as a fraction of the dry weight there was also a reduction in collagen concentration. Uldbjerg's group showed that the collagen content was significantly reduced as early as ten weeks into pregnancy, when measured as a percentage of the wet weight (Uldbjerg et al., 1983b; Uldbjerg et al., 1985). Kleissl et al. found an overall reduction in the concentration of cervical collagen per unit weight of protein (Kleissl et al., 1978). In the mouse it has also been shown that the cervical collagen concentration drops during pregnancy, reaching its lowest at term (Rimmer, 1973) with a similar situation in rats (Kokenyesi and Woessner, 1990). Another study in rats equated the fall in collagen concentration with the reduction in tensile strength and increase in extensibility of the cervix (Zarrow and Yochim, 1961). Findings supporting the same circumstance in the human have been made, the proportion of collagen being higher in women in labour with unripe cervices compared to those in labour with favourable cervices (Uldbjerg et al., 1983b; Ekman et al., 1986). In fact it was shown that the lower the concentration of collagen the faster the dilatation of the cervix proceeded (Uldbjerg et al., 1983b).

Von Maillot and Zimmerman could not find a significant change in the amount of collagen when expressed as a fraction of the dry weight of the cervical tissue (von Maillot and Zimmermann, 1976).

Despite the difficulty of assessing the mass of the whole human cervix at term, Ito et al. claim that they found the total collagen in the pregnant cervix to be similar to that in the nonpregnant cervix (Ito et al., 1979).

Although the bulk of the evidence suggests that the concentration of cervical collagen falls in pregnancy it has been reported that the absolute quantity of collagen in the cervix increases in the sheep, the rat and the mouse (Fosang and Handley, 1988; Golichowski, 1980; Kokenyesi and Woessner, 1990; Rimmer, 1973).

The changes in the concentration of collagen are accompanied by changes in solubility and changes in its structural integrity.

The proportion of collagen soluble in aqueous solution has been shown to increase from about 30% of the total collagen in the nonpregnant cervix to over 50% in the pregnant cervix (von Maillot and Zimmermann, 1976). Danforth found that the water soluble fraction of the solid material in human cervix was raised from 5% in nonpregnant tissue to 84% postpartum (Danforth et al., 1974), and that the proportion of insoluble collagen in the solid extract of the cervix fell dramatically between the nonpregnant and the postpartum samples. Kleissl et al. did not find a difference between term pregnant and nonpregnant women with regard to the percentage of collagen, relative to the total protein, found in the soluble fraction of the cervical tissue. In the same fraction, however, they did observe a greater degree of collagen breakdown products in the samples from pregnant patients (Kleissl et al., 1978). They also noted that the amount of collagen in the insoluble fraction of the cervical tissue fell from 75% of the total protein to 35%. Although Ito observed very little solubility in acetic acid his group found that the proportion of the

total collagen appearing in an acetic acid solution containing pepsin, was almost 94% in term pregnant women compared with only 51% in nonpregnant controls (Ito et al., 1979), and that the degree of insoluble collagen was much lower at term. Uldbjerg's group obtained similar results, little extractability in acid but large increases in the presence of pepsin, and a significant reduction in the fraction of collagen that was insoluble (Uldbjerg et al., 1983b). In a subsequent publication Uldbjerg found no difference in the extractability of cervical collagen in acetic acid plus pepsin as the result of pregnancy, and no change in the proportion of the collagen that was intact (Uldbjerg et al., 1985) despite the reduction in total collagen, mentioned above, that they reported in this paper. He suggested this indicated that the collagen lost was removed rapidly once degraded, however this is at variance with the findings of Kleissl et al.

The crosslinks within the collagen are important in terms of stability (Veis, 1980) and the fewer there are the more easily degraded the collagen. Old collagen contains more crosslinks than new and is therefore less soluble (Uldbjerg and Ulmsten, 1990). This suggests that the reduction in the level of insoluble collagen is due to the replacement of old collagen with new collagen. Examination of the crosslinks has shown that the material containing the most crosslinks persists longest in term cervix, the collagen with fewer crosslinks probably having been digested (Kleissl et al., 1978). The lower the number of crosslinks the more susceptible the protein is to collagenase digestion (Veis, 1980).

Fosang and Handley studied collagen synthesis in the sheep as the conversion of ^{3}H-proline to hydroxyproline and found that although as a whole the term cervix doubled its capacity for collagen

synthesis compared with nonpregnant, when measured relative to the wet weight or the dry weight there was no difference (Fosang and Handley, 1988).

Proteoglycans

Glycosaminoglycan levels in the cervix have been shown to increase dramatically in pregnancy. Danforth et al. measured the concentration of hexosamine, present in all GAGs, in relation to the dry weight of the tissue and found substantially more GAGs in postpartum cervix than nonpregnant cervix (Danforth et al., 1974). The proportions of different GAGs changed, chondroitin sulphate and dermatan sulphate approximately doubling but keratan sulphate seemingly increasing 8-fold (Danforth et al., 1974). However, keratan sulphate was not determined directly but by assuming that the fraction of GAGs that did not contain uronic acid was keratan sulphate, since this glycosaminoglycan is known to be uronate free. The same group also observed the appearance of an unidentified component and proposed that it may have been another GAG since its degradation products were rich in sugar residues (Danforth et al., 1974). An increase in the proportion of hyaluronate was reported in the human cervix at term and postpartum by von Maillot et al., who also showed an increase in the proportion of keratan sulphate and a decrease in chondroitin sulphate and dermatan sulphate (von Maillot et al., 1979). They saw no change in unsulphated chondroitin. Others have not even been able to demonstrate the presence of keratan sulphate (Uldbjerg et al., 1983a).

Golichowski assessed GAGs by measuring uronate levels. He did so because uronic acid is only found in significant quantities in

GAGs whereas hexosamine is also found in glycoproteins. Keratan sulphate does not contain uronate but he suggests that the cervix is unlikely to contain this GAG (Golichowski, 1980) because other similar connective tissues do not. A large increase in uronate per cervix was measured in rats as pregnancy developed but the concentration of glycosaminoglycan (uronate) in terms of the dry weight of the tissue did not change. He also found that there was an increase in the proportion of hyaluronic acid to total GAG during pregnancy. Assessment of collagen levels allowed him to show that the ratio of collagen to GAG fell slightly during pregnancy (i.e. proportionately more GAG to collagen) (Golichowski, 1980). A study in rats showed that the concentration of dermatan sulphate increased relative to collagen as pregnancy continued (Kokenyesi and Woessner, 1990) and von Maillot's work also revealed a similar trend in human cervix, the GAG to collagen ratio showing an increase in favour of the GAGs (von Maillot et al., 1979).

Junqueira measured GAGs in human cervix using electrophoresis and found a small increase in the proportion of chondroitin sulphate but a decrease in dermatan sulphate and no change in heparan sulphate (Junqueira et al., 1980). Uldbjerg assessed hexosamine to quantify GAGs and observed a drop in the concentration of sulphated glycosaminoglycans, in terms of tissue wet weight, in late pregnancy with similar results for hyaluronic acid (Uldbjerg et al., 1983b). However, from his results it is apparent that the concentration of collagen fell by a greater proportion than did the GAGs, thereby making the ratio of GAGs to collagen increase.

Danforth measured the total hexosamine levels then isolated the GAGs and assessed the hexosamine values once more. He then

assumed that the difference between the two figures constituted the level of glycoprotein that was present since hexosamine is also associated with these proteins (Danforth et al., 1974). The results indicated that there was a drop in the amount of glycoprotein present in pregnant tissue (per mg dry, defatted weight). Others have also indicated that the level of glycoproteins increases in the pregnant state (Uldbjerg and Ulmsten, 1990).

Cervical tissue from sheep was incubated with ^{35}S-sulphate in order to examine its incorporation into sulphated proteoglycan. The results indicated that at term there was far greater proteoglycan synthesis than in the early pregnant and nonpregnant ovine cervix (Fosang and Handley, 1988). The same group carried out similar experiments using ^{3}H-glucosamine to assess hyaluronate synthesis and once again found that term cervix produced much more hyaluronic acid than nonpregnant tissue.

While it may not be altogether clear what is actually happening with regard to the glycosaminoglycans it is obvious that some changes are occurring. Additionally, the plasma concentration of an enzyme that is capable of degrading GAGs, β-D-*N*-acetylglucosaminidase, has been shown to increase with gestation and to increase slightly more just before term in humans (Takenaka et al., 1991). This supports the idea that remodelling of the cervical tissue occurs throughout pregnancy and that there may be a significant increase in connective tissue degradation just prior to delivery.

It is suggested that hyaluronate's hydrophilic properties, its relatively low level of interaction with protein and its extended structure may promote the appearance of large gaps between the collagen fibres (Golichowski, 1980). Chondroitin sulphate has been

shown to bind to collagen and much more strongly than keratan sulphate (Öbrink, 1973), thus lower levels of chondroitin sulphate and higher levels of keratan sulphate may result in loosening of the connective tissue.

Water

Danforth et al. found the nonpregnant cervix to be approximately 74% water and the postpartum cervix to be 78% water (Danforth et al., 1974). Uldbjerg et al. measured the water content of nonpregnant cervix as almost 81%, the late pregnant cervix as 86% and postpartum tissue as 87% water (Uldbjerg et al., 1983b). Both these groups assessed the water content by the difference in the wet weight and the dry weight (dried to a constant weight). Kleissl weighed the wet tissue and extracted the collagen followed by lyophilisation of the collagen extract and residual tissue. This revealed 73% of the nonpregnant cervix to be water and 80% of the intrapartum cervix to be water (Kleissl et al., 1978). Thus it appears that there is a small increase in the proportion of the cervix that is water.

Collagenolysis

The breakdown of collagen requires the action of collagenase or leucocyte elastase as intact collagen molecules are resistant to general proteolysis. However, once the site-specific cleavage is performed by collagenase the products can denature at body temperature and the collagen is amenable to digestion by other proteases present in the tissue (Uldbjerg and Ulmsten, 1990).

Collagenase has been reported in the human cervix as free, active enzyme, inactive enzyme complexed with α_2-macroglobulin or

as an inactive form bound to an unidentified low molecular weight fragment (latent collagenase) (Kitamura et al., 1979; Kitamura et al., 1980). A procollagenase has also been described and it is therefore likely that this form also exists in the cervix (Rajabi et al., 1991; Uldbjerg and Ulmsten, 1990; Clark and Cawston, 1989).

Measurement of collagenase activity has been carried out using synthetic polypeptides. These, however, are liable to degradation by other enzymes and also by collagenase complexed with α_2-macroglobulin (Kitamura et al., 1979; Rath et al., 1987). It is therefore more appropriate that collagen be used as the substrate and many investigators are now doing this.

Kitamura et al. (Kitamura et al., 1979), relating their results to the wet weight of the tissue, found an increase in the degradation of a synthetic peptide (DNP-peptide) in extracts of pregnant human cervix compared to nonpregnant. Collagenase-α_2-macroglobulin complex activity and free enzyme activity increased but latent collagenase activity was unchanged (latent enzyme was activated with aminophenylmercuric acetate). Free, active collagenase was also shown to increase using collagen itself as the substrate, and there was no change in latent collagenase. Uldbjerg's group used DNP-peptide to assess the overall collagenolytic activity of human cervix and found it increased from the nonpregnant to the term state (Uldbjerg et al., 1983b). Rath has studied collagen breakdown by extracts from human cervix and found that collagenolytic activity was higher postpartum than in early pregnant or nonpregnant women and that there was no difference between the latter two groups (Rath et al., 1988a; Rath et al., 1987). He subsequently published more material showing that human cervical extracts contained collagenase activity,

but that any increase was not evident even in the early stages of labour (2-3cm dilatation). However, a significant increase had occurred by the time the cervix had dilated to 6-8cm. This paper also showed collagenase activity to have dropped again to prelabour levels immediately after delivery (Osmers et al., 1990).

Another group has looked at the collagenase activity at term in women using biopsies taken at caesarean section. They were from women in labour with dilated cervices (4-8cm) or women not in labour with closed cervices (0-2cm). These workers collected cervical and lower uterine segment tissue and found no differences in enzyme activity in each in the absence of labour, suggesting that both may behave similarly during labour. In the group of patients in active labour, lower uterine segment biopsies were regarded as safer and easier to obtain. The results produced using those samples showed that there was a thirteen fold increase in the level of degradation of the collagen substrate associated with labour and cervical dilatation (Rajabi et al., 1988).

It is apparent that there is an increase at the end of pregnancy in collagenase/collagenolytic activity. Since the fibroblast is the major cellular component of the cervix and is known to produce collagenase (Clark and Cawston, 1989; Osmers et al., 1992) it was considered the most likely candidate to provide this collagenase. However, a study on the origins of the collagenase detected in the cervix has been carried out recently, using *in situ* hybridisation and immunohistochemical techniques. Cervical tissue was treated to extract the mRNA, which was then probed for fibroblast procollagenase mRNA but this *in situ* hybridisation did not reveal the presence of any such messenger molecules. The immunohistochemical part of the study used an

antibody specific for human leucocyte collagenase that was previously shown did not cross react with fibroblast collagenase. This provided evidence that the polymorphonuclear cells that infiltrated the cervix contained granules incorporating collagenase and also showed the presence of collagenase in the connective tissue, outwith the cells (Osmers et al., 1992). Okamura et al. showed an increase, with gestation, in the activity of polymorphs in cervical mucus (Okamura et al., 1988). These results lend support to the observations of Junqueira who found polymorphs in the cervix with the surrounding tissue apparently dissolved (Junqueira et al., 1980). Uldbjerg examined leucocyte elastase by radioimmunoassay and observed an increase during pregnancy which appeared to continue postpartum (Uldbjerg et al., 1983b). A Japanese group studied elastase activity in human cervical mucus and also the histological localisation of this enzyme in the cervix during pregnancy. They found elastase levels rose during pregnancy, especially at term, and showed, using immunohistochemistry, that it was not present in the cervical stroma of unripe cervix but was present at term (Kanayama and Terao, 1991).

Additionally, polymorph collagenase has been shown to selectively degrade type I collagen over type III, type I making up 70% of the cervical collagen. Fibroblast collagenase degrades each type of collagen equally (Horwitz et al., 1977).

Mammalian collagenases are unable to degrade the protein core of proteoglycans (Poole, 1980). Therefore, in order to breakdown the connective tissue there must be other enzymes to digest these structures. Tissue proteinases have been shown to have this ability, elastase, cathepsins and metalloproteinases among them (Roughley and Barratt, 1977; Roughley, 1977; Keiser et al., 1976; Dingle et al.,

1977; Sapolsky et al., 1974; Sapolsky et al., 1976). It is generally accepted that proteinases are secreted in an inactive form (Poole, 1980) and there is evidence that the latent enzyme is activated by limited proteolysis, collagenase being a good example (Vaes, 1972; Eeckhout and Vaes, 1977; Werb et al., 1977; Horwitz et al., 1976). Thus, the proteases may perform the dual functions of activating collagenase, or other latent enzymes, and breaking down the partially degraded collagen and connective tissue matrix.

Rath has studied protease activity in human and guinea pig cervix and although protease activity was detected there was no measurable increase in pregnancy. Guinea pigs in late pregnancy showed no increase compared to nonpregnant animals (Rath et al., 1989), and human cervix from first trimester patients showed no increase over nonpregnant values (Rath et al., 1987). He has, however, shown that there is significantly more protease activity in samples from human cervix taken after normal spontaneous deliveries (Rath et al., 1988a). Others have also shown that there are proteases in the extracts of human cervical tissue (Ito et al., 1980; Mori et al., 1981) and that activity may be increased postpartum (Hutchins and Parkin, 1981).

There is evidence that enzyme inhibitors are produced by cervical cells in sheep (Raynes et al., 1988b) and rabbits (Sakyo et al., 1986b) and by many human tissues (Vater et al., 1979; Stricklin and Welgus, 1983; Murphy et al., 1981; Welgus and Stricklin, 1983; Dean and Woessner, 1984; Mercer et al., 1985) therefore they may also have a role to play in the complex processes of collagenolysis.

Summary

The following table (Table 1.1) summarises the changes that have already been described.

Table 1.1 Summary of changes associated with the processes of cervical ripening

Collagen	Less regimented structure. Increased solubility. Reduced concentration. Replacement of old collagen with new collagen (fewer cross-links).
Proteoglycans	Increased concentration of GAGs (GAG:Collagen ratio increased in favour of GAGs). Alterations in the proportions of individual GAGs. Increase in concentration of large GAG hyaluronate.
Collagenolysis	Increased collagenase. increased Elastase. Increased proteases (samples taken postpartum).
Water	Increased water content (may be due to hydrophilic properties of hyaluronate).
Fibroblasts	Fibroblast activation: source of new collagen, GAGs and collagenase and proteases.
Leucocytes	Infiltration of cervix by leucocytes: source of collagenase, elastase and proteases.

Methods Used Clinically to Promote Cervical Ripening and Labour — Clues to Local Mechanisms Involved in Cervical Ripening

Membrane Stripping

The placing one or two fingers inside the uterine cavity and separating the fetal membranes from the lower part of the uterus by sweeping the fingers through a full circle has been employed to induce labour and cervical ripening. This is not a particularly useful method of cervical ripening when the cervix is totally unripe and the patient is nulliparous, the cervix being insufficiently dilated to make it possible. Where the patient is multiparous and effacement of the cervix has begun it is often more successful, although the success rate is inconsistent (Trofatter, 1992). The theory as to its efficacy as a method of inducing the required changes in cervical state relies on the release of endogenous prostaglandins (Porto, 1989) from the membranes, decidua or the cervix itself, or possibly all three, and is based on the fact that prostaglandin levels measured in plasma increase following vaginal examination or amniotomy (Mitchell et al., 1977; Sellers et al., 1980) presumably through mechanical disturbances. Hillier and Coad have shown that stretching the human cervix can cause the release of PGE_2 and $PGF_{2\alpha}$ (Hillier and Coad, 1982).

Amniotomy

This deliberate rupturing of the membranes is often used to promote labour (Shime et al., 1984; Gibb et al., 1982; Hauth et al., 1980). However, this is only appropriate where the cervix is already in a favourable condition because the mother is then committed to

deliver and if the cervix is unripe when amniotomy is performed maternal and fetal complications may ensue (Satin and Hankins, 1989; Calder, 1980; Calder, 1990).

Foreign Body Mediated Dilatation

The insertion of objects into the cervical canal in order to dilate it is claimed to have been mentioned in the writings of Hippocrates 400 years BC (Trofatter, 1992; Johnson, 1989). However, there are problems associated with using mechanical force to increase the diameter of the cervix. These are mainly concerned with damaging the tissue, by tearing it (Hulka and Higgins, 1961; Hulka et al., 1974), which can result in permanent injury (Johnstone et al., 1976) leading to cervical incompetence and a higher risk of abortion or preterm delivery in future pregnancies (Margolis and Goldsmith, 1972; Pnatelkis et al., 1973; Papaevangelou et al., 1973; Pickering and Forbes, 1985).

In the past many items have been used in attempts to dilate the cervix including fingers, sponges, bark and canvas, but probably the best known were derived from seaweed (Johnson, 1989). The seaweeds in question were *Laminaria japonica* or *digitata* and the basis of their action seems to rely on the absorption of fluid and subsequent expansion, resulting in a gradual increase in diameter (Margolis and Goldsmith, 1972). These dilators are referred to as tents, a term defined medically as a conical expansile plug of soft material used for dilating an orifice or for keeping a wound open (Johnson, 1989).

The use of such dilators has not been particularly popular in Britain but they are used by American, Japanese and Scandinavian

clinicians (Johnson, 1989). The reasons for not using them stem from the fear that by introducing foreign bodies into the endocervix and leaving them *in situ* for prolonged periods there is an increased risk of infection, however the risk does seem to be minimal, when properly handled, and with the introduction of synthetic dilators the sterility of the products can be guaranteed (Killick et al., 1985; Johnson, 1989; Porto, 1989).

Apart from Laminaria tents there are also available Dilapan and Lamicel tents. Dilapan tents were designed to act like Laminaria but to be free of the stigma of infection and lack of consistent action. They are made of a hydrophilic polymer and expand as they absorb water in a predictable manner. Lamicel is a polyvinyl alcohol sponge impregnated with magnesium sulphate. It does not exert enough force to dilate the cervix and its mode of action is not clear (Johnson, 1989). Although it is commonly believed that it may partially depend on dehydrating the tissue this is disputed by Johnson.

In general, the mechanism behind the dilatation caused by cervical tents is unclear. Laminaria have been shown to result in an endocervical lumen with a greater diameter than the expanded dilator itself (Stubblefield, 1980). Thus, there must be some additional factor involved and the main contenders appear to be the prostaglandins that are released by virtue of the mechanical disturbance (Olund et al., 1984; Ye et al., 1982) or by the presence of a foreign body that provokes an inflammatory reaction (Johnson, 1989) similar to that considered to take place in natural cervical ripening (Liggins, 1981).

Lamicel contain magnesium sulphate, which is known to cause relaxation of smooth muscle (Johnson, 1989), but the smooth muscle content of the cervix is considered to be negligible. Even though there

have been reports of muscular/contractile function in cervical tissue the level was minimal and would be very unlikely to participate to any significant degree in dilatation of the cervix (Hillier and Karim, 1970; Najak et al., 1970; Uldbjerg and Ulmsten, 1990; Danforth and Evanston, 1954).

So, even now it is still unclear how these dilators bring about their effects but their use continues and it is interesting that in a trial comparing PGE_2 and laminaria for the dilatation of the cervix prior to termination up to 16 weeks of pregnancy, the laminaria tents appeared to produce better results (Killick et al., 1985).

The use of a Foley catheter to bring about cervical dilatation has also been examined. The catheter, with a balloon attached, is passed through the cervix into the uterine cavity and the balloon inflated. The catheter is then fastened to the thigh with tape in order to produce a downward force to pull it through the cervix and this has been shown to cause dilatation of the cervix (Porto, 1989). Calder et al. established a technique of combining the Foley catheter with prostaglandin therapy (Calder et al., 1976).

A group working in this area have designed a device consisting of two balloons one of which sits inside the uterus and one intravaginally, (i.e. one at either end of the cervix). Their device is intended to close off the cervix in order to retain an application of prostaglandin containing gel administered through the catheter, so that the effects of the PG are confined to that organ. The balloons were inflated, the gel passed into the endocervix and the catheter taped to the patients thigh. They found that cervical ripening occurred and that the double balloon device on its own may have produced the ripening effect (Atad et al., 1991). Observations have been made which

suggested that the presence of the gel only (with no prostaglandin) in the endocervical canal may produce cervical ripening (Keirse et al., 1983).

Pharmacological Techniques

The use of pharmacological agents will be discussed in the next section.

Summary

The induction of cervical ripening is not a modern problem and some of the methodology employed today dates back centuries. The most obvious technique is that of inserting expanding plugs into the cervix and is still in use. However, there are now products with predictable physical (swelling) characteristics that the clinician can be sure are sterile and therefore less likely to cause infection. Some of these dilators have been shown to be too weak to stretch the cervix or shown to cause dilatation of the cervix to a greater diameter than themselves. Membrane stripping, amniotomy and the dilators are all considered to rely to some extent on the endogenous production of prostaglandins, mechanical disturbance of the tissue resulting in prostaglandin production. The role of prostaglandins will be more fully discussed in the next section.

Control of Cervical Ripening

The factors controlling the changes that occur in the cervix during pregnancy are complex and not fully understood. Hormones, including oestrogen, progesterone and relaxin, have been implicated as have the products of arachidonic acid metabolism, principally the prostaglandins.

The following outlines what is known about those factors and their effects on the cervix and cervical ripening.

Prostaglandins

Prostaglandins were first discovered in the 1930s in human seminal fluid and in extracts of the accessory sex glands of the ram. They were found to have a stimulatory action on isolated uterine and intestinal smooth muscle but to cause a drop in blood pressure following intravenous injection and it was von Euler, working in Sweden, who identified the active component as lipid soluble and introduced the term prostaglandin (Kurzrok and Lieb, 1930; Von Euler, 1935; Bowman and Rand, 1980).

Prostaglandins are derived from 20 carbon, polyunsaturated fatty acids and are included in the group of biological factors known as the eicosanoids (Smith, 1986). The main precursor of the prostaglandins is arachidonic acid, a major fatty acid found in cell membranes and abundant in reproductive and fetal tissue (Crawford, 1983; Keirse, 1978) within lipid pools such as the phospholipids, cholesterol esters or glycerol esters (Irvine, 1982; Blackwell and Flower, 1983).

Because of their low serum concentrations ($<10^{-11}$M), the lack of a specific organ for their synthesis and the fact they are produced by many tissues, the occurrence of catabolic mechanisms in the lung, liver and kidney which prevent prostanoids from building up in the circulation and because they have been found to act both on the cells in which they are synthesised and often on the cells which lie in the surrounding area, the prostaglandins are regarded as local hormones (Smith, 1986).

Prostaglandin Synthesis

The first stage in prostaglandin synthesis involves the release of arachidonic acid from the membrane stores and the enzymes responsible for this are the phospholipases (phospholipase A_1, A_2, C and D) (Blackwell and Flower, 1983; Stryer, 1980; Dennis, 1987; Van den Bosch, 1980). Once free, the arachidonic acid can be metabolised via three pathways 1) by cyclooxygenase to prostaglandins, 2) by lipoxygenase to leukotrienes or 3) by epoxygenases to epoxides. The element considered in this thesis is the first of these.

The original naming of the prostaglandins was based on their partitioning into ether (PGE series) or into phosphate buffer (PGF series, from the Swedish *fosfat*) and the numbers that are included in the identification of prostaglandins indicate the number of double bonds contained in the carbon chains attached to the cyclopentyl portion of the molecules (Bowman and Rand, 1980). Figure 1.3 represents prostaglandin E_2. The E group of prostaglandins is now defined as those which possess a keto group at position 9 and a hydroxyl group at position 11.

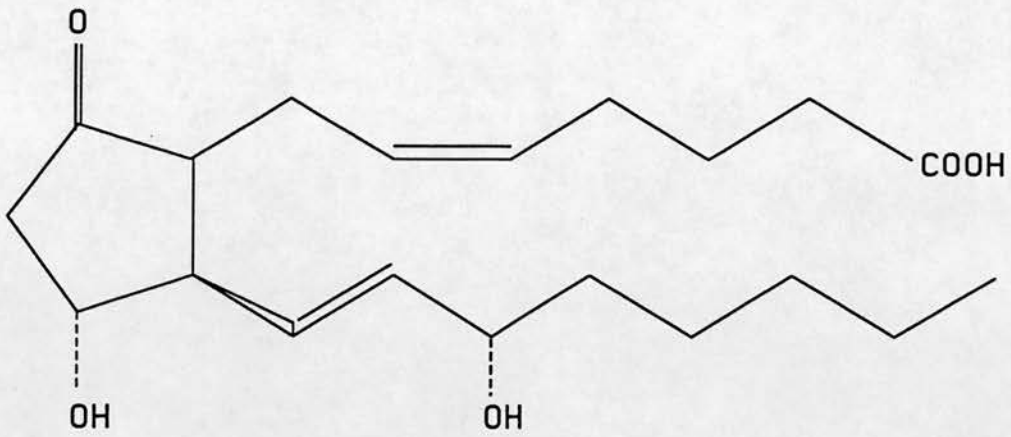


Figure 1.3 Prostaglandin E_2

Cyclooxygenase (prostaglandin endoperoxide synthase or PGH-synthase) converts arachidonic acid to the prostaglandin endoperoxide PGH_2 which is further metabolised to various different products by several enzymes (Smith, 1986; Kulmacz, 1987; Coleman et al. 1990). The prostaglandins that have been most important in cervical ripening are PGE_2 and $PGF_{2\alpha}$ (Keirse, 1979; Ulmsten, 1986; Calder, 1986), however, $PGF_{2\alpha}$ is much less potent than PGE_2 and would only be used in clinical situations where PGE_2 is unavailable (Calder, 1990).

PGH_2 is converted to PGE_2 by PGH-PGE isomerase and to $PGF_{2\alpha}$ by a reduction process that was originally thought only to occur without the involvement of an enzyme, enzyme inhibition or absence resulting in $PGF_{2\alpha}$ formation (Bakhle, 1983; Smith, 1986). It has been shown that $PGF_{2\alpha}$ synthesis does take advantage of enzymic catalysis also (Lin and Jarabak, 1978; Watanabe et al., 1985). PGH_2 can also converted to prostacyclin (PGI_2) and to thromboxane (TxA_2) by specific isomerases (Smith, 1986).

Prostaglandin Receptors

Prostaglandins act via receptors in cell membranes. Different receptors exist for each of the prostaglandins and several types can be found on the same cell (Feigen and Chapnick, 1979; Coleman et al., 1980; Coleman et al., 1984; Carsten and Miller, 1980; Kennedy et al., 1982; Kennedy et al., 1983). Once the PG is bound to a receptor a second messenger system is brought into operation, both the adenylate cyclase and the phosphatidylinositol pathways having been found to be involved (Kozawa et al., 1992; Elgendy and Hausman, 1990; Goureau et al., 1992; Toriyama et al., 1992). In fact, it has been shown that PGE_2 can activate both mechanisms and it has been proposed that there are two distinct PGE_2 receptor subtypes, one linked to each of these two second messenger systems (Kozawa et al., 1992; Goureau et al., 1992; Toriyama et al., 1992). $PGF_{2\alpha}$ appears to be linked to the phophatidylinositol pathway and both it and PGE_2 increase intracellular calcium ion levels (Goureau et al., 1992; Toriyama et al., 1992; Toriyama et al., 1990). Inhibition or stimulation of cAMP can occur depending on the tissue and the prostaglandin. Regulation of the action of PGs also depends on the up and down regulation of receptor numbers, the activity of other molecules in the cell membranes and on modification of intracellular calcium concentrations (Robertson, 1986).

Prostaglandin Degradation

In the main, metabolism of prostaglandins to their inactive metabolites occurs via the enzyme 15-hydroxy-prostaglandin dehydrogenase (PGDH) which reduces the hydroxyl group attached to the 15th carbon atom to a ketone group. This is regarded as the rate

limiting step in PG catabolism and results in the loss of most of the activity of the prostaglandins. The 15-keto-prostaglandin is then further reduced and oxidised by different enzymes (Hansen, 1976). The lungs liver and kidneys are rich in PGDH and prostaglandins in the circulation are metabolised on their passage through these organs (Bowman and Rand, 1980).

The Cervix and Prostaglandins

Clinical Experience

For some time the ripening effects of prostaglandins on the cervix have been recognised (Shepherd et al., 1976; Calder et al., 1977), first becoming apparent to obstetricians using them for the induction of labour at term. Since the mid 1970s there have been many reports of cervical ripening associated with the use of prostaglandins and they are now widely used in obstetrical and gynaecological procedures to promote cervical softening and dilatation (MacLennan and Green, 1979; MacKenzie and Embrey, 1977; Wiqvist et al., 1986; Ulmsten, 1988a; Elder, 1988; Lyndrup et al., 1991; Brindley and Sokol, 1988; Rath et al., 1982; MacLennan, 1981).

PGE_2 and $PGF_{2\alpha}$ are the prostaglandins used to improve the status of the cervix but PGE_2 is by far the more commonly adopted of the two because it is more potent, shows greater specificity and is toxicologically more acceptable (Calder, 1990; MacKenzie and Embrey, 1979; Brindley and Sokol, 1988). Much of the work on $PGF_{2\alpha}$ has been carried out by MacLennan in Australia because of legal restrictions on the use of PGE_2 (MacLennan, 1981).

The prostaglandins employed clinically are either synthetic analogues, such as Cervagem™ (16,16-dimethyl-trans-Δ^2-PGE, methyl ester), or the naturally occurring prostaglandins. Because of the metabolism of the endogenous prostaglandins analogues were developed to prolong their activity and allow lower doses to be administered. However, the use of prostaglandins for cervical ripening is restricted in term pregnancies to the natural forms because of a lack of knowledge with regard to the effects of the analogues on the

fetus. The synthetic derivatives are only used at term in cases of stillbirth.

Various routes of administration have been used over the years with varying degrees of success. In early work using prostaglandins for the induction of labour, the stimulant effect on the uterus was the desired effect. They were given intravenously, orally and as vaginal pessaries. Gastrointestinal problems (nausea, vomiting and diarrhoea), pyrexia and a painful inflammatory reaction at the site of the intravenous infusion were common side effects (Barr and Naismith, 1972; Karim and Sharma, 1971; Embrey et al., 1974). However, this procedure was intended to activate uterine contractions and was actually only useful in cases where the cervix was ripe. Current practice for the promotion of cervical ripening tries to limit the stimulation of uterine activity, ideally not provoking any response until the cervix is ripened. To this end the best route of administration seems to be the instillation of the prostaglandin endocervically (Calder, 1990; Ekman et al., 1983), however, because siting of the PG within the cervical canal and its retention there can be problematical (Ulmsten, 1988a) the vaginal route is often used. This requires a higher does of prostaglandin (Ekman et al., 1983) and because of greater systemic absorption may result in a slight increase in side effects (Ekman et al., 1983; O'Brien et al., 1986; Ulmsten et al., 1983).

Prostaglandin Production by the Cervix

It has been shown that the cervix does have the capacity for eicosanoid synthesis, *in vitro* at least. Ellwood and co-workers demonstrated that the ovine cervix was capable of prostaglandin synthesis (Ellwood et al., 1979) and then went on to show similar

findings in the human (Ellwood et al., 1980). They found that pregnant human cervix in a perfusion system produced PGE_2, $PGF_{2\alpha}$, PGFM (the metabolised form of $PGF_{2\alpha}$) and prostacyclin (PGI_2, as determined by stable metabolite levels) but little thromboxane. Another group, from Sweden, incubated human cervix with radiolabelled arachidonic acid and observed its conversion to PGE_2, $PGF_{2\alpha}$ and thromboxane but did not detect any prostacyclin metabolite. They did find evidence of the production of two unknown compounds which they claimed were higher in term pregnant tissue compared to first trimester tissue, but they found no correlation between either the unknown compounds and cervical ripening or the prostaglandins and cervical ripening (Christensen et al., 1985). Tanaka et al. observed, predominantly, prostacyclin synthesis by human cervical homogenates incubated with arachidonic acid and noted increased activity in postpartum tissue compared with nonpregnant (Tanaka et al., 1981). They also found small amounts of PGE_2 but no changes were detected. Wallis and Hillier measured PGE_2, $PGF_{2\alpha}$ and prostacyclin production by nonpregnant human cervix and found E and F to dominate, at similar levels, with a lesser degree of prostacyclin output (Wallis and Hillier, 1982). Although Ellwood found PGFM to be produced by the human cervix (Ellwood et al., 1980) there is no direct evidence, such as an immunohistological study, for the presence of PGDH in this tissue.

Human cervical strips have been shown to produce prostaglandins in response to passive mechanical stretching and the authors suggest that this could also be the case *in vivo* when uterine contractions pull the cervix (Hillier and Coad, 1982). Anderson and Turnbull showed that the uterus is contracting, whether or not the

mother is aware of it, several weeks before the onset of labour (Anderson and Turnbull, 1969). This provides a potential mechanism whereby the physical forces exerted by the uterus could stimulate prostaglandin production by the cervix and therefore play a part in prostaglandin induced remodelling of the cervical connective tissue.

Human cervix incubated with arachidonic acid has also been shown to produce HETEs (hydroxyeicosatetraenoic acid) (Saeed and Mitchell, 1982b; Flatman et al., 1986) products of the lipoxygenase pathway. However, it has been shown that their precursors HPETEs (hydroperoxyeicosatetraenoic acid) can also be produced by PGH synthase (cyclooxygenase) (Hecker et al., 1987). Other researchers have also established that HETE production occurs in human cervical biopsy material (Heidvall et al., 1992).

There is evidence that receptors for PGE exist in the cervix (Giannopoulos et al., 1985). It is not known whether the numbers increase at term but it has been suggested that women who do not respond to prostaglandin therapy may have a receptor deficiency (Ulmsten, 1988a).

The non steroidal anti-inflammatory aspirin is an inhibitor of prostaglandin synthesis and has been found to prolong pregnancy and labour in humans (Lewis and Schulman, 1973). Indomethacin, another prostaglandin synthesis inhibitor, has been shown to suppress contractions of the uterus provoked by stretching (Manabe et al., 1983). It has been suggested the habitual use of such agents during pregnancy, as reported by Collins and Turner (Collins and Turner, 1975), may be a contributing factor in cases of an unripe cervix at term (Calder, 1980). Ledger et al. found that inhibiting

prostaglandin synthesis with mefenamic acid prevented cervical ripening from taking place in the sheep (Ledger et al., 1984).

Prostaglandins in Amniotic Fluid

The production of prostaglandins by tissues closely allied to the cervix may also have an influential effect on its state. An association between PGE_2 and $PGF_{2\alpha}$ levels in amniotic fluid and the ripeness of the cervix has been observed by Calder, higher concentrations of the prostaglandins coinciding with a riper cervix (Calder, 1980). Other workers have also observed that arachidonic acid and prostaglandins appear in larger quantities in the amniotic fluid in the last stages of pregnancy. This is suggested to be due to an increase in PG synthesis rather than a decrease in catabolism since the level of the inactive metabolites as well as the active prostaglandins increases (Keirse et al., 1974; Keirse et al., 1977b; Keirse et al., 1977a; Reddi et al., 1984). There have also been reports of increased levels of lipoxygenase products in the amniotic fluid during gestation and in preterm labour (Romero et al., 1987a; Romero et al., 1987b; Romero et al., 1989d).

Study of amniotic fluid and its ability to influence prostaglandin synthesis has suggested that there are substances in it able to stimulate and inhibit PG production and that the occurrence of these compounds changes during pregnancy (Cohen et al., 1985; Rehnström et al., 1983; Saeed et al., 1982).

Prostaglandins from the Fetus and Membranes

Prostaglandins found in the amniotic fluid could be derived from the amnion and the chorion. The chorion possesses PGDH (prostaglandin dehydrogenase) activity to a greater degree than any

other tissue whereas the amnion has little or no capacity for enzymic prostaglandin metabolism (Keirse et al., 1978; Okazaki et al., 1981). Both have been found to produce prostaglandins and HETEs and though different researchers reported differing results it seems that PGE_2 is certainly produced by the amnion and the chorion, with some debate over prostacyclin (Dembélé-Duchesne et al., 1981; Casey et al., 1984; Skinner and Challis, 1985; Olson et al., 1983a; Christensen and Gréen, 1983; Saeed and Mitchell, 1982a).

The fetus itself is also a source of prostaglandins (Falkay et al., 1980). Fetal prostaglandins have been measured in the first voided urine of neonates (urine collected immediately following delivery, therefore produced *in utero*) and shown to be greater during labour than in caesarean sections in the absence of labour (Casey et al., 1983a). Human neonatal urine has also been found to contain inhibitors and stimulants of *in vitro* PG synthesis (Strickland et al., 1983; Casey et al., 1983b).

The placenta is another tissue which has been shown to be capable of producing cyclooxygenase and lipoxygenase derived products (Duchesne et al., 1978; Dembélé-Duchesne et al., 1981; Saeed and Mitchell, 1982a; Walsh et al., 1985; Mitchell and Grzyboski, 1987), as is the decidua (Okazaki et al., 1981; Mitchell and Grzyboski, 1987). Some work has suggested that prostaglandin production by decidual tissue may rise in labour (Skinner and Challis, 1985), however, other reports have not shown any differences (Okazaki et al., 1981; Olson et al., 1983b).

Summary

Thus the prostaglandins that are most commonly detected in significant quantities are PGE_2 and $PGF_{2\alpha}$ and these are probably the most important PGs in cervical ripening. The source of these prostaglandins is likely to be the cervix itself and cells of the immune system involved in the inflammatory response. However, the initiation of PG production may be provoked by the production of PGs by the fetus and fetal membranes, which may continue to contribute to PGs present in the cervix. The uterine body may also be a source of PGs for cervical ripening.

Mechanism of Prostaglandin Action on the Cervix

The effects of prostaglandins on cervical tissue have been studied in order to probe the mechanisms whereby they promote cervical ripening.

While the muscular content of the cervix is low it has been shown that it can respond to PGE_2 and $PGF_{2\alpha}$. Hillier and Karim found PGE_2 inhibited spontaneous contractile activity and observed inconsistent stimulatory and inhibitory responses to $PGF_{2\alpha}$ (Hillier and Karim, 1970; Najak et al., 1970). The significance of this is unclear; however, it is possible that the relaxing properties of PGE_2 may play a minor role in cervical dilatation.

The ripening effect of PGE_2 has been shown to be unaffected by the use of agents that suppress uterine contractions (Insull et al., 1989; Goeschen et al., 1985) which supports the findings that the uterine musculature is not an essential element in cervical ripening (Liggins, 1978).

It has been suggested that exogenously delivered prostaglandins stimulate endogenous prostaglandin production (Uldbjerg and Ulmsten, 1990). Gréen at al. observed uterine contractions in women following administration of $PGF_{2\alpha}$ to induce the termination of pregnancy and found that they continued and increased, even when the plasma levels of the prostaglandin were falling. They also looked at PGEM and PGFM plasma levels following $PGF_{2\alpha}$ administration and found both increased (Green et al., 1981). They suggest that early uterine activity, and presumably cervical ripening, were due to the administered prostaglandin and subsequently endogenous production took over. The inhibition of cyclooxygenase has been shown to increase the time between $PGF_{2\alpha}$ treatment and abortion (Souka et al., 1983), supporting the theory that stimulation of endogenous PGs takes place.

Prostaglandin Actions on Cervical Connective Tissue

Prostaglandins have been shown to affect the connective tissue of the cervix. Biopsies taken from first trimester human cervices before and after treatment with PGE_2 showed marked disruption of the regular collagen fibres only fifteen hours after intracervical application of the prostaglandin (Uldbjerg et al., 1981). These same workers also noted an increase in GAGs, and in vesicle enrichment and migration to the plasma membrane of fibroblasts. MacLennan's group (MacLennan et al., 1985) obtained similar results with $PGF_{2\alpha}$ in rabbits. They also observed the infiltration of "giant cells" which they were unable to identify but found they were multinuclear and stained basophilic on hematoxylin and eosin staining. This may be a different cell type to those found by Junqueira (Junqueira et al., 1980) and

Rath (Rath et al., 1988b), possibly indicating a general inflammatory cell influx.

The production of hexosamine-containing substances (GAGs/proteoglycans) by a rat fibroblast cell line was shown to increase under the influence of $PGF_{2\alpha}$ (Murato et al., 1977). Hyaluronic acid was the most abundant stimulated component found by these authors and therefore hyaluronic acid synthetase was examined. The activity of this enzyme was also seen to increase and the effect of $PGF_{2\alpha}$ was blocked by actinomycin D (blocks mRNA transcription), suggesting that the prostaglandin was inducing the enzyme.

A study by Cabrol et al. used hysterectomised and ovariectomised rats with the cervix left *in situ*. They found that treatment with PGE_2 caused an increase in hyaluronic acid in relation to the dry weight. This was also the case when the animals were given oestrogen and progesterone supplements. Additionally, they observed that there was an overall increase in the level of hyaluronate, heparan sulphate, dermatan sulphate and chondroitin sulphate in the animals on steroid supplements compared to the others (Cabrol et al., 1987). A later study by Cabrol's team, using intact, near term rats, examined the effects of a cyclooxygenase inhibitor, a lipoxygenase inhibitor and a dual cyclooxygenase/lipoxygenase inhibitor (Cabrol et al., 1990). All three resulted in an increase in the water content of the cervix, a factor indicative of cervical ripening. Inhibition of only prostaglandins resulted in an increase in hyaluronic acid, leading the authors to suggest that this indicated that lipoxygenase products may have been responsible for the increase. The sum total of all the GAGs was reduced in the presence of the dual inhibitor only, suggesting that

both cyclooxygenase and lipoxygenase derived material is involved in GAG control. They saw a drop in heparan sulphate levels when prostaglandin synthesis was inhibited but not lipoxygenase activity, and no effect on dermatan sulphate of dual inhibition but increased dermatan sulphate concentration when each enzyme pathway was inhibited selectively.

Norström found opposing effects of PGE_2 in first trimester tissue and term tissue (Norström, 1982). In early pregnant human cervical tissue PGE_2 promoted collagen synthesis but inhibited proteoglycan synthesis, and in later pregnancy collagen synthesis was reduced and proteoglycan synthesis increased.

Comparisons of postpartum cervical samples from 1) women with unripe cervices, given PGE_2, 2) women with unripe cervices but who spontaneously laboured and were not given PGE_2 and 3) women who spontaneously laboured and had ripe cervices, indicated that in the PGE_2 treated group the collagen content was similar to the spontaneously delivering women and lower than those with unripe cervices (Ekman et al., 1986). This work also appeared to show a higher level of non extractable collagen in the untreated, unripe cervices, possibly signifying a greater degree of connective tissue remodelling in the presence of PGE_2.

As mentioned previously cervical tissue can produce products of arachidonic acid metabolism other than prostaglandins. A study on the effects of arachidonic acid and prostanoids on collagen dissolution (*in vitro*) in human cervix explants, showed that addition of prostaglandins to the cultures had no effect on hydroxyproline release (a measure of collagen breakdown) into the culture medium but that arachidonic acid caused an increase. These authors found

that when they used high levels of arachidonate (10^{-5}M) there was no effect on collagen breakdown but they only examined the effects of PGs at similar, high concentrations and did not look at the effects of lower levels of prostaglandins. Inhibition of PG synthesis also inhibited collagen breakdown but did not affect the stimulatory properties of arachidonic acid. The amount of hydroxyproline released into the medium was reduced in the presence of a phospholipase inhibitor (Wallis and Hillier, 1982). These results suggest that while prostaglandins are involved, other arachidonate metabolites are probably acting within the cervix to promote the degradation of the connective tissue matrix.

Experiments with rats showed that treatment *in vivo* with $PGF_{2\alpha}$ or arachidonic acid, towards the end of pregnancy, both resulted in a greater degree of collagen solubility than measured in control animals (Hillier and Wallis, 1981). The increase in collagen solubility and the appearance of hydroxyproline in culture medium is thought to indicate increased collagenolytic/proteolytic activity.

In contrast, work on rabbit cervical fibroblasts provided evidence of a stimulatory action of PGE_2 and $PGF_{2\alpha}$ on collagenase activity but this did not show a similar response to arachidonic acid, both in the presence and absence of indomethacin (cyclooxygenase inhibitor). This paper also showed that prostacyclin did not have a significant effect on collagenase activity (Goshowaki et al., 1988). The lack of a response to arachidonic acid in these experiments may indicate a requirement for other cell types, such as macrophages or neutrophils, that may have been present in the explants of cervical tissue but absent from the fibroblast cultures. However, the purity of these fibroblast cultures is questionable since the cells were in

primary culture derived from rabbit cervix (Sakyo et al., 1986a) and not a cell line. The authors suggest that the lack of an effect of arachidonic acid and indomethacin indicates that endogenous prostaglandins are not involved in cervical ripening (Goshowaki et al., 1988).

Collagenase activity and DNP-peptide hydrolytic activity (DNP-peptide is a substrate for collagenase) have also been reported to be increased in samples of cervical tissue taken postpartum from women given PGE_2 compared to samples taken from women that were not given the prostaglandin (Uldbjerg et al., 1983c).

Rath's group could not detect an increase, or decrease, in collagenolytic or general proteolytic functions when they examined biopsies taken from human cervix that were pretreated with the PGE analogue sulprostone (Rath et al., 1987). Similarly, they could not find any change in collagenase or proteolytic activity in the cervices of guinea pigs after intracervical application of sulprostone (Rath et al., 1989). In both these publications the authors found no evidence of collagen breakdown products using electrophoretic analysis.

Uldbjerg observed an 80% increase in the activity of collagenolytic/proteolytic enzymes in human cervical tissue following treatment with a local application of PGE_2, however, they did not find any change in the level of elastase in the tissue (Uldbjerg et al., 1983c).

Since the process of cervical effacement and dilatation begins with the relaxation of the tissue nearest the uterine body, the endocervix, it is possible that the contradictions found experimentally lie, at least in part, in differing techniques for obtaining tissue. This could, for example, mean that as the dilatation progresses down the

cervix and more and more of the organ becomes integrated into the contours of the uterus, that samples obtained at a slightly later stage are in effect closer to the most active regions of the cervix and may themselves be becoming more active with regard to the ripening process. Thus some workers may find little or no evidence of some processes while others find them to be strongly active, simply because they have taken their samples from different parts of the organ.

Summary

From the work described it is evident that the cervix is capable of producing prostaglandins in its own right and that there is a link between the eicosanoids and cervical ripening. The cells responsible for PG production within the cervix have not been identified but the fibroblast must be the most likely source. The tissues surrounding the cervix also possess the machinery for eicosanoid synthesis, so a plentiful supply of endogenous prostaglandins is readily available to participate in the ripening process. The facts that cervical tissue has the ability to produce eicosanoids and that prostaglandins have been shown to have fundamental, restructuring effects on the connective tissue suggests that they are intimately involved in cervical ripening whether through direct or indirect mechanisms. An autocrine function is possible since fibroblasts are producers of collagen, GAGs and collagenase. The prostaglandin receptors must have some function lending support to a role for endogenous prostaglandins. The part played by other eicosanoids may also be crucial in cervical ripening.

Another question which is raised when studying cervical ripening is what the initial stimulus for increased PG synthesis is.

Since the fetus is the best judge of its maturity it is most likely to be a fetal product, related to gestational age and maturity.

The Cervix and Progesterone

Progesterone Withdrawal Hypothesis

Progesterone levels in women have been shown to rise as pregnancy develops, early in gestation the principal source being the corpus luteum and by the end of the first trimester production has shifted to the placenta (Buster and Simon, 1989; Liggins, 1983; Csapo et al., 1972). In some species the corpus luteum remains as the main source of progesterone and an essential element for the continuation of pregnancy, for example in rabbits, goats and rats (Liggins, 1983), its removal resulting in the ending of gestation.

In humans there is not considered to be a significant fall in serum progesterone levels prior to parturition (Anderson et al., 1985; Smit et al., 1984; Hanssens et al., 1985), despite findings showing such a trend (Turnbull et al., 1974) and the strong adherence to this idea by Csapo (Csapo, 1975). Turnbull's group observed an increase in the plasma oestradiol/progesterone ratio (Turnbull et al., 1974). The importance of progesterone in pregnancy maintenance is not disputed and has led others to suggest that local regulation of hormone levels may be involved. Such changes may be undetectable when studying peripheral blood levels (Milewich et al., 1977; Gibb et al., 1978; Mitchell et al., 1982). Some work has shown that a progesterone withdrawal effect may be produced locally via the production of oestrogen by placental and fetal tissue, which was found to inhibit the formation of progesterone (Gibb et al., 1978; Mitchell et al., 1982; Das et al., 1985; Townsley, 1975; Grimshaw et al., 1983). Challis et al. found progesterone synthesis capabilities to be greater in human chorion and decidua from women at term, not in

labour (caesarean sections), than in the same tissues from women with spontaneous, term labour (Challis and Vaughan, 1987). They did not find significant differences in the ability of amnion, chorion and decidua from the two groups of women to synthesise oestrone, but their results may indicate a slightly greater capacity in tissue from spontaneously labouring patients. Their results lend support to a paracrine regulatory mechanism rather than a systemic one.

Inhibition of progesterone synthesis in pregnant ewes, using inhibitors of 3β-hydroxysteroid dehydrogenase, has been shown to precipitate labour and delivery (Taylor et al., 1982) and to cause an increase in the level of $PGF_{2\alpha}$ without affecting oestrogen. In a study on cervical ripening in sheep Ledger et al. inhibited progesterone synthesis and observed an increase in prostaglandins E and F metabolites in blood drawn from the utero-ovarian vein and no change in oestrogen concentration (Ledger et al., 1984). They observed an increase in uterine activity and significant cervical ripening. Furthermore, they administered an inhibitor of prostaglandin synthesis to one group of animals, concurrently with the progesterone synthesis inhibitor, and found that prostaglandin levels did not rise to the same extent and that the change in uterine activity and cervical ripening did not occur either (Ledger et al., 1984).

A study on hormone levels in women undergoing induction of labour did not detect any difference in the presence of progesterone in the circulation between those with unripe and those with ripe cervices (Zuidema et al., 1986), nor in the ratio of progesterone to oestrogen.

Prostaglandin synthase (PGS) mRNA was examined in the endometrium of ewes. Throughout the oestrus cycle and in early pregnancy no differences were seen in PGS mRNA concentrations. In

ovariectomised animals treatment with oestradiol caused a fall in mRNA levels whilst progesterone did not. Treatment with both steroids did not cause a reduction in mRNA content either, so although progesterone did not appear to have any effect alone it did block the effect of oestrogen (Salamonsen et al., 1991).

Human cervical tissue in culture (nonpregnant) has been shown to release hydroxyproline into the medium (a measure of collagen breakdown) and this activity can be reduced by the inclusion of progesterone in the culture system (Wallis and Hillier, 1981).

Cervical cells from pregnant rabbits were found to produce an inhibitor of collagenase and progesterone added to the culture medium resulted in an increase in the output of this inhibitor (Sakyo et al., 1986b).

Rajabi et al found high concentrations of progesterone (10^{-3}-10^{-4}M) to inhibit collagenolytic activity in medium from cultured, pregnant guinea pig cervix cells (Rajabi et al., 1991), in agreement with other studies on progesterone (Halme and Woessner, 1975; Jeffrey et al., 1971). However, at lower levels (10^{-7}-10^{-9}M) they observed a stimulatory effect. The *in vitro* work mentioned previously used 10^{-6}-10^{-8}M progesterone. Procollagenase mRNA levels in the guinea pig cells were raised by as much as four fold in the presence of the lower concentrations of progesterone (optimum stimulation at 10^{-8}M), with no apparent effect at higher concentrations (Rajabi et al., 1991). In addition, this team found that the stimulatory effect of oestradiol on procollagenase mRNA could be blocked by the inclusion of progesterone (10^{-4}M) in the culture medium.

The state of pregnancy is that of an essentially foreign body inside the mother and there is therefore a need to suppress the

immune system to prevent rejection. Progesterone has immunosuppressive properties (Stites and Siiteri, 1983) and there is evidence that high local concentrations can reduce inflammation and rejection of grafted tissue (Siiteri et al., 1977) while systemic administration may not (Krohn, 1954; Medawar and Sparrow, 1956; Simmons et al., 1968; Watnick and Russo, 1968).

The lack of severe, general immunosuppression in pregnancy may indicate that there is local suppression of rejection by high concentrations of progesterone in the direct vicinity of the reproductive organs. Cervical ripening and parturition being regarded as inflammatory type processes (Liggins, 1981) lends support to this idea, since they would be suppressed by locally raised progesterone levels. The observation of progesterone withdrawal in some species and the theory that local hormone regulation may be crucial in humans fits in well with this, so that at term the immunosuppression would disappear and parturition would ensue, following the rapid softening and dilatation seen in the last stages of cervical ripening.

Progesterone withdrawal has been shown to permit the infiltration of the pregnant ovine uterus by leucocytes (Staples et al., 1983), a strong indication of an inflammatory reaction, and a similar infiltration has been observed naturally in the human cervix at term (Junqueira et al., 1980).

Evidence from the Progesterone Antagonist RU486

In recent years the development of drugs with actions antagonistic towards those of progesterone has led to the development and licensing of RU486 for termination of first trimester pregnancy.

There are several of these compounds, often called antiprogestins, and they are steroidal derivatives (Kelly, 1988), thought to reversibly antagonise the effects of progesterone at the receptor level (Baulieu, 1989; Baulieu, 1987). RU486 also binds to glucocorticoid receptors, with antiglucocorticoid effects, but has no affinity for mineralocorticoid receptors (Baulieu, 1989). Studies have shown the existence of progesterone receptors in the human cervix. The levels did not vary during the menstrual cycle (Sanborn et al., 1976; Sanborn et al., 1978). The antiprogestins have been shown to cause abortion in guinea pigs (Elger et al., 1987a; Batista et al., 1991), rats (Bosc et al., 1987; Garfield et al., 1987), and women (Baulieu, 1989; Couzinet et al., 1986; Ulmann and Dubois, 1989), with greater efficacy apparent in early pregnancy. Some of these steroids have been shown to make the uterus highly sensitive to prostaglandin treatment (Bygdeman and Swahn, 1985; Swahn and Bygdeman, 1988; Cameron et al., 1986; Haluska et al., 1987) and combinations of the two chemicals have been found to improve the treatment-expulsion time and to permit lower dosing of each than would be required if used alone (Elger et al., 1987a).

In cycling guinea pigs the corpus luteum is destroyed by $PGF_{2\alpha}$ (Horton and Poyser, 1973; Poyser, 1976) if conception does not occur, and serum progesterone levels drop. In guinea pigs given antiprogestins but not mated, the expected fall in serum progesterone at the end of the luteal phase did not occur (Elger et al., 1987a; Elger et al., 1987b) and it was suggested that the antiprogestins blocked stimulation of uterine prostaglandin production *in vivo* (Elger et al., 1987a). Others have observed a stimulatory effect of antiprogestins

on uterine prostanoid production *in vitro* using human and rat tissue (Kelly et al., 1986b; Kelly et al., 1985).

Apart from the use of RU486 as a method of medical termination of pregnancy, as opposed to surgical termination, there are now studies being carried out to determine its value as a method of postcoital contraception or a once a month contraceptive pill. In women given the antiprogestin on the day prior to the date of expected menstrual bleeding this has already been shown to result in suppression of β-hCG in 80% of women (Dubois et al., 1988; Ulmann and Dubois, 1989).

However, this thesis is concerned with cervical ripening and examining the ripening action of antiprogestins on this process (Chwalisz and Elger, 1986; Chwalisz et al., 1987; Frydman et al., 1988).

It has been shown that the human cervix becomes easier to dilate following RU486 treatment (Rådestad et al., 1988; Heidvall et al., 1992), with similar results in the rat (Stiemer and Elger, 1990) and guinea pig (Elger et al., 1987a). In the guinea pig the cervix was more compliant at 60 days gestation following antiprogestin treatment than it was at 44 days (Elger et al., 1987a), which may indicate the triggering of a process associated with the rapid changes seen in the cervix at term as opposed to those which are involved in the general remodelling in the earlier stages of pregnancy. The endocervical diameter has been shown to increase in early pregnant women pretreated with RU486 (Lefebvre et al., 1990), with no apparent influence of parity, which may again indicate that the antiprogestin activates/permits the mechanisms of rapid change to function in the cervix, since it has been suggested that the

restructuring of the cervix in earlier pregnancies may in some way be retained.

Stiemer and Elger observed a slight increase in the water content of the pregnant rat cervix (day 18) following antiprogestin treatment, similar to the levels they found in the untreated, late pregnant (day 20) rats (Stiemer and Elger, 1990). Cabrol, likewise, detected an increase in the water content of the rat cervix following RU486 treatment (Cabrol et al., 1991). Stiemer also observed a swelling of the cervix when examined histologically and considered there to be an increase in the percentage of collagen in the sections from animals treated with antiprogestin compared with the controls (Stiemer and Elger, 1990).

A histological study in the guinea pig using the antiprogestin ZK98.299 showed several similar changes in the cervical ultrastructure consistent with intrinsic cervical ripening (Hegele-Hartung et al., 1989). They observed an increase in oedema/ground substance associated with dissociation of the regularly arranged collagen fibres. Fibroblast activation was noted as was the infiltration and activation of leucocytes. Neutrophils, macrophages and mast cells were described and the neutrophils appeared to degranulate while the macrophages were rich in phagocytosed material. Their observations in late pregnant, antiprogestin treated animals were so similar to their control animals at term that they described them as one.

Experiments using pregnant guinea pig cervical cells in culture provided results opposite to those expected from the above work. They showed that progesterone caused a slight increase in collagenase activity and found that RU486 blocked this stimulation but also that

it reduced the collagenase activity to a level significantly lower than the control value (Rajabi et al., 1991).

The only significant difference in rat cervical GAGs was seen in the hyaluronic acid content, which showed a slight increase in RU486 treated animals compared with their control counterparts. Heparan, dermatan and chondroitin sulphates were also measured but did not show any changes under the influence of the antiprogestin (Cabrol et al., 1991). To examine the effect of prostaglandins on antiprogestin induced cervical ripening Cabrol et al. administered the cyclooxygenase inhibitor diclofenac to rats as well as RU486. They did not detect any effect of prostaglandin inhibition on RU486 induced hydration of the cervix or the increase in hyaluronic levels (Cabrol et al., 1991), but did find that in the dual treatment group there was a drop in the cervical concentration of heparan sulphate. However, they did find that the blockade of prostaglandin synthesis prevented the preterm delivery they observed following RU486 treatment alone. Chwalisz et al. gave guinea pigs the antiprogestin ZK98.299 plus indomethacin (prostaglandin synthesis inhibitor) and did not detect any interference with the ripening effect of the antiprogestin (Chwalisz et al., 1987).

The prostaglandin output of human cervical tissue *in vitro* was found to be unaffected by oral pretreatment with RU486 (Rådestad et al., 1990), and a study of plasma PGEM and PGFM indicated that their concentrations did not change in RU486 treated women either (Hill et al., 1990). Cabrol's work with rats and the dual treatment of antiprogestin plus prostaglandin synthesis inhibitor did not show up any effect of RU486 on serum PGE_2 or leukotriene B_4. They did show that the prostaglandin output was significantly reduced by diclofenac

and that the leukotriene was unchanged in its presence (Cabrol et al., 1991).

In terms of softening and dilatation of the cervix Stiemer and Elger found RU486 to be the most effective of the antiprogestins that they tested (Stiemer and Elger, 1990).

Recently, RU486 (mifepristone) was granted a product license for clinical use in the United Kingdom. It is licensed to be issued on a named patients only basis for the induction of medical termination and is used in conjunction with a prostaglandin. Further studies are being carried out to evaluate its usefulness as a method of contraception, for example, as a 'once a month pill' and for cervical ripening at term.

Summary

The immunosuppression produced by progesterone and the increased levels associated with pregnancy have led to the conclusion that it is important to the maintenance of the pregnancy. The termination of gestation by the antiprogestin RU486 supports this, despite the fact that circulating progesterone levels remain high in the human throughout pregnancy and do not fall at term as observed in other species. The softening of the cervix following treatment with RU486 suggests that progesterone is a significant factor in cervical ripening. The role progesterone plays is not clear, but it may act through inhibition of any of the mediators of an inflammatory response, such as the prostaglandins or any of the cytokines involved. It is also possible that progesterone has direct effects on the cells of the cervix. The steroid has been reported to affect collagenase production by the cervix which may indicate a direct action on the

fibroblasts. Progesterone may also have indirect effects on the cervix through actions on the myometrium, maintaining its quiescence and thereby reducing the mechanical stresses on the cervix which in turn may reduce stimulation of prostaglandin production.

The Cervix and Oestrogen

The serum levels of the oestrogens (oestrone, oestradiol-17β and oestriol) have been shown to increase during pregnancy (Buster and Simon, 1989; Turnbull et al., 1974; Tulchinsky et al., 1972). A distinct, rapid increase in amniotic fluid oestrogen levels has been claimed in the last two to three weeks of pregnancy (Challis and Olson, 1988). It has been shown that in primigravid women with a deficiency in placental sulphatase there is little serum oestrogen (France and Liggins, 1969), and that the pregnancy can be prolonged and cervical ripening may not take place (France et al., 1973). However, in multigravid women with the same sulphatase deficiency low serum oestrogen does not necessarily mean that cervical ripening and labour do not occur and these patients may proceed to a normal vaginal delivery. This has been explained by suggesting that some of the changes that take place in the cervix during previous pregnancies are retained in some way and permit normal functioning at term (Liggins, 1978), thus implying that the oestrogens' role is in the remodelling process that occurs throughout gestation. Treatment of pregnant macaques with dexamethasone, which inhibits the production of oestrogen, results in an increase in the length of gestation (Novy and Walsh, 1983). Also noteworthy is the fact that in hypertensive women , where oestrogen levels may be low, ripening of the cervix can occur prematurely (Liggins, 1978).

Several authors claim to have observed a ripening effect of oestradiol on the cervix or to have shown that it causes changes in cervical tissue consistent with the softening seen at term (Gordon and Calder, 1977; Pinto et al., 1964; Pinto et al., 1965; Tromans et al.,

1981; Gordon, 1981; Stewart et al., 1981). In the rat it has been associated with an increase in water content and weight of the cervix (Zarrow and Yochim, 1961), and in the sheep intravenous infusion of oestradiol has been shown to cause cervical softening (Fitzpatrick and Dobson, 1981).

Other researchers have found oestradiol to have no effect or to be inhibitory. Given vaginally, it did not promote ripening of early pregnant human cervix (Anthony et al., 1984) and two further groups could not find any benefit in the use of oestradiol with an unfavourable cervix (Thiery et al., 1978; Pedersen et al., 1981). Luther did not find that intramuscular administration improved the state of the cervix prior to prostaglandin treatment (Luther et al., 1980) nor did Williams' group observe any improvement with direct application to the cervix (Williams et al., 1988).

In the rat oestrogen caused inhibition of collagenase activity in the uterus (Woessner, 1979) but in culture it did not have any effect on enzyme biosynthesis (Jeffrey et al., 1971). Explants of human cervix were seen to exhibit a reduction in collagenolysis in oestradiol's presence (Wallis and Hillier, 1981). Rabbit cervical cells in culture produced greater amounts of a collagenase inhibitor under its influence (Sakyo et al., 1986b) but the same group also found oestradiol did not affect collagenase production or general protein synthesis (Sakyo et al., 1987). In guinea pigs, Rajabi and co-workers found that collagen breakdown increased in cervical tissue treated in culture with oestradiol (Rajabi et al., 1991). They also found that oestradiol could stimulate procollagenase production by guinea pig cervical cells (Rajabi et al., 1991). This effect was blocked by the cyclooxygenase inhibitor indomethacin and by the anti-oestrogen

tamoxifen. In addition, production of the mRNA molecule for procollagenase was shown to be stimulated by oestradiol when it was included in the culture medium of cells from day 50 pregnant guinea pigs (Rajabi et al., 1991).

Receptors for oestrogen have been found in the cervix (Sanborn et al., 1975; Sanborn et al., 1978), and this steroid has been shown to cause changes in the numbers of other membrane receptors, increasing the level of expression of those for oxytocin (Soloff, 1975; Nissenson et al., 1978; Fuchs et al., 1983b). Oestrogen may also raise α-adrenergic and reduce β-adrenergic receptor numbers (Roberts et al., 1981). In women there was no change in PGE_2 and $PGF_{2\alpha}$ receptors throughout the menstrual cycle but in ovariectomised hamsters given oestradiol supplements there was a decrease in prostaglandin receptors (Wakeling and Wyngarden, 1974). In ovariectomised ewes given oestrogen there was a reduction in the concentration of prostaglandin synthase mRNA in endometrial tissue (Salamonsen et al., 1991).

Indirect Actions of Oestrogen

Although uterine activity has been shown not to be necessary for cervical ripening to take place it is likely that the stresses it places on the cervix play some part in the process. Oestrogen has been shown to promote the synchronisation of uterine muscle activity by stimulating the formation of gap junctions between myometrial cells (Bosc et al., 1987). Prostaglandin production by human amnion and decidua has been shown to be stimulated by oestrogen (Olson et al., 1983a; Kelly and Abel, 1980; Kelly and Abel, 1981) and it has been suggested that oestrogen may cause uterine stimulation by diverting

PG synthesis from the inhibitory prostaglandin prostacyclin to the stimulatory prostaglandins such as PGE_2 and $PGF_{2\alpha}$ (Challis and Olson, 1988).

Oestrogen has been shown to inhibit the production of progesterone by human chorion (Mitchell et al., 1982) and it has been suggested that this could be important in altering the progesterone/oestrogen ratio, the two steroids having opposing effects to each other in the uterus (Challis and Olson, 1988).

DHAS and Oestrogen Synthesis in Pregnancy

The androgen dehydroepiandrosterone sulphate (DHAS) is produced by both the fetus and the mother (Mochizuki and Tojo, 1980) and converted to oestrogen by the placenta (Gant et al., 1971). At term the conversion of DHAS to oestradiol from each source is thought to be similar (Siiteri and MacDonald, 1966; Tulchinsky and Korenman, 1971), with the level of DHAS in umbilical cord blood (Parker et al., 1982) and maternal circulation increasing towards the end of pregnancy (Madden et al., 1976).

Plasma hormone levels in women undergoing induction of labour with ripe cervices have been compared to levels in women undergoing induction of labour where the cervix was not ripe and have shown that circulating oestradiol and oestriol levels were the same in each group, but that the level of DHAS was significantly higher in blood from women with ripe cervices (Zuidema et al., 1986).

In rhesus monkeys there is a rise in DHAS with gestation and this is augmented further near term (Serón-Ferré et al., 1983), similarly there is an increase in DHAS in baboon serum which shadows gestational age (Townsley and Pepe, 1977).

Over a prolonged period (2-3 weeks) Mochizuki administered DHAS to women, intravenously and on repeated occasions (Mochizuki and Tojo, 1980) and noted that cervical ripening in the treated women was improved when compared to the control subjects. His work also involved measuring hormone and collagenase levels and showed that oestradiol concentration increased in the cervix, to a greater degree than in the blood, and there was a significant increase in collagenase activity in the medium taken from cultured explants of cervix. DHAS has been shown to increase collagenase and gelatinase released from rabbit cervical cells in culture (Sakyo et al., 1986a; Sakyo et al., 1987; Mori et al., 1981) but the same effect was not observed with dehydroepiandrosterone (DHA) or oestradiol (Sakyo et al., 1986a). Sakyo also showed that DHAS and oestradiol did not affect collagen synthesis (Sakyo et al., 1987).

Interestingly, there appear to be a large number of specific DHAS binding sites in the plasma membrane of human cervical fibroblasts (Ohno et al., 1991) which may represent receptors for DHAS. The same paper claims that DHAS induces the mobilisation of the prostaglandin precursor arachidonic acid.

Summary

Thus, the oestrogens would appear to have some role in the mechanisms of cervical ripening probably through actions on the cervix itself where it promotes collagenase activity and collagen breakdown, but also indirectly by coordination of uterine muscle contractions and stimulation of prostaglandin production by the fetal membranes. It is not clear which of the oestrogens is most important but oestradiol and fetal DHAS must be contenders.

The Cervix and Relaxin

Cervical Ripening

Relaxin has also been implicated as a participant in cervical ripening. It is a polypeptide hormone first discovered in 1926 and identified in 1930 and considered to have a similar structure to insulin (Sherwood, 1988). In women it is produced by the corpus luteum, placenta and decidua (Weiss et al., 1976; Fields and Larkin, 1980b; Bigazzi et al., 1980), and levels are highest in the first trimester of pregnancy (Eddie et al., 1986). Unlike other species there is no surge prior to parturition in the human (Quagliarello et al., 1980).

Relaxin has been shown to cause softening of the pubic symphysis in guinea pigs (Frieden and Hisaw, 1951) and mice (Steinetz et al., 1965), and to cause cervical dilatation in several species with an apparent requirement that the tissue is first primed with oestrogen (Zarrow et al., 1956; Zarrow and Yochim, 1961; Fields and Larkin, 1980a). As a method of inducing cervical ripening in humans it was not studied until relatively recently because it was not available in a pure enough form, most work having been done with a crude extract of porcine ovary (Larkin et al., 1983). More recently porcine relaxin has been purified and used in clinical experiments in order to examine its effects on human cervix.

Much of the human work has been done by MacLennan (MacLennan et al., 1980; MacLennan, 1981; MacLennan et al., 1986). His groups found that women given purified porcine relaxin showed a greater improvement in cervical score than the controls over a 15 hour period. They observed that 2mg of relaxin, given vaginally, produced

similar ripening to 25mg of intravaginal $PGF_{2\alpha}$ and that intracervical application of relaxin provided no advantage over intravaginal application. Treatment of rabbits with vaginal gel containing $PGF_{2\alpha}$ or relaxin provided histological evidence that the relaxin significantly affected the connective tissue structure and that these changes in the connective tissue were similar to those seen under the ripening influence of the prostaglandin (MacLennan et al., 1985). The collagen bundles appeared to break down and the ground substance to increase.

Evans' group found that a 2mg dose of porcine relaxin, intracervically, resulted in a greater degree of ripening of the cervix than observed in control women, but a 4mg dose did not (Evans et al., 1983), the reasons for which are not clear. In pigs, relaxin has been shown to cause increased cervical extensibility and an increase in the wet weight (O'Day et al., 1989).

Hwang et al. observed the effects of administering a monoclonal antibody against relaxin to rats on days 20-22 of pregnancy. They found that the animals where relaxin had been inhibited by the antibody had lower cervical wet weight, dry weight and water content, and they were also less compliant than the control cervices (Hwang et al., 1989).

It is interesting to note that relaxin (porcine and human extracts) has been found to have a restraining effect on the uterus, apparently inhibiting contractions (McCarthy et al., 1957; Eichner et al., 1958; Downing and Sherwood, 1985; Szlachter et al., 1980). However, in a study on both human and rat myometrial tissue, recombinant human relaxin blocked the contractile activity of rat myometrium in an organ bath, in a dose dependent manner, but had

no effect on spontaneous or induced contractile function in human tissue (Petersen et al., 1991), which was in agreement with other findings (Decker et al., 1958; Kelly and Posse, 1956; McGaughey et al., 1958). The differing effects may be due to the relaxin extracted from tissue being relatively impure and therefore containing contaminants which caused the observed effects. It seems paradoxical that relaxin should inhibit uterine activity towards the end of pregnancy while at the same time promoting cervical ripening. However, in rats and pigs there is a drop in serum levels of relaxin following the peak near term which would allow uterine activity to increase (Downing and Sherwood, 1985; Gooneratne et al., 1983) and delivery to follow.

Mechanisms Involved

Relaxin has been found to inhibit the *in vitro* incorporation of tritiated proline by cervical tissue from women (Wiqvist et al., 1984), and this was taken to be an indication of reduced collagen synthesis. In ovariectomised rats given progesterone and oestrogen supplements there is an increase in collagen solubility associated with relaxin treatment, as well as increases in the total of individual GAGs in the cervix and a fall in the collagen concentration (Downing and Sherwood, 1986).

Relaxin treatment of guinea pigs has been found to increase the water solubility of proteoglycans in the pubic symphysis (Sherwood, 1988) and the charge density of connective tissue was reduced following relaxin treatment (Joseph et al., 1952), suggesting that there were changes in the proteoglycan structure. Hwang's experiments with a monoclonal antibody directed against relaxin also showed the

level of GAGs to be lower in the cervices from treated rats than from the control animals (Hwang et al., 1989)

Relaxin has been shown to activate mouse cervical fibroblasts (Leppi and Kinnison, 1971) and to increase proteolytic activity in the pubic symphysis, including that of collagenase (McDonald and Schwabe, 1982; Steinetz and Manning, 1967; Sherwood, 1988). Human amnion and chorion in culture have been shown to release increasing amounts of plasminogen activator and collagenase into the medium when incubated with relaxin (Koay et al., 1986; Koay et al., 1983). The infiltration of rabbit cervical tissue by unidentified giant cells occurred following treatment with relaxin which may be similar to the leukocytic infiltrate seen during labour (MacLennan et al., 1985).

Indomethacin has been shown to block relaxin induced reduction in cervical tone in rats, which implies a role for prostaglandins in its action (Kennedy, 1976)

The mode of action of relaxin is unclear but high affinity binding sites for the porcine derived material have been found in pig, guinea pig and rat cervical tissue (Mercado-Simmen et al., 1982; Gates et al., 1981; Weiss and Bryant-Greenwood, 1982). *In vitro* it has been shown to raise cAMP levels in rat and human cervix (Cheah and Sherwood, 1980; Norström and Wiqvist, 1985).

Obviously it would be preferable to study the effects of relaxin using the purified, human form of the protein. It has now been identified and is produced by Genentech. Currently there is relatively little information on the effects of recombinant human relaxin in humans and it may be some time before there are enough data to draw any conclusions about its role in cervical ripening and

parturition in women. However, such studies are now being carried out in Edinburgh, Glasgow and Oxford.

Summary

Relaxin extracts have been reported to promote cervical ripening clinically, biochemically and histologically. However, preliminary results from the recent clinical study in Edinburgh, using recombinant human relaxin suggest that it has no effect on cervical ripening (A Calder, Pers. Comm.). While this requires fuller examination it may indicate that the active agent in earlier studies was a contaminant or artifact of the extraction process.

The Cervix and Oxytocin

Oxytocin has been used in obstetrics for many years to induce and augment labour (Faris and Kahlenberg, 1954; Embrey and Anselmo, 1962). It is a neurohypophysial peptide hormone of nine amino acids (some regard it as an octapeptide because its two cysteine residues oxidise to form cystine). It is a highly potent uterine stimulant and lends its name to a class of drugs with similar uterine effects (oxytocic agents), including prostaglandins. The contraction of the uterus and the ejection of milk from the breast are the main functions associated with oxytocin (Bowman and Rand, 1980).

Receptors for this peptide hormone have been identified by several researchers in rat, sheep, rabbit and human uterus (Fuchs et al., 1983b; Crankshaw et al., 1982; Auron et al., 1984; Fuchs et al., 1982). Fuchs et al. have also provided evidence of their presence in fallopian tubes and cervix (Fuchs et al., 1984), the levels they reported in the cervix being less than 1% of those in the uterus. The uterine receptor concentration has been shown to rise during gestation (Fuchs et al., 1984), peaking during early labour (Fuchs et al., 1982; Fuchs et al., 1984). The sensitivity of the myometrium to oxytocin also rises during pregnancy (Bowman and Rand, 1980). Since, oestrogen has been shown to promote an increase in oxytocin receptor numbers (Soloff, 1975; Nissenson et al., 1978; Fuchs et al., 1983b) it is suggested that a shift in the oestrogen/progesterone ratio may be involved in permitting oxytocin derived contractions and increasing the sensitivity of the uterus to the hormone (Brindley and Sokol, 1988; Bowman and Rand, 1980).

Prostaglandins increase uterine sensitivity to oxytocin (Calder, 1990), reports having been made of $PGF_{2\alpha}$ potentiating oxytocin-induced contractions (Gillespie, 1972; Husslein et al., 1981; Fuchs et al., 1983a). In addition, there is evidence that oxytocin itself can stimulate the production of PGs by uterine tissue (Fuchs et al., 1982; Roberts et al., 1976; Mitchell et al., 1975; Fuchs et al., 1981; Husslein et al., 1981; Husslein et al., 1983).

Fuchs et al. studied plasma PGFM in women given oxytocin and found that in those in whom the induction was successful PGFM levels rose and the cervix dilated, but in those where it was unsuccessful the prostaglandin level did not rise, and despite uterine contractions the cervix failed to dilate (Fuchs et al., 1983a; Husslein et al., 1981).

The clinical use of oxytocin is primarily concerned with causing uterine contractions and it does not appear to have a direct effect on cervical ripening (Wiqvist et al., 1986; Valentine, 1977; Wilson, 1978; Auron et al., 1984). An *in vitro* study using human cervical tissue showed that oxytocin was able to cause contraction of the cervix, an effect that was reversed by the addition of PGE_2 to the tissue bathing fluid (Najak et al., 1970).

Any ripening of the cervix that does occur during the induction of labour using oxytocin is likely to be due to the increased myometrial activity rather than a direct effect of oxytocin on the cervix (MacKenzie, 1981).

Clinicians regard attempts to induce the birth process with oxytocin when the cervix is in an unfavourable condition as unwise (Calder, 1990; Owen and Hauth, 1992) and recommend the prior use of prostaglandins and amniotomy (Calder, 1990).

Summary

The function of oxytocin in the uterus is primarily on the myometrium, producing contractions. It may thus indirectly cause physical stresses in the connective tissue of the cervix and play a minor role in ripening the cervix. Oxytocin cannot be regarded as a primary inducer of cervical ripening.

Chapter 2

Techniques Employed in the Study of the Cervix

Techniques Employed in the Study of the Cervix

The Guinea Pig as a Model

The guinea pig was selected for the work presented in this thesis because of its similarities to the human in terms of its physiology during pregnancy.

This animal undergoes a luteoplacental shift whereby the production of progesterone, and therefore the continuation of pregnancy, does not depend on the corpus luteum (Elger, 1979; Illingworth and Challis, 1973; Heap and Deanesly, 1964), similar to women. In addition, the guinea pig also delivers its young in the presence of high circulating levels of progesterone (Hegele-Hartung et al., 1989; Elger and Hasan, 1985).

Pharmacologically the human and guinea pig show similar trends, oestrogen failing to cause abortion (Bacic et al., 1970; Elger and Hasan, 1985) and different prostaglandins showing similar potency profiles when used to induce the termination of pregnancy (Elger, 1979). The rat showed a significantly different abortifacient response to various prostaglandins when compared to the guinea pig while the rhesus monkey was similar to the guinea pig (Elger, 1979), which indicates that the rat may not be a good choice of model for comparison with humans. A primate model would probably be most analogous to the human situation, however, on a practical and cost basis this is inappropriate.

Another indication of the similarity among human, primate and guinea pig was shown by Rot who assessed the effect of human interleukin-8 on the chemotaxis of neutrophils from various species

(Rot, 1991). This work indicated that an optimal response from monkey and guinea pig neutrophils required approximately three times the concentration of human IL-8 needed with human neutrophils, but that rat cells required one hundred times that of the human cells.

Other Species and Techniques

Many different species have been used in experiments studying the cervix and the data obtained from them can give an insight into the processes that take place during the transformation of the cervix, but it should always be borne in mind that the human situation cannot necessarily be extrapolated from such studies.

Problems arise when the overall physiology of the animal in question is compared to that of the human and it becomes obvious that there are fundamental differences, such as the drop in plasma progesterone levels prior to parturition known to occur in sheep and rats (Taylor et al., 1982; Bassett et al., 1969; Stiemer and Elger, 1990), but which does not occur in humans (Anderson et al., 1985).

Even within the same species different approaches have been used. For example the guinea pig cervix has been studied by *in vivo* treatment with various drugs, such as steroids and prostaglandins and the effects assessed on the biochemical, physical and histological alterations they induce in the tissue. Other work has been done with primary cell culture or tissue culture techniques in order to examine the effects on cellular metabolism. These techniques have also been applied to other animals including humans.

Histological Characteristics

The pioneering work carried out by Danforth, examining the histological structure and delineation of the cervix, reopened the discussion about its intrinsic nature, muscular or fibrous, the former being the accepted option at that time (Danforth, 1947). Danforth's improved fixation and staining procedure indicated a predominantly fibrous organ and since then the cervix has come to be regarded as being fibrous in nature (Danforth and Evanston, 1954; Danforth, 1983; Uldbjerg and Ulmsten, 1990). The use of histological methodology, including immunohistochemistry, has been employed to reveal the underlying changes that occur during cervical ripening which permit the physical characteristics to alter so significantly. Such techniques are valuable tools in the elucidation of how the manipulations the clinician may carry out, physical or pharmacological, affect the structure of the cervix (Junqueira et al., 1980; Rath et al., 1988b; Yoshida and Manabe, 1990; Saito et al., 1981; MacLennan et al., 1985; Hegele-Hartung et al., 1989; Minamoto et al., 1987). One of the difficulties with such work is that the composition of the cervix is not uniform and changes as the uterine body is approached, the degree of musculature increasing with proximity to the corpus. This latter effect provoked heated literary discussions between Danforth and Hughesdon in the early 1950's (Hughesdon, 1952; Danforth and Evanston, 1954). Thus, the results obtained from material taken from a cervix for study may depend on the region from which it was dissected.

Physical Characteristics

The mechanical properties of the cervix are the most obvious indicators of ripening. Models employed in such avenues of research have involved the measurement of the physical characteristics of the cervix, how they can be affected by drugs and also how physical manipulations of cervical tissue can affect it. For example, work has been done looking at the ability of the cervix to contract (Najak et al., 1970). These authors detected contractile activity in strips of cervix in an organ bath, both spontaneous and drug induced. Conrad and Ueland (Conrad and Ueland, 1983) and Koob and Ryan (Koob and Ryan, 1980) have reviewed the ways in which some have attempted to quantify the changes in the mechanical attributes of the cervix that occur with pregnancy by examining the ability of isolated tissue (*in vitro*) to stretch and determining the point at which the resistance of the material to deformation is lost, and also by looking at the changes in elasticity of the cervix *in vivo* by assessing the degree to which it allows the passage of a measuring probe. Ledger et al. studied the effect of the contracting uterus on the mechanical functions of cervical ripening by surgically isolating the cervix from the uterine body while leaving both *in situ* in a pregnant sheep (Ledger et al., 1985), as did Stys et al. but, on this occasion, by subduing the uterine activity chemically (Stys et al., 1978). Stiemer and Elger (Stiemer and Elger, 1990) examined the forces required to dilate the rat cervix in their assessment of antiprogestins and endocrine hormones and their role in cervical ripening, as well as utilising histological methodology and thus combining more than one technique. Also in this area of cervical study there has been work done to examine the response of the cervix

to stretching (*in vitro*) in terms of prostaglandin production (Hillier and Coad, 1982).

Biochemical Characteristics

As well as the histological and physical studies of the cervix there have been studies concerned with the biochemical composition and biochemical output of the cervix at various different times, for example nonpregnant, early pregnant and late pregnant. In such studies different approaches have been used which serve to introduce further complications, making the analysis of the results difficult. This can be illustrated by the following examples. Some authors have assessed the degree of hydration of cervical tissue, an apparently simple measurement. However, on studying the literature there are differences in the way the results are reported, for example, as the lyophilised weight (Stiemer and Elger, 1990), as the oven dried weight (Uldbjerg et al., 1983b) or as the dried and defatted weight (Danforth et al., 1974). As well as water there are many reports on the amount of collagen in the cervix. The difficulties arise in this work because of the different processes used to extract the collagen. The degree of solubility of the collagen has been reported as its dispersal into acetic acid (Kleissl et al., 1978), into acetic acid plus digesting enzyme (Uldbjerg et al., 1983b), and into sodium chloride or guanidine chloride (Danforth et al., 1974). The picture is then obscured even more by the lack of consistency of authors in quoting the amounts of material they have detected, be it collagen or GAGs or other proteins, in terms of the wet weight of the tissue or the dry weight of the tissue. There have been studies that examined biochemical factors associated with tissue that had been sampled and examined immediately. There

have also been studies of cervical tissue maintained in culture for several days, looking at how differing culture conditions have affected the production of such substances. In addition, biochemical measurements have been made to assess the effects on tissue placed under physical stress while maintained *in vitro* for a few hours (Hillier and Coad, 1982), thus bringing together the two methodologies, mechanical and biochemical. Reports of collagenase output by explants of cervix maintained in culture have been made (Wallis and Hillier, 1981; Raynes et al., 1988a) but so have reports of collagenase extracted from biopsied material not sustained in culture (Rajabi et al., 1988; Rath et al., 1988a; Osmers et al., 1990; Kitamura et al., 1979), and to add further to the confusion there have been reports of collagenase activity in the medium of cultured cells derived from the cervix (Rajabi et al., 1991; Sakyo et al., 1986a; Goshowaki et al., 1988).

One of the problems of the experimental methods used is the sampling procedure. To obtain cervix tissue from women at term of pregnancy or in labour is ethically difficult and presumably not of great interest or significance to the delivering mother. The risk to the fetus also makes it unlikely that novel pharmacological manipulations will be carried out just for scientific research, the use of such intervention being restricted to cases where no alternative options are available or where a pregnancy is being terminated. Thus, human models for studying the cervix have most often been in nonpregnant women, early pregnant women or just after the birth, with severe limitations regarding the examination of the effects of drug treatments on cervical constitution prior to the disruptive passage of the fetus.

Cell Culture

The use of cell culture has become more and more popular in recent years, not least because of pressure to reduce the number of experiments on animals. The absolute value of such work, however, still requires to be confirmed in living creatures and we must not become solely reliant on it, since a living system rarely, if ever, operates in such an isolated manner, many factors influencing its normal functioning. As far as the cervix is concerned the first cell line that was ever developed was the HeLa cell line which was derived from the cervix of an American woman in the 1950s. Worth remembering is the fact that the very nature of a cell line is its transformed state which allows it to go on dividing continually, and that many, including HeLa, were produced from excised tumour material. The final point concerning cell lines derived from cervical tissue is that although there are several in existence there is a distinct lack of a line with its origins in the stromal tissue, those available being of epithelial origin and thus likely to be of limited use in studying the changes that occur in the connective tissue of the cervix.

Until a cell line is produced from stromal tissue and it retains the characteristics of its origins it would appear that the best lines of enquiry will involve human or animal material, both in primary culture systems or as part of the whole animal. Any results generated from cell lines will require to be confirmed using such alternatives as already outlined. Thus, there will always be a place in this type of research for animal and human models.

Current Understanding, Aims and Objectives

Thus in conclusion the cervix and cervical ripening are complex in nature. The cervix itself is composed predominantly of a connective tissue matrix consisting of collagen embedded in a network of proteoglycans. The main cellular component of this organ is the fibroblast, a collagen and glycosaminoglycan (GAG) producing cell type. There are also smooth muscle cells present but the numbers are so low that they are not thought to play a significant role in cervical function.

The nonpregnant cervix is rigid and closed, having a highly organised histological appearance where the collagen fibres tend to run in large parallel bundles held together by the proteoglycan network.

Cervical ripening is the process by which the normally unyielding cervical tissue softens to permit the passage of the fetus. Current thinking regards cervical ripening as an inflammatory based process. The histological changes are dramatic. The connective tissue becomes less regimented in its organisation and there is an infiltration of leucocytes. Collagen concentration in the ripening cervix falls as does the number of crosslinks between the fibres, thought to indicate the gradual replacement of older collagen with new collagen. The overall concentration of glycosaminoglycans increases. Those species of glycosaminoglycan which proffer less rigidity showing an increase while those which endow the connective tissue with greater tensile strength showing a reduction. An increase in the large, hydrophilic GAG hyaluronic acid is thought to increase the water content of the cervix. The leucocytes that infiltrate the cervix appear

to be the source of collagenase found in the ripening cervix, this enzyme being essential for the degradation of collagen.

Prostaglandins are undoubtedly important factors in cervical function, their ability to promote cervical compliance having been recognised in the 1970s. PGE_2 and $PGF_{2\alpha}$ are the prostaglandins in clinical use; PGE_2 is the most potent. It has been shown that the cervix itself is capable of producing prostaglandins and that inhibition of prostaglandin synthesis can prevent cervical ripening taking place. The presence of prostaglandins in the surrounding tissues (i.e. the uterus, fetal membranes and amniotic fluid) may also influence the cervix. PGE_2 and $PGF_{2\alpha}$ have been shown to produce histological and biochemical changes in the cervix consistent with cervical ripening, for example, increased GAG synthesis, alterations in collagen solubility (indicative of replacement of old collagen with new collagen which is amenable to rapid breakdown) and increased collagenolytic activity.

Progesterone is crucial to the continuation of pregnancy. In several species parturition is preceded by a fall in serum progesterone, however, in the human there is no significant reduction in circulating progesterone at term. Progesterone is considered to be an anti-inflammatory steroid and it is likely that the concentration of progesterone in and around the reproductive tissues is even higher than in the circulation during gestation. Progesterone has been associated with reduced collagen breakdown and the inhibition of progesterone synthesis has been found to result in increased prostaglandin production, as measured in the circulation and in blood drawn form the utero-ovarian vein in the sheep. Significantly, the development of drugs with actions antagonistic to those of

progesterone (antiprogestins) has led to the introduction of Mifepristone (in combination with prostaglandin therapy) for the induction of medical terminations. This drug acts on both the uterus and the cervix. It has been found to soften the human cervix. In animal studies antiprogestins have been shown to increase hydration of the cervix and increase hyaluronic acid production. Histologically the cervix takes on characteristics similar to those seen in natural cervical ripening. Following progesterone withdrawal Leucocyte infiltration of the uterus has been observed and antiprogestins have been linked to an increased presence of active fibroblasts and white cells.

Oestrogen levels in serum rise during pregnancy. This steroid possesses generally opposing effects to those of progesterone. While some workers have observed cervical ripening effects others have not, both in human and animal studies, and cervical ripening can take place in conditions where the serum oestrogen concentration is low. The role of oestrogen may be in the coordination of uterine contractions through the stimulation of gap junction formation and prostaglandin synthesis. Such uterine activity while not essential to cervical ripening is probably beneficial. DHAS levels rise at term, around half originating from the fetus. DHAS is converted to oestrogen by the placenta but it has been found to promote cervical ripening and stimulate the release of arachidonic acid through a direct action.

Relaxin causes softening of the pubic symphysis in guinea pigs and activates mouse cervical fibroblasts and enzyme production in the pubic symphysis. It has been shown to be involved in cervical ripening in several species but the results in human studies have been less

conclusive. The most recent studies, using recombinant human relaxin, have again found no evidence of a ripening effect of relaxin in humans (A Calder, Pers. Comm.).

The neurohypophysial peptide oxytocin, while an important tool to the obstetrician in stimulating uterine contractions and inducing delivery of the fetus, appears to have little or no direct effect on cervical ripening.

The most important factors in cervical ripening seem to be progesterone withdrawal and the action of prostaglandins. Related to these and to the perception of cervical ripening as an inflammatory process it is inevitable that other mediators of inflammation would be significantly involved in the changes that take place in the cervix.

The development of the antiprogesterone drugs has lead to the introduction of RU486 (mifepristone) for the induction of early terminations. This drug is now used in the UK in conjunction with PGE as the efficacy of the treatment is significantly improved by this combination. RU486 promotes cervical ripening and this project was designed to examine the relationship between progesterone and the prostaglandins, and to study other factors that may be important in cervical ripening and therefore influenced by the antiprogestin. To do this tissue from as near to term as possible was necessary and an animal model was required, the availability of human cervical material at this stage of gestation being very restricted. The guinea pig was chosen for this purpose because of its similarities to the human. The aim was to develop a culture system using cells derived from the guinea pig cervix using material from control animals and from animals pretreated with RU486. These cultures would then permit the *in vitro* manipulation of the environment in which the cells would be

maintained. Such manipulations would provide information on the capacity of the cervix to produce prostaglandins, the mechanisms involved and any influence RU486 may have.

The infiltration of the cervix at term by leucocytes reinforces the inflammatory nature of the ripening process and the interleukin IL-8 is a chemotactic factor likely to be involved as it possesses strong attraction and activation properties towards neutrophils. Therefore, it was intended that available human material would be used to produce tissue cultures to permit the examination of the ability of the human cervix to produce this chemokine. In addition, human cervical tissue would be used to study the immunohistological distribution of IL-8 in the cervix.

Chapter 3

Tools for the Analysis of the Involvement of Cytokines and Second Messengers in Cervical Ripening

Tools for the Analysis of the Involvement of Cytokines and Second Messengers in Cervical Ripening

This chapter reviews the cascades involved in inflammatory, and other, cell activation and in the actions of their products, including cytokines.

Accounts are given of agents that provoke or otherwise interfere with these cascades, thus making them useful experimental tools. They will be used to study the prostaglandins and their relationship with the cervix by incorporating them into the guinea pig culture system described in the next chapter.

Nonspecific Activation of the Immune System

Endotoxin is a lipopolysaccharide(s) (LPS) (Westphal, 1984) and represents a group of compounds composed of lipid and sugar residues found associated with the surface membrane of gram negative bacteria (Raetz, 1990). LPS nonspecifically activates the immune system (Morrison and Ryan, 1987) and activates the complement cascade (Vukajlovich et al., 1987). LPS has been found to provoke the release of prostaglandins and leukotrienes (Morrison and Ryan, 1987), interleukin-8 and other cytokines and inflammatory mediators, mainly from monocytes and macrophages but also from keratinocytes, endothelial cells and choriodecidual cells (Matsushima et al., 1992; Kelly et al., 1992; Strieter et al., 1989; Doebber et al., 1985).

The component of LPS that is responsible for its stimulatory actions is lipid A, a molecule unique to gram negative bacteria

(Galanos et al., 1985) and there are suggestions that some form of receptor for lipid A exists. The evidence for this relies on the ability of a naturally occurring form of lipid A (from *R. sphaeroides*) to act as an antagonist to LPS (Takayama et al., 1989) but not provoke a response itself (Strittmatter et al., 1983), and work showing that genetic mutations in mammalian cells can affect the response to LPS and lipid A (Morrison, 1986; Hopkins-Sibley et al., 1988). Some evidence exists which shows a saturable and apparently specific binding of a radiolabelled lipid IV_A probe, a precursor of lipid A produced in *E. coli*. These findings indicated that the probe binds to macrophages but not to fibroblasts (Hampton et al., 1988), however, the protein responsible for this association has not been identified. Because of competition for the binding sites observed between the lipid IV_A and acetylated-LDL (low density lipoproteins), which binds to a scavenger receptor (Brown and Goldstein, 1983), it has been suggested that this may in fact be a defensive mechanism whose function is to remove LPS from the circulation (Raetz, 1990). Specific LPS binding sites have been identified (Lei and Morrison, 1988). LPS is also claimed to activate G protein (Daniel-Issakani et al., 1989). Since all known situations where G protein activation occurs are via a membrane receptor linkage (Stryer and Bourne, 1986) this supports the existence of a specific LPS receptor (DeFranco, 1987). LPS is claimed to inhibit the enzyme adenylate cyclase through its stimulation of G protein (DeFranco, 1987).

Because of its fatty acid component it has been suggested that LPS may not act solely through a receptor but become an integral part of the cell membrane by inserting its hydrophobic region into the membrane (DeFranco, 1987), similarly to its natural position in the

bacteria from which it is derived. Such an action may allow it to interact with other molecules associated with the membrane and affect intracellular events.

While the details of how LPS stimulates cells are not well understood it is apparent that it can promote cellular activity. It has also been found, in some instances, to have a priming effect on cells allowing them to respond to phorbol esters by making certain proteins more susceptible to phosphorylation by protein kinase C (Rosen et al., 1989; Aderem et al., 1988). It is suggested that lipid A in some way mimics an endogenous mediator or directly affects a signal transduction cascade having been found to activate phosphatidylinositol turnover and protein kinase C and to affect G protein (Rosoff and Cantley, 1985; Wightman and Raetz, 1984; Daniel-Issakani et al., 1989). One difficulty associated with the receptor molecule being the same or related to that of acetylated LDL is that acetylated LDL does not possess the same actions as LPS (Raetz, 1990), however, neither does the lipid A molecule from *R. sphaeroides* which appears to bind to a receptor and block the effects of LPS.

So, it is apparent that LPS has a stimulatory activity in cells of the immune system but less clear is such activity in other cell types, particularly the main cell type found in the cervix, the fibroblast. It is also clear that LPS is able to induce the release of inflammatory mediators such as prostaglandins and cytokines.

The Protein Kinase C Pathway

PKC is dependent on calcium and phospholipids for its phosphorylative action (Takai et al., 1979a) and is activated endogenously by diacylglycerol (DAG), following cell surface receptor binding and activation of the phosphatidyl inositol signal transduction system (Takai et al., 1979b; Kishimoto et al., 1980). DAG has been found to increase the affinity of PKC for calcium making an increase in intracellular calcium levels unnecessary for its activation (Kishimoto et al., 1980). Phosphatidylserine is essential in PKC activation (Kaibuchi et al., 1981; Takai et al., 1979b). Synthetic forms of DAG are also capable of inducing PKC activation *in vitro*, bypassing the membrane receptor molecules and the breakdown of phosphatidyl inositol. The DAG molecules are thought to intercalate into the cell membrane (Kaibuchi et al., 1982; Kaibuchi et al., 1983; Kishimoto et al., 1980).

Phorbol myristate acetate (PMA) belongs to a group of similar compounds (phorbol esters) which have tumour promoting properties and are often used to stimulate cells in culture.

Phorbol esters are thought to act at the cell surface (Nishizuka, 1984) and have been shown to activate the enzyme protein kinase C (PKC) (Castagna et al., 1982; Yamanishi et al., 1983).

The phorbol esters have also been shown to be dependent on the presence of calcium and phospholipid in order to bind to and activate PKC (Kikkawa et al., 1983; Ashendel et al., 1983; Sando and Young, 1983; Leach et al., 1983). In intact cells the phorbol esters are thought to intercalate into the membrane and mimic DAG, having a similar structure (Nishizuka, 1984). Unactivated PKC is a soluble

enzyme, however, when intact cells are treated with phorbol ester and the cells disrupted the PKC is found strongly associated with the membrane (Nishizuka, 1984; Kraft et al., 1982).

Thus the phorbol esters are thought to interact with protein kinase C, resulting in its activation and the propagation of cellular signal transduction via this pathway. The measured effects of such an action may give an insight into the mechanisms which control those factors endogenously.

Protein Dephosphorylation

Phosphatases are intracellular enzymes which control processes as diverse as metabolism, cell division, contractility and membrane transport and secretion. They are classified as protein phosphatase 1 and protein phosphatase 2, depending on their preference for the α or β subunit of phosphorylase kinase, then further subdivided primarily by their dependence on divalent cations (Cohen, 1989). Their function is to remove phosphate groups from other intracellular proteins, the removal of phosphate often resulting in a change in the function of the protein in question from its active state to its inactive state or vice versa (Cohen, 1989; Alberts et al., 1983).

Okadaic acid is a relatively new tool in biochemical analysis. It was first isolated from marine sponges, from which it derives its name (*Halichonria okadaii*) (Tachibana et al., 1981), and has been a major cause of gastrointestinal disturbances through the consumption of contaminated shellfish.

This molecule has a polyether fatty acid structure and was found to promote tumour growth in mouse skin (Cohen et al., 1990). It has been shown to be an inhibitor of protein phosphatase activity (Takai et al., 1987; Bialojan and Takai, 1988).

Okadaic acid is a specific inhibitor of protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) (Bialojan and Takai, 1988; Haystead et al., 1989), and has been found to posses little or no effect on other phosphatases and protein kinases, including protein kinase C (Takai et al., 1987; Bialojan and Takai, 1988; Haystead et al., 1989; Suganuma et al., 1988).

The mechanism through which it exerts its effects is unclear but it is hydrophobic and thought to enter cells relatively easily, and it has been suggested that okadaic acid may interact with the C-terminus of PP1 and PP2A rather than the catalytic centre (Cohen et al., 1990; Bialojan and Takai, 1988).

Thus okadaic acid is a tool which may be used to examine the role protein phosphorylation plays in cellular functioning and the effects attributable to exogenously applied materials.

Calcium Influx

Calcium is an important factor in many physiological processes including neurone membrane depolarisation, the efficient functioning of enzymes, muscle contractions and as a second messenger in membrane receptor signal transmission.

Most of the available evidence shows a stimulatory effect of the calcium ionophore A23187 on the production of prostaglandins (Hsu and Goetz, 1993; Ibe and Raj, 1992; Houmbard et al., 1991; Cabre et al., 1993). This molecule causes an increase in intracellular calcium concentration. However, experiments carried out using human tissue (fetal membranes/placenta) and A23187 revealed an inhibitory effect of the ionophore (R Leask, Pers. Comm.). The role of calcium in prostaglandin production therefore required further investigation and in addition, the role of calcium on the effects of the other materials employed in this study were examined.

The name ionophore was the generic term given to molecules that bind cations and transport them across membranes (Pressman et al., 1967). There are families of ionophores with differing selectivity towards the biologically active cations and A23187 belongs to the family of carboxylic ionophores (Pressman, 1976). This particular ionophore has a specificity for divalent cations (i.e. Ca^{2+} and Mg^{2+}) over the monovalent cations (i.e. Na^{+} and K^{+}) (Reed and Lardy, 1972; Pfeiffer et al., 1974) and will not therefore alter the balance of sodium and potassium ions across the plasma membrane (Pressman, 1976). The action of A23187 is thought to depend on its reduced form being lipid soluble and able to pass across the lipid bilayer of the cell. When it reaches the edge of the membrane the polar environment results in

the loss of the hydrogen ion and traps the ionophore in the polar-nonpolar interface. Calcium in the intercellular fluid binds to the ionophore which changes its properties and permits it to move back into the polar membrane. It can then cross to the internal surface of the cell membrane where the calcium is released and replaced with hydrogen which restarts the cycle (Pressman, 1976). Thus A23187 will swap two H^+ ions for each Ca^{2+} (Alberts et al., 1983). A23187 will also allow the flux of calcium ions from membrane bound intracellular stores and the cytoplasm (Reed and Lardy, 1972).

Indomethacin

Prostaglandins were discussed in Chapter 1. Their synthesis depends on the enzyme cyclooxygenase, also known as prostaglandin synthetase, and its catalysis of the conversion of arachidonic acid to the prostaglandin endoperoxide PGH_2. Therefore the role of prostaglandins or stimulants of their synthesis can be examined through the inhibition of the enzyme cyclooxygenase.

Indomethacin is a non steroidal anti-inflammatory drug (NSAID). This group of drugs are well known for their anti-inflammatory, antipyretic and analgesic properties and includes aspirin (acetylsalicylic acid) and ibuprofen (Bowman and Rand, 1980).

Indomethacin is a potent inhibitor of cyclooxygenase and therefore prevents the production of prostaglandins (Bowman and Rand, 1980). Indomethacin is over one hundred times more potent, with regard to cyclooxygenase inhibition, than aspirin (Horuk, 1994).

Summary

The following table (Table 3.1) summarises the action of the drugs already described (LPS, PMA, OA, A23187 and indomethacin).

Table 3.1 Summary of drug actions

LPS	G-protein activation. Activation of immune system cells.
PMA	Protein kinase C activation via substitution for DAG (DiAcylGlycerol).
Okadaic acid	Phosphatase inhibitor resulting in maintenance of phosphorylated state of cellular proteins.
A23187	Increases free calcium by transporting calcium into the cytosol.
Indomethacin	Inhibitor of PGH-synthase (cyclooxygenase) resulting in inhibition of PG synthesis.

Cytokines

Cytokine is the generic name given to molecules such as the interleukins, tumour necrosis factors and interferons. They are chemical mediators which generally act close to their site of synthesis to coordinate immune/inflammatory responses to tissue damage or the invasion of the host by foreign antigens. Their influence on the cervix is of interest as it may provide further evidence that inflammatory processes could be the mainstay of cervical ripening.

Interleukin-1

IL-1 is a polypeptide of approximately 31,000 Da (Daltons) which is cleaved to give an active molecule of 17,500 Da. It exists in two forms IL-1α and IL-1β (Dinarello et al., 1973; March et al., 1985). IL-1β is the predominant form measured in blood and body fluids, while IL-1α appears to be embedded in the cell membrane (Dinarello, 1988; Kurt-Jones et al., 1985). Although the two show only 26% amino acid homology in the human they are both composed of β-folded sheets (Dinarello, 1988) and are both recognised by the same IL-1 receptor (Kilian et al., 1986; Dower et al., 1986). There is one section of the molecules, where the amino acid homology is high, that is thought to contain the receptor recognition site (Dinarello, 1988).

IL-1 is produced by a variety of cell types including fibroblasts, lymphocytes, macrophages/monocytes and polymorphonuclear cells. It is a major player in inflammatory reactions and was originally called endogenous pyrogen because of its ability to cause fever (Dinarello, 1988; Atkins, 1960). It is also produced by cells in the brain and is

thought to promote sleep (Dinarello, 1988), presumably accounting for tiredness associated with illness and activation of the immune system. Interleukin-1 has many and varied actions (Tabibzadeh, 1991b; Dinarello, 1988), mostly concerned with inflammation and tissue repair (Krane et al., 1985; Evêquoz et al., 1984; Dayer et al., 1986).

IL-1 has been shown to stimulate prostaglandin E synthesis by rat glomerular mesangial cells and human lung fibroblasts (Pfeilschifter et al., 1989; Elias, 1988; Elias et al., 1987). The rat cells also showed increased phospholipase A_2 release (Pfeilschifter et al., 1989). IL-1β was more potent than IL-1α in stimulating both PGE_2 synthesis and PLA_2 in rat mesangial cells (Pfeilschifter et al., 1989), but in lung fibroblasts the effect of each on prostaglandin output was similar (Elias et al., 1987). Prostacyclin synthesis by human endothelium was stimulated by both forms of IL-1, again to a similar degree (Endo et al., 1988).

Interleukin-1 and the Reproductive Tract

Interleukin-1 is thought to stimulate collagenase and proteolytic enzymes production by rabbit cervical cells (Goshowaki et al., 1988; Ito et al., 1987) and by other cell types (Krane et al., 1985; Evêquoz et al., 1984; Dayer et al., 1986).

IL-1 has also been shown to have chemotactic and activating effects on cells of the immune system (Dinarello, 1988; Hunninghake et al., 1987; Klempner et al., 1978; Klempner and Dinarello, 1979; Sauder et al., 1984), however, there is now evidence that this cytokine has the ability to stimulate the production of another interleukin, IL-8, (Larsen et al., 1989b; Matsushima et al., 1988; Strieter et al.,

1989) which exerts strong chemotactic/activating functions, particularly on neutrophils, and that such properties attributed to IL-1 may in fact have been the result of contamination by IL-8 (Peveri et al., 1988; Matsushima et al., 1988). Streiter et al. claim to have observed no significant chemotactic activity with culture medium containing recombinant IL-1β (Strieter et al., 1989), and Yoshimura's group reported similar findings using purified, natural IL-1 (α and β forms) and their recombinant counterparts (Yoshimura et al., 1987a). Hyaluronic acid in the cervix has been shown to increase during pregnancy (Fosang and Handley, 1988) and it has been reported to stimulate IL-1 production by human monocytes (Hiro et al., 1986). Hyaluronic acid has itself been found to have a chemotactic effect on neutrophils as has the exudate from hyaluronic acid conditioned monocytes. The authors suggested that the monocytes released a neutrophil chemotactic factor into the conditioning medium (Mazzone et al., 1986). This may have been IL-1, which subsequently stimulated IL-8 release.

The proinflammatory actions of interleukin-1, whether direct or indirect, may be of importance in the process of cervical ripening if it is assumed to be an inflammatory type reaction.

Human menstruation has also been likened to an inflammatory reaction, showing similar characteristics (Tabibzadeh, 1991b). The presence of IL-1 in plasma has been reported to vary during the menstrual cycle and to peak in the secretory phase (Cannon and Dinarello, 1985). IL-1 mRNA has also been identified in reproductive tissue, both mouse and human uterine material showing its presence with *in situ* hybridisation (Takács et al., 1988; Kauma et al., 1990). A bioassay identified the production of IL-1 by decidual cells (Romero et

al., 1989c) and this cytokine has also been found in amniotic fluid (Tamatani et al., 1988). Endometrial epithelium and stroma has been shown to possess specific binding sites for interleukin-1 (Tabibzadeh et al., 1990; Tabibzadeh, 1991a).

Interestingly, there is evidence that IL-1 is produced by placental tissue obtained after vaginal delivery in much greater quantities than placental tissue from elective caesareans (Taniguchi et al., 1991). IL-1β was the predominant form measured in culture medium in both groups. This suggests that IL-1 may be also be involved in labour. The same group found a seventeen fold increase in interleukin-1 output by tissue derived from women in labour but where intrauterine infection was present. It has been suggested that microbial invasion of the uterus may be involved in the precipitation of preterm labour by the instigation of an inflammatory response (Taniguchi et al., 1991; Uldbjerg and Ulmsten, 1990; Romero et al., 1988; Lamont et al., 1990; Romero et al., 1989a; Romero et al., 1989b), the many factors produced, including prostaglandins and cytokines, resulting in parturition.

This obviously supports the idea that cervical ripening is an inflammatory based process, and there is evidence of interleukin production by cervical tissue. Ito et al. claimed to have found IL-1 production in cervical explants from pregnant rabbits but no such protein synthesising activity in nonpregnant animals (Ito et al., 1988). They also showed that the IL-1 they isolated was capable of stimulating collagenase production by rabbit cervical fibroblasts and proposed that interleukin-1 was involved in cervical ripening and dilatation.

Tumour Necrosis Factor-alpha (TNF)

Tumour necrosis factor is another polypeptide that is associated with inflammation and immunoregulation (Tabibzadeh, 1991b). It is synthesised as a 26,000Da protein and processed to a mature form with a molecular weight of 17,000Da (Jue et al., 1990). It is also often referred to as cachectin or given the suffix "α", and is related to lymphotoxin-α (also known as TNFβ) (Male et al., 1987). The two tumour necrosis factors were shown, using a human cervical carcinoma cell line, to share the same receptor (Aggarwal et al., 1985). Where the acronym TNF is used in this text it refers to TNFα.

Although TNF was originally thought to be a macrophage product there is now *in situ* hybridisation and immunohistochemical evidence that it may be produced by other cell types, such as smooth muscle (Barath et al., 1990), monocytes (Sariban et al., 1988; Ruco et al., 1989), neutrophils (Vilcek and Lee, 1991), breast tissue (Spriggs et al., 1987), lymphoid tissue (McCall et al., 1989) and epidermal cells (Oxholm et al., 1988).

The name tumour necrosis factor may be slightly misleading since this molecule has many other biological functions and they have been found to overlap with those of IL-1 (Balkwill and Burke, 1989; Le and Vilcek, 1987), such as the stimulation of granulocytes, B-lymphocytes and T-lymphocytes and inducing fever.

The complex network of cytokines involved in inflammatory-/immuno-modulation shows areas of overlap, as mentioned above, but there also appears to be cooperation between different components of this system resulting in synergistic responses of greater magnitude than their simple sum.

Fibroblasts have been shown to produce PGE_2 in response to TNF (Dayer et al., 1985), and a combination of both IL-1 and TNF was shown to stimulate PGE_2 production to a much greater extent than anticipated by adding their effects together (Elias et al., 1987; Elias, 1988; Elias et al., 1988a). However, Elias also noted that fibroblast proliferation was inhibited by the two cytokines in combination, despite each individually stimulating proliferation (Elias, 1988; Elias et al., 1988a). He found that inhibition of prostaglandin synthesis blocked the inhibitory effects of IL-1 + TNF treatment (Elias, 1988; Elias et al., 1988a) and suggested that the response of the cells may indicate some involvement of these cytokines in the regulation of fibrotic activity in inflamed tissue. This work also indicated that the cytokines in question were not causing cell death. Pfeilschifter et al. also observed PGE_2 stimulation in rat mesangial cells treated with TNF, plus an increase in the activity of phospolipase A_2 (Pfeilschifter et al., 1989). They also observed that the combination of IL-1 and TNF resulted in greater stimulation than their individual effects added together.

Prostacyclin synthesis by endothelial cells in culture has been shown to increase following treatment with TNF (Endo et al., 1988; Kawakami et al., 1986), and Endo et al. observed an additive increase in prostaglandin synthesis when IL-1 and TNF were combined in the same culture medium (Endo et al., 1988).

Fibroblasts have also been shown to produce collagenase in response to TNF (Dayer et al., 1985) and to increase their synthesis of glycosaminoglycans (Elias et al., 1988b). The GAG study found that hyaluronic acid (HA) was the predominant molecule but also found chondroitin sulphate, dermatan sulphate and heparan sulphate. TNF

appeared to increase HA synthesis and decrease the production of the other GAGs, possibly by promoting HA synthetase activity. Indomethacin inhibition of prostaglandin synthesis did not affect the GAG response to TNF (Elias et al., 1988b), thus indicating separate control mechanisms.

As with IL-1, TNF has been shown to promote the synthesis of mRNA for the chemotactic/stimulatory cytokine IL-8 (Matsushima et al., 1988; Larsen et al., 1989b; Strieter et al., 1989)

TNF and the Reproductive Tract

In terms of reproductive biology the analogy of menstruation to an inflammatory reaction (Tabibzadeh, 1991b), and similarly parturition, has led to studies of TNF in reproductive tissue. TNF has been found in human placental and decidual supernatants, as well as in amniotic fluid and decidual tissue itself (Casey et al., 1989; Jäättelä et al., 1988). Uterine tissue was shown to contain TNF mRNA and placental cells to posses receptors for the cytokine (Tabibzadeh, 1991b; Eades et al., 1988). Thus, TNF's presence in such tissues supports a role for it in reproductive physiology possibly through inflammatory style processes that regulate menstrual function and the mechanisms of parturition.

Interleukin-8

IL-8 is a 72 amino acid peptide belonging to a supergene family of peptides with potent leucocyte chemotactic properties. It has been placed within the chemokine subgroup of the cytokine group of mediators (Tanaka et al., 1993; Lindley et al., 1993). Structurally it comprises a β-sheet and two α-helices (Clore and Gronenborn, 1992). The family of proteins to which it belongs includes platelet basic protein/connective tissue activating protein/β-thromboglobulin (PBP/CTAP/β-TG), platelet factor 4 (PF4), growth regulated gene product (GRO), and macrophage derived inflammatory protein-2 (MIP-2), (for a review see Baggiiolini et al. (Baggiolini et al., 1994) Baggiolini and Sorg (Baggiolini et al., 1992), Matsushima and Oppenheim (Matsushima and Oppenheim, 1989)).

The literature shows that interleukin-8 was initially identified by several separate laboratories and given a different name by each based on the experiments they carried out. The cells used to produce the IL-8 were usually stimulated to do so with lipopolysaccharide and some of the alternative names assigned to IL-8 include, Monocyte Derived Neutrophil Chemotactic Factor (MDNCF) (Yoshimura et al., 1987b), Neutrophil Activating Factor (NAF) (Peveri et al., 1988) and Monocyte derived Neutrophil Activating/Attracting Protein/Peptide (MONAP/NAP) (Schröder et al., 1987). Another peptide, T-Lymphocyte Chemotactic Factor (TCF), has also been shown to have the same amino acid sequence and properties as IL-8 (Larsen et al., 1989a). Since it appears to be a multipotent compound, acting on several cell types, agreement was reached that this compound should be renamed interleukin-8 (IL-8) (Westwick et al., 1989).

A receptor for interleukin-8 has been shown to exist and this shows the binding characteristics of a single receptor type (Samanta et al., 1989; Grob et al., 1990). However, another group has reported the detection of a high and a low affinity binding site for IL-8 on polymorphs (Besemer et al., 1989). Different authors have claimed both a highly significant binding of IL-8 to red blood corpuscles (RBCs) (Darbonne et al., 1991) and negligible binding of IL-8 to RBCs (Besemer et al., 1989). Evidence is now available that the Duffy blood group antigen is in fact the reported receptor for IL-8 on RBCs (Horuk, 1994). The prospect of RBCs sequestering IL-8 in the blood stream is attractive since this would limit the activation of neutrophils to the site of IL-8 production and also help to maintain the concentration gradient that is considered essential to its chemotactic properties.

Several human cell types have been shown to produce IL-8, among them monocytes/macrophages (Yoshimura et al., 1987b), dermal fibroblasts and keratinocytes (Larsen et al., 1989b), endothelial cells (Strieter et al., 1989) and possibly more relevant to this thesis, chorion and decidual cells (Kelly et al., 1992). IL-8 has been detected in amniotic fluid, greater quantities in fluid from women at term, in labour than women at term but not in labour (Romero et al., 1991). Romero's group also noted a further increase in amniotic fluid IL-8 levels in term pregnant women with bacterial infections detected in their amniotic fluid (Romero et al., 1991). Bacterial endotoxins have been shown to inhibit the chemotactic effect of IL-8 on neutrophils (Bignold et al., 1991), however, greater numbers of neutrophils have been observed in the amniotic fluid of women in whom microorganisms were detected in the same fluid (Romero et al., 1991).

As its various original names implied, interleukin-8 attracts neutrophils and also causes them to release the contents of their granules. The *specific* granules, which contain collagenase, are those stimulated to become active by IL-8, however, it has also been shown to stimulate the release of elastase from the azurophil granules, under certain conditions (Peveri et al., 1988). The importance of IL-8 in cervical ripening has not been examined before, however, the view that leucocyte collagenase is crucial in the breakdown of cervical collagen (Osmers et al., 1992; Rath et al., 1988b) implies that some factor must cause the observed leucocyte infiltration of the cervix (Junqueira et al., 1980; Minamoto et al., 1987) and degranulation of those leucocytes. Interleukin-8 presents itself as an excellent candidate and there is now some evidence that a chemotactic substance is produced by rabbit cervical cells, probably fibroblasts (Uchiyama et al., 1992).

Interleukin-8's effects are thought to involve increased intracellular calcium (Thelen et al., 1988; Bacon et al., 1989), an inhibitory effect on cAMP and the activation of protein kinase C (Bacon et al., 1989).

There is evidence that other cytokines may be involved in the regulation of IL-8 synthesis. In particular, interleukin-1 and tumour necrosis factor were shown by Matsushima et al. (Matsushima et al., 1988), Larsen et al. (Larsen et al., 1989b) and Strieter et al. (Strieter et al., 1989) to cause rapid induction of IL-8 mRNA expression. Additionally, IL-8 and prostaglandin E_2 have been found to act cooperatively, PGE_2 reducing the concentration of IL-8 required to cause neutrophil invasion of rabbit skin (Colditz, 1990).

If cervical ripening is regarded as an inflammation based process, then the cytokines must be an integral part of that system and IL-8 is an attractive candidate for a central role therein.

The Medical Research Council is currently pursuing the patent rights for interleukin-8 with regard to its use in the promotion of cervical ripening.

Overview

The process of cervical ripening is complex. The structural changes require the coordination of the degradation and rebuilding of the connective tissue, and this appears to rely on an inflammatory type activity. The fibroblasts become active and produce collagen and GAGs to replace the old highly structured material. They can also produce the collagenase essential for the breakdown of collagen. Similarly the cervix is infiltrated by polymorphonuclear cells stimulated to produce the only two enzymes capable of initiating the demise of the collagen molecule, collagenase and elastase. These cells also contain other proteases that are able to complete the digestion of the collagen molecules and also activate latent forms of collagenase. The factors controlling cervical ripening are likely to be common mediators of inflammation such as the prostaglandins and the cytokines. These factors may be produced by constituent cells, such as the fibroblasts, and by recruited leucocytes. An auto/paracrine system may then be set up, under the ultimate control of hormonal mediators produced by the fetus and related to the gestational age, culminating in the rapid removal of the resistance of the cervical connective tissue to dilatation as parturition progresses to the delivery of the child.

This thesis examined the prostaglandins and the regulation of their production in the cervix, including the possible effects of progesterone and the cytokines IL-1 and TNF, and the production and regulation of the chemokine interleukin-8.

Chapter 4

Materials and Methods

Materials and Methods

The reagents mentioned in this chapter are contained in appendix I at the end of this thesis.

The Guinea Pig Study

Preparation of the Cervical Cells for *in vitro* Culture

The Guinea Pigs

Pregnant Duncan Hartley guinea pigs were sacrificed at forty nine to fifty two days gestation (mean ± se, 50.4 ± 0.3 days). The length of gestation was determined from the date of the previous litter in conjunction with the weight change of the animal on a weekly basis. Full term gestation in these animals is 65 days. Those animals assigned to be treated *in vivo* with the antiprogestin RU486 were given a standard injection of 10mg in 0.1ml ethyl oleate 24 hours prior to being killed. The animals were killed by cervical dislocation.

Dissection

The dissection table was prepared by cleaning with disinfectant solution and swabbing with 70% isopropanol (IPA). Several layers of paper towelling were spread on the work surface and sprayed with IPA. The guinea pig was weighed (1470 ± 32g) and placed on its back on the dissection table, the abdomen was cleaned of any loose material and sprayed with IPA.

Using equipment pretreated with disinfectant (Shield, as directed by manufacturer) and washed with IPA the skin was removed

from the guinea pig abdomen and the exposed muscle sheet cleared of any fur. The muscle was cut and the abdomen covered with aluminium foil. The uterus was removed through a hole in the foil and a cut was made through the vagina ensuring that the cervix was above the line of the incision.

The lower part of the uterus, including the cervix, was removed and placed in 50ml cold tissue collection buffer (see later).

The remaining part of the uterus was opened and the fetuses decapitated to ensure they were dead. The fetuses and the placentae were counted and weighed. The mean (± sem) weight of the fetuses was 184 ± 14g and of the placentae 22.4 ± 1.4g (n=21).

In a class II tissue culture cabinet the cervix was washed in fresh PBS and the extraneous tissue removed using scissors. The upper limit of the cervix was taken to be the point at which the septum separating the two uterine horns began (see figure 4.1).

Enzyme Based Cervix Cell Dispersal

Trypsin Pre-Digestion

The minced cervix was placed in a trypsin solution for 15 minutes at room temperature with occasional mixing by shaking or pipetting. The tissue was then centrifuged at 1400g (2,500rpm, Sorvall RT6000B) for 10 minutes (4°C) and the supernatant discarded.

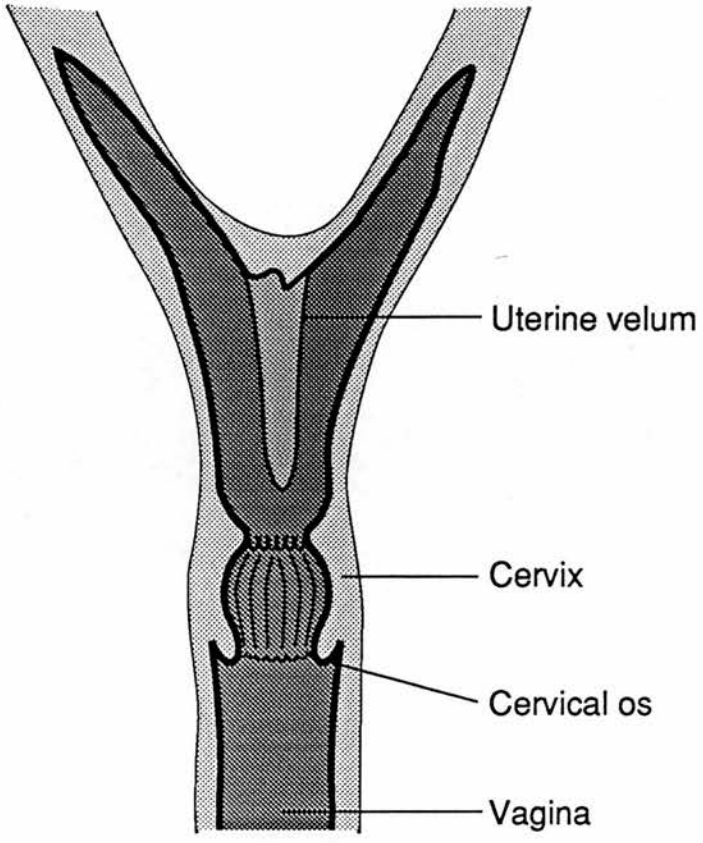


Figure 4.1 Diagrammatic representation of the guinea pig cervix.

The mean (± sem) weight of the cervices was 268 ± 24mg (n=7). The cervix was then cut longitudinally using a scalpel blade and chopped into a mince with scalpel blades to be enzymatically/mechanically dispersed. The dispersal process was based on that of Rajabi et al. (Rajabi et al., 1991).

Main Digestion

After resuspending in 50ml prewarmed collagenase/DNAse digestion medium the tissue was incubated at 37°C for 60 minutes. At intervals during the incubation the tissue was forced through a large bore pipette, several times, in order to dislodge any cells that were loosened by the action of the enzymes. This accelerated the process of dispersal and reduced the exposure time of the cells to the digesting enzymes.

After an hour at 37°C the digesting tissue mixture was filtered through a 150μm sterile nylon mesh. The enzymes in the cell containing fraction, which passed through the mesh, were inhibited by the addition of approximately 5ml FBS and centrifuged at 2,500rpm for 20 minutes (4°C). The cell pellet was resuspended in 5-10ml complete medium and kept on ice. The tissue captured by the filter was placed in fresh, prewarmed digestion medium (50ml) and incubated for a further hour as described previously. This process was repeated until all the cervical tissue had been dispersed, usually three, one hour long, incubations were sufficient.

Cell Quantification and Viability

The resuspended cell fractions were combined and the number of cells quantified using a haemocytometer. The average (± sem) number of cells released by this process was $22 \times 10^6 \pm 1.3 \times 10^6$ (n=16). Trypan blue exclusion was used to assess cell viability, which was always greater than 80% in the cultures used.

Guinea Pig Cervix Culture Development

Initial attempts at producing cultures of guinea pig cervical cells were based on the idea that the main cellular component of the cervix is the fibroblast, and that fibroblasts are relatively easily grown, being renowned for contaminating cultures of dispersed tissues where they are not the desired cell type but become the predominant one.

In the first instance the cervix of a guinea pig was finely minced using scalpel blades then incubated in complete medium at 37°C (95% air/5% CO_2) for several days. The flasks were then examined for signs of cellular outgrowths, emanating from the small pieces of tissue. This method did show promise in the early stages and isolated groups of cells grew in the culture vessels. After two weeks there were 30 x 10^6 cells in total spread out in several flasks. These cultures were split by trypsinisation in an attempt to stimulate replication. They were also plated out at 10^6 and 5 x 10^5 cells per well in cluster plates precoated with collagen to determine whether or not this would improve cell adherence and thus also encourage growth, however, this offered no improvement over the uncoated polystyrene. The cells were maintained in culture flasks for several weeks but they failed to divide enough to produce confluent cultures suitable for experimental use.

Further attempts at maintaining guinea pig cervical tissue in culture were based on both mechanical and enzymatic dispersal, until the method finally used in the main experiments was developed.

Following on from the first method the cervix was again cut into small pieces using scalpel blades but then treated with a digestion medium containing collagenase and DNAse at 37°C for 40 minutes. This technique was based on a digestion process used successfully by

others in the Centre for Reproductive Biology (R Kelly, Pers. Comm.). This technique was based on the method published by Jones et al. (Jones et al., 1989).

The cells that were released from the cervix by this process were permitted to grow in a culture flask in complete medium and early results were encouraging as more cells seemed to adhere to the culture flasks than with the previous method. Visually the cells were fibroblast-like. Again, the difficulty encountered with this method arose from the fact that although the cells were viable they did not produce confluent cultures. Variations of this technique were employed where the digestion time was increased to 60 minutes and mechanical dispersal was encouraged by introducing continuous stirring with a magnetic stirrer. This made little difference to the results.

Since the methods described above were not producing enough cells from the initial stages a Virtis 23 homogeniser was tested. This piece of equipment basically consisted of a steel blade on the end of a rotating spindle that pulverised the minced cervix into a soup of exceptionally small fragments. A sonic homogeniser was avoided for the purposes of this work since it would have destroyed the cells and it was hoped that the cellular content of the cervix would remain intact by using the Virtis homogeniser.

Because the soup produced by this method was already a fine suspension of tissue no enzymes were used. Instead of trying to produce cultures from which cells would be propagated, the cell clumps were seeded directly into culture plates. Since there had only been sparse adherence of cells previously different substrates were tried in the wells in an effort to find a better growing surface. A

protein blotting membrane, polypropylene capillary matting and a synthetic lens tissue were used. The rationale was that a different surface may have been more attractive to the cells and especially with the capillary matting that the cells would have become attached within the fine mesh and become better established. This would at least have allowed these cultures to be used experimentally. However, this method did not produce good results, the homogenisation possibly being too violent and destroying too many cells, suggested by the presence of many small fragments that were clearly not cells.

The next obvious step was to reduce the damage to the cells caused by the homogenisation procedure, and this was done by reducing the speed of the rotor and the length of time the cervical tissue was exposed to it. Also at this stage the enzyme mixture was reintroduced (40 minutes, 37°C). The mixture was agitated during the digestion process and any undigested material was further digested in fresh digestion medium. This did prove to be slightly more successful as cells were found but still there remained many small unidentified fragments and the different substrates did not offer any improvements over the polystyrene surface of the culture dishes themselves.

Abandoning the homogenisation completely, because of the damage caused to the cells and the poor results, and placing the minced cervix in a trypsin solution prior to the main digestion proved to be a highly successful alteration. From such experiments it was evident that to digest the minced cervix completely would have taken 4-5 hours and would presumably have been extremely harsh on the cells, thus the removal of the released cells from the digestion mixture and replacement of the undigested material in fresh digestion medium

reduced the proteolytic damage, almost eliminated the mechanical damage and gave excellent yields of cervical cells (approximately 20 x 10^6 cells per cervix). Another advantage of this method was that the cultures were each representative of the cervix as a whole and were not confined to fibroblasts, so any resident macrophages or other white blood cells would be included in the experiments that followed. Because the process was relatively severe, very likely to have removed some of the surface molecules from the cells, and because the cells were likely to have been stimulated to produce prostaglandins, at least by the mechanical disruption, they were left to recover for 65-70 hours.

This last method was adopted for the guinea pig cultures employed in the work for this thesis and is described in greater detail elsewhere.

Guinea Pig Cervix Cell Culture Used Experimentally

Plating Out of Cells

The dispersed cells were seeded into 48 well culture plates (Costar) at 10^5 cells per well in a volume of 1ml complete medium. The 48 well format allowed for 8 different treatments per plate with 6 replicates of each. The plates were made of polystyrene, each had a lid and was described by the manufacturer as of low evaporation design which should have improved the accuracy of the system.

Progesterone Effect

Because of the high levels of progesterone in the pregnant animal part of the study was designed to examine the role of

progesterone. Therefore approximately half the total number of wells were seeded with complete medium containing progesterone at a concentration of 10^{-6}M. This was done as soon as possible in order to reduce the time during which the cells were in a progesterone free medium to the minimum possible.

The culture plates were incubated at 37°C in a 95% air/5% CO_2, humidified atmosphere for approximately 65-70 hours. This allowed time for the cells to adhere to the polystyrene dish and to recover from the relatively harsh digestion procedure. Figure 4.2 shows how the cells looked at this stage under an inverted microscope.

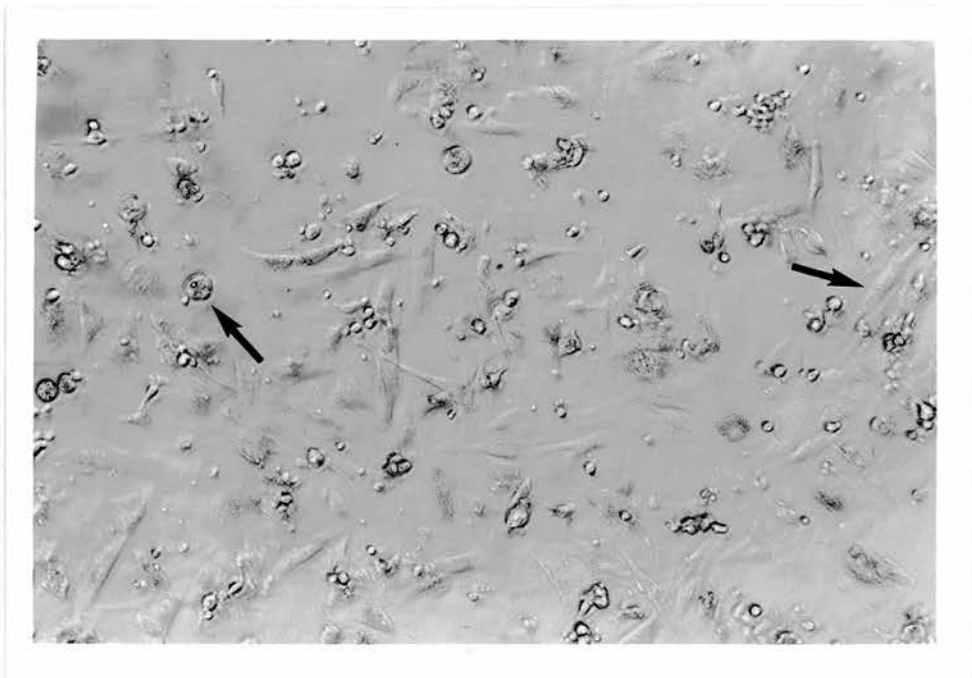


Figure 4.2 Guinea pig cervix cells in culture. The arrow on the right indicates an example of a fibroblast, the arrow on the left indicates an example of another cell type present in the cultures.

They appear to adhere well to the plates. Fibroblast-like cells constitute the majority, however, other types are evident. Some clumps of cells are also present although which type they are and the reason for clumping is not clear. It may be that the digestion did not separate all cells, leaving small numbers together which went on to produce small colonies within the whole culture.

Experimental Procedure

At the end of the recovery period the cells were used in experiments to examine the effects of various treatments on the production of prostaglandins. The medium was removed from each well and the cells washed with 1ml sterile PBS (Northumbria Biologicals). The PBS was removed and replaced with the medium containing the compound being tested (treatment medium, 1ml/well). The culture dishes were then returned to the incubator and a humid, 95% air/5% CO_2 atmosphere at 37°C for a further 24 hours. The treatments for cells seeded in 10^{-6}M progesterone were made up in medium which contained progesterone.

Each of the primary cultures comprised a control set of wells where nothing was added to the medium, a control for the progesterone containing wells where only progesterone was added to the medium and a variety of different treatments (6 wells each).

Cells Lost By Washing

The number of cells lost by the removal of the original medium and the washing process was examined by counting the number of cells in the fluid removed from the wells. This fluid was centrifuged as the original preparation had been and the number of cells found to be equivalent to approximately 10^4 cells lost per well.

Cells Remaining at the End of the Experiment

To determine the number of cells in the final cultures, after experimentation, the wells were washed with PBS and trypsin used to free the cells from the culture dish and each other. This was not particularly successful as many cells remained attached to the culture plates which meant that the cells counted did not represent the numbers in the wells. This continued to be the case even when a strong solution of trypsin plus EDTA was employed. Attempts to remove the cells from the plate mechanically were not successful. Using a plastic scraper the surface of the well was scraped but there were still cells apparently adhering to the plastic. This made assessing cell numbers difficult, however, 7-8 x 10^4 cells were released per well by these methods. While the actual number of cells in each well was probably higher an accurate assessment was not possible.

Sample Storage

Following the experimental treatment incubation period the medium was harvested. Half of it was mixed with methyloximating solution (0.5ml medium + 0.25ml methyloximating solution) and the methyloximation of the prostaglandins in the sample allowed to take place at room temperature, overnight (samples treated in this way

were said to have been 'moxed'). The moxed samples were stored at 4°C. The remainder of the medium was frozen and stored at -20°C. All the samples from these cultures were stored in 1ml polypropylene tubes in racks in a 96 well format and each tube was capped (Alpha Laboratories).

Standardisation of Assayed Values of Prostaglandins

Estimation of the quantity of prostaglandins present was carried out in the following way. The samples were assayed in aliquots and for the purposes of this example a 20µl aliquot will be assumed. The absolute figure obtained from the assay was a measure of the quantity of PG (in picogrammes) in the aliquot (20µl). This was a fraction of the moxed sample of 750µl (500µl medium plus 250µl MOX), introducing a multiplication factor of 37.5 (750µl/20µl) to give the amount of PG in 750µl moxed medium (which is the same as that in 500µl of the unmoxed medium). To relate this to the total volume of treatment medium, that is 1000µl, a second multiplication factor of 2 was used (1000µl/500µl). So, in practice the absolute figure was multiplied by 75 to give a measure of picogrammes of prostaglandin per 1000µl per 10^5 cells, using the seeding value for the number of cell present. Where different aliquot volumes were used the multiplication factors were adjusted accordingly.

The Human Study

The aim of this study was to examine prostaglandin and interleukin-8 production *in vitro*, by cells derived from the cervices of women at different reproductive stages and following treatment with and without prostaglandin and RU486 therapies.

Cervical Tissue

Human cervical explants were obtained from five groups of women. Three groups were from pregnant women undergoing surgical termination of pregnancy (including women treated with prostaglandin or RU486), one was from postmenopausal women hospitalised for dilatation and curettage to investigate uterine bleeding, and the fifth group of tissues were obtained from premenopausal women undergoing hysterectomy where there was no disease of the cervix. The pregnant and post menopausal women's cervices were biopsied using a Tru-Cut™ needle. This produced plugs of cervix about 10-15mm in length and 2mm in diameter (approximately 10-15mg). The hysterectomy tissue was obtained at operation and cervical tissue not required for pathological examination was available for study. Tissue samples were collected in saline at 4°C by the operating theatre staff.

The division of the tissue was as follows,

Group 1 was from women given the prostaglandin E analogue Cervagem™ (16,16-dimethyl-trans-Δ^2-prostaglandin E_1, methyl ester) prior to surgical termination. Cervagem was given as a 1mg pessary 2-

3 hours before surgery to aid cervical dilatation. These women were nulliparous.

Group 2 was from parous women not given cervagem pretreatment before surgical termination.

Group 3 was from the hysterectomy patients. These women were premenopausal and were not pregnant. They were at various stages of the menstrual cycle and there were insufficient data to examine any stage specific differences.

Group 4 samples were from the postmenopausal women being treated for unexplained uterine bleeding.

Group 5 were from women attending for surgical termination that agreed to pretreatment with the antiprogestin RU486 48 hours before the operation. Difficulties were encountered in obtaining needle biopsies from these patients. A possible explanation is that the cervix, which was not as rigid as in the other groups because of the effect of the RU486, was more likely to slide round the needle rather than into it, thereby often making the biopsies inadequately small.

Results from one of the human cervices were excluded because of discrepancies in the tissue weights. A further biopsy was discarded because bacterial contamination was evident after 24 hours incubation. Such contamination is not unexpected in cervical tissue as there are several opportunities for exposure to microorganisms to occur. An obvious occasion would be between removal of the tissue and dissection in the tissue culture cabinet while the other main source of contamination would be from a vaginal infection.

This study had the approval of the local ethics committee. RU486 (Mifepristone) is only available in the United Kingdom under a named patient basis and only for medical terminations. Therefore, the

use of RU486 in this study required the approval and cooperation of the manufacturer (Roussel) who supplied the drug for experimental use.

Culture Method Employed

All the biopsy material made available was dissected under sterile conditions in a clean air cabinet within about an hour of excision from the patient. Each tissue was rinsed in fresh PBS several times and when necessary any blood clots that were attached to it were gently removed in order to reduce contamination of the cultures with cells other than those from the cervix itself.

The needle biopsy tissue, when large enough, was cut longitudinally and one piece was placed in 4% formaldehyde fixing solution for 24 hours. Then it was processed in a Shandon 2LE tissue processor which sequentially moved the tissue through increasing concentrations of ethanol (from 70% to absolute alcohol) to dehydrate it, followed by immersion in histoclear (clearing agent, miscible with both alcohol and paraffin wax) to replace the alcohol. The histoclear was then replaced with molten wax. Once the cervix was impregnated with wax it was embedded in a wax block for later immunohistochemical investigation. The remaining tissue was cut into four pieces (approximately 1-3mg each) and used in tissue culture (see below), two of the pieces were assigned control medium and the other two to a treatment medium, thus each cervix provided enough material to examine the effects of only one treatment.

The hysterectomy tissue was cut into small pieces similar in size to those obtained with the needle biopsies. However, because this

sampling method resulted in a much larger piece of cervical tissue for experimentation more than one treatment could be tested on each individual cervix. Generally 16-20 small segments of tissue (approximately 2-3mg each) were taken from the biopsy material and four pieces were assigned to each treatment including the control. With these tissues it was also possible to place a relatively large piece in formaldehyde for fixation and wax embedding.

The experiments were carried out in 48 well plates as used for the guinea pig cultures. The tissue was placed in the well with 250µl of the appropriate medium. This was enough to cover the tissue but still allow it to remain close to the fluid:air interface. The plates were incubated in a humid atmosphere of 95% air/5% CO_2 at 37°C for 24 hours and the spent treatment medium was then harvested and replaced with fresh treatment medium. The plates were returned to the incubator for a further 24 hour period. The medium was harvested into two separate tubes one to be frozen and used in a later interleukin-8 assay and one to be treated with MOX for later prostaglandin assay. The wet weight of the tissues was then recorded using a Cahn 1200 electronic balance.

Standardisation of Assayed Values of IL-8 and Prostaglandins

Microscopic examination of the cultures suggested that a degree of tissue debris was present in the wells of the culture dishes, therefore before assay the medium was centrifuged at 10,000g for 30 seconds in order to sediment any particulate material and reduce the likelihood of interference in the assay. The medium was diluted prior to assay by a factor of ten, using BSA/tween buffer, and 25-40µl samples of this diluted culture medium (made up to 100µl with PBGS) were used for assay.

Calculation of the total quantity of IL-8 in the medium was performed as follows. This example assumes an aliquot of 25µl of the diluted medium. The absolute figure from the assay was a measure of the quantity of IL-8/ml (in nanogrammes), taking into account the fact that the sample was assayed in 100µl. In effect, only 2.5µl of that 100µl was culture medium, thus introducing a multiplication factor of 40. To standardise the results the quantities of IL-8/ml were divided by the mass of the tissue from which they were derived, giving a final quantification in ng IL-8/mg tissue/ml.

Quantification of the PGE_2 in the medium was performed as follows. This example assumes an aliquot of 20µl of already diluted medium (as for IL-8). The absolute figure from the assay was a measure of the amount of PGE_2 in the assayed aliquot (in picogrammes). The aliquot effectively contained 1.33µl of the original medium. Therefore, the figures obtained were multiplied by 750 (1000µl/1.33µl) to give pg/ml and divided by the weight of the tissue (mg), resulting in units of pg/mg/ml. The results are presented in ng/mg/ml.

Assays

Prostaglandins and interleukin-8 were assayed by radioimmunoassay (RIA), both utilising basically the same methodology. These RIAs rely on competition between an antigen and a radio labelled form of the same antigen for a specific antiserum. The antibody/antigen complex is then bound by a second antiserum directed against the immunoglobulins of the species in which the first antiserum was raised, for example rabbit, and the excess antigen (labelled and unlabelled) washed away. Depending on how much of the antigen was in the sample being assayed the level of radioactivity in the resulting complex will vary.

All RIAs were carried out in polypropylene tubes (Sarstedt) in assay racks (Amersham). Separation of bound and free antigen was effected by the addition of a magnetite-DAR (second antibody) complex (magnetic particles coated with antiserum raised in donkeys and directed against rabbit immunoglobulins) which was supplied by Dr R.W. Kelly.

The magnetite was sedimented by placing the racks of tubes in suitable magnets (Amersham).

The gamma radioactivity in each tube was measured using a Nuclear Enterprises gamma counter (NE1600).

Assay results were calculated using a Macintosh computer and Assayzap software (Biosoft, Cambridge, UK) written by Dr P. Taylor of the MRC unit in which most of this work was carried out. Assayzap used four parameter, weighted logistics to fit a curve to the standard points.

Prostaglandins

Assays for prostaglandins were established ones developed by Dr R.W. Kelly (Kelly et al., 1986a; Kelly et al., 1989). The polyclonal antisera were raised in rabbits against haptenised, methyloxime derivatives of the various prostaglandins. Adding a methyloxime group to the PG improves its stability and therefore reduces the likelihood that the antisera will cross react with other prostaglandins or its own metabolites. This introduced the requirement that the collected samples were also treated with methyloximating solution (MOX) to convert any prostaglandins contained therein to their Moxed form (Figure 4.3). Another benefit of this process was that the samples were then more stable when stored at 4°C.

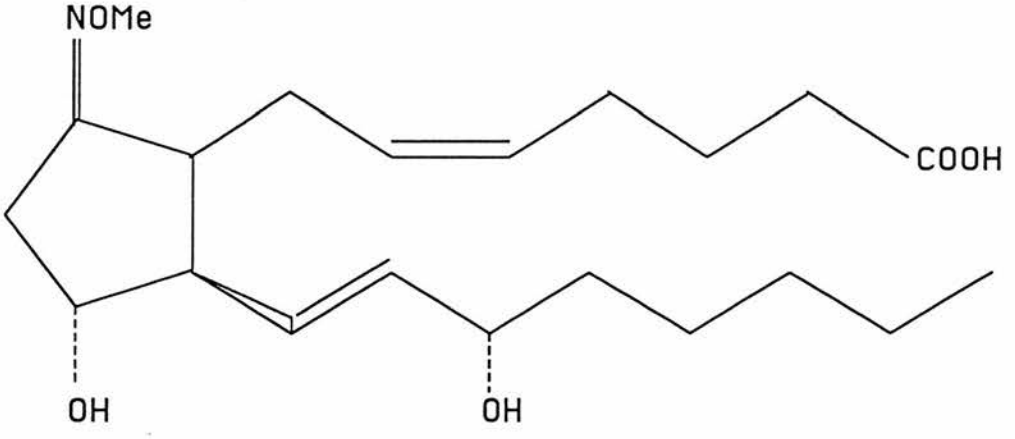


Figure 4.3 Moxed form of PGE_2

Prostaglandins were methyloximated and coupled to tyrosine through an imide linkage. This conjugate was iodinated using chloramine T oxidation for use in the assays (Kelly et al., 1989).

Standard curves were reconstituted from freeze dried aliquots of the relevant prostaglandins using PBGS. Serial dilutions were made to produce the range of standards required (10 standards, 10pg/ml to 5120pg/ml). These solutions were prepared freshly, shortly before use and were not stored in the reconstituted form.

Quality controls were made up from aliquots of moxed medium containing known amounts of the prostaglandin in question.

The antiserum was stored freeze dried at -20°C and aliquots were diluted 1:100 with PBGS and kept at 4°C. The working strength used in assays was between 1:20,000 and 1:150,000 again diluted with PBGS.

The stock iodine labelled PGs were stored in glass at 4°C and diluted when required in PBGS to give approximately 20-25,000 counts per minute per 100µl.

Samples of harvested medium were assayed in aliquots of 10-40µl made up to 100µl with PBGS. Dilutions were performed using a Hamilton Microlab 1000. The medium from the human cervix cultures required to be prediluted by a factor of ten using PBGS before the samples were aliquoted using the Microlab.

The standard curve was constructed by adding 100µl of the serial dilutions to the relevant assay tubes.

100µl of antiserum were added to all but the total counts and the NSB (Non Specific Binding) tubes and 100µl of labelled PG were added to every tube. The NSB tube had 200µl of PBGS to allow for the dilution produced by the addition of antiserum and labelled PG in the other tubes. The total binding (B_0) tubes contained no PG other than the radiolabelled form and therefore the sample volume of 100µl was

replaced with PBGS. The contents of the tubes were thoroughly mixed.

All samples, standards, total counts, NSBs and B_0s were in duplicate.

The assay mixture detailed above was incubated overnight at room temperature.

Following incubation, 250µl of the second antibody preparation was added to each tube and the tubes agitated to disperse the magnetic particles evenly through the reaction mixture. The second antibody was allowed to react for 30 minutes then the racks were placed on the magnets to precipitate the magnetic particles (30 minutes). The rack (plus magnets) were then inverted to drain off the excess buffer (antiserum and labelled PG) then the pellet resuspended in 250µl of wash buffer. The magnetic particles were precipitated once more for 30 minutes followed by draining of the supernatant and 30 minutes inverted on several layers of absorbent paper to allow any remaining buffer to drain out of the tubes.

The average binding capacity of the antiserum in these assays was 19% and the non specific binding was 1%. The intra-assay coefficient for the PGE_2 assay was 6% (at 100pg/ml) and the inter-assay coefficient was 7.6% (n=32). $PGF_{2\alpha}$ inter-assay coefficient was 8.8% (n=32) and the intra-assay coefficient was 9.3% (at 100pg/ml). In both assays the sensitivity was 2pg/tube (100µl sample/standard per tube).

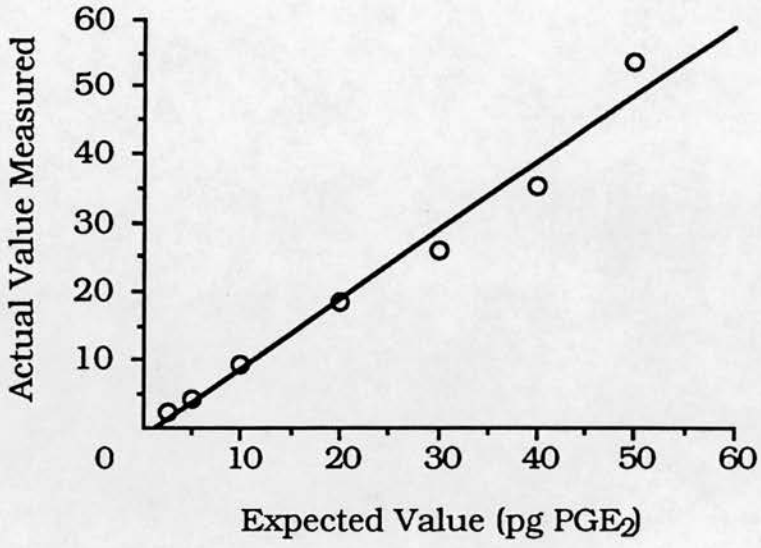


Figure 4.4 A typical curve from a prostaglandin assay produced by Assayzap, using 4 parameter, weighted logistics to fit the line to the duplicate data points.

Assay Interference

To examine the interference of hydrophilic factors contained in the culture medium the following experiment was carried out with PGE_2.

Guinea pig culture medium, that had been stored at -20°C, was pooled and a portion treated with MOX as described elsewhere. A second portion (20ml) was run down a Sep-Pak™ C-18 cartridge to remove lipophilic molecules, including prostaglandins. The cartridge was pretreated by running 5ml methanol then 5ml of water down it. This resulted in the particles of the cartridge being surrounded by a layer of methanol. As the medium flowed past the methanol the lipophilic molecules in the medium preferentially partitioned into the alcohol phase. The phenol red pH indicator from the medium remained in the column and the fluid flowing out was colourless. This

was stripped medium. The column was next flushed with 2ml methanol then 2ml water and these elutions pooled. This fraction should have contained the molecules partitioned into the methanol around the C-18 particles.

Stripped medium was spiked with PGE_2 and diluted to produce a range of concentrations. The spiked samples and some of the unadulterated stripped medium were treated with MOX as was the pooled medium.

The samples spiked with PGE_2 correlated well with the expected values (Figure 4.5) having a correlation coefficient (r) of O.99 ($p \leq 0.001$).

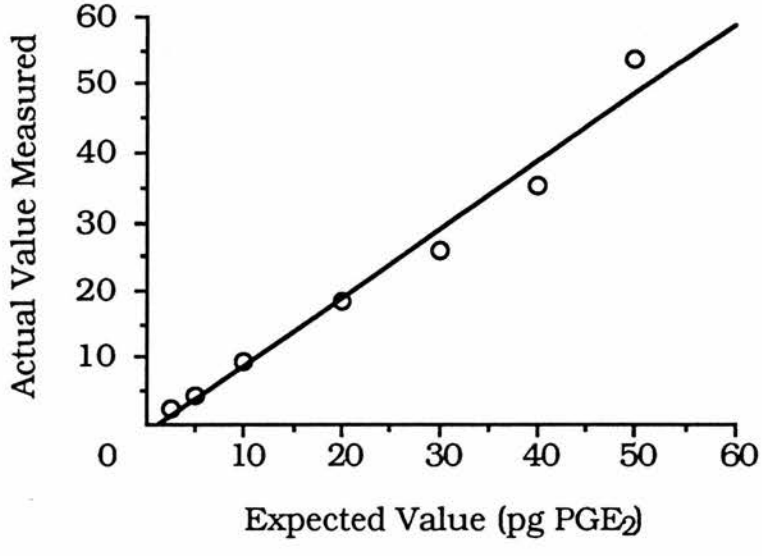


Figure 4.5 Correlation between the amount of PGE_2 added to the stripped medium and the amount detected in the radioimmunoassay.

Stripped medium did not contain any measurable PGE_2, as anticipated.

The sample of moxed medium contained 628pg PGE_2/ml and the fluids eluted from the column after the stripping process contained 2791pg PGE_2/ml. Since this latter fraction was only 4ml, derived from 20ml pooled medium, the equivalent value relative to the moxed medium was 558pg PGE_2/ml, which represented a recovery of 89%.

The correlation between spiked, stripped medium and the expected values for those samples indicated that hydrophilic molecules in the culture medium or released by the guinea pig cells did not interfere with the assay. The stripping process was obviously successful as witnessed by the fact that the material left on the column contained almost as much PGE_2 as the unstripped medium. The slight loss may indicate that a larger volume of methanol and water should have been used to clear the sep-pak, however, all the phenol red appeared visually to be eluted by the method used.

Interleukin-8

For the IL-8 radioimmunoassay a polyclonal rabbit antiserum (ICN Flow) was used with recombinant interleukin-8 (Collaborative Biomedical Products) labelled with iodine (^{125}I, Amersham), using chloramine T oxidation.

Standard curves were reconstituted from freeze dried aliquots of recombinant IL-8 using standards buffer (50% PBGS and 50% BSA/Tween buffer). Serial dilutions were made to produce the range of standards required (9 standards, 0.097ng/ml to 25ng/ml). These solutions were prepared freshly, shortly before use and were not stored in the reconstituted form.

Quality controls were included in the assays and were prepared from a stock medium containing IL-8.

The antiserum was stored at -20°C. Short term it was diluted 1:100 with PBGS and kept at 4°C. The working strength used in assays was 1:1000 again diluted with PBGS.

The stock iodine labelled IL-8 was stored in glass at 4°C and diluted when required in PBGS to give approximately 15-20,000 counts per minute per 100µl.

The assay procedure was similar to that for the prostaglandins, but there were some modifications. All but the total counts tubes contained 100µl of BSA/tween buffer and this was added before the samples. The second antibody added was increased to 500µl and it was found that the washing step could be omitted. Thus following the 30 minutes allowed for the second antibody to bind to the primary antibody the tubes were drained of supernatant and inverted on absorbent paper for a further 30 minutes.

Samples of harvested medium were generally diluted by a factor of ten with BSA/tween buffer and assayed in aliquots of 25-40µl made up to 100µl with PBGS. Dilutions were performed using a Hamilton Microlab 1000. Before the initial dilutions were made the samples were centrifuged for 30 seconds at high speed (10,000g) in a Biofuge micro centrifuge. This was carried out to ensure that any particulate matter from the cervical biopsies, that may have been taken up when the medium was harvested, was excluded from the assay.

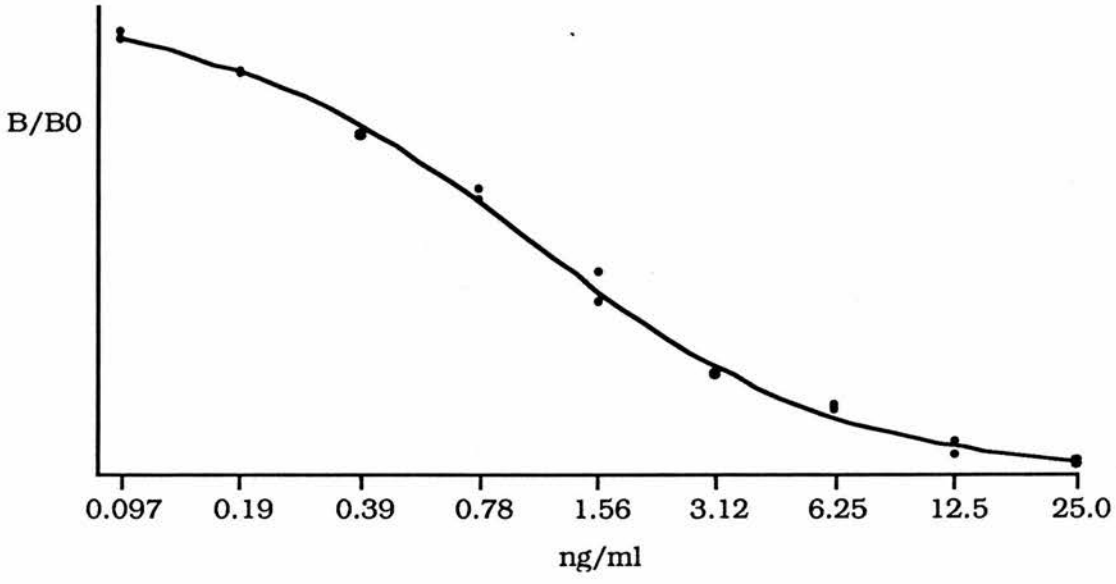


Figure 4.6 A typical curve from an interleukin-8 assay produced by Assayzap, using 4 parameter, weighted logistics to fit the line to the duplicate data points.

Where the difference between duplicates was greater than 10% the sample was re-assayed. The average B_0 of the antiserum in these assays was 17% and the non specific binding was 1.6%. The intra-assay coefficient for the IL-8 assay was 5.9% (at 3.12ng/ml) and the inter-assay coefficient was 11.6% (n=9). For this assay the

sequential dilution of culture medium from human cervix, up to 8 fold dilution, gave a correlation with expected values of R=0.994 ($p \leq 0.01$). The sensitivity of the assay was determined as the point distinguishable/separable from the B_0 by two times the standard deviation. The sensitivity was 0.195ng IL-8/ml and the value for unused culture medium was below this.

Kit Elisa/RIA Comparison

Because the use of a kit to assay all the samples involved in this work would have been prohibitively expensive the radioimmunoassay described above was developed. In order to ensure that the radioimmunoassay was in agreement with the ELISA kit (Quantikine) with regard to the assay of IL-8 in the medium harvested from cultures, samples were assayed by both methods and compared.

Correlation between the two sets of data was calculated and the coefficient (r) was 0.968 ($p \leq 0.05$), indicating that the two assay methods do in fact produce parallel results.

Statistical Analysis

Statistical analysis of the data was performed with the aid of a Macintosh computer and software from Clear Lake Research (CLR Anova). This program carried out between and within subject comparisons of variance and follow up comparisons assigning significance to the results using several optional tests. For this work the Newman-Keuls test was used and in order to be consistent throughout the same test was used in each analysis where such testing was performed. This test reduces the likelihood of spurious positive claims of significant differences. Statistical constraints permit such follow up analyses when the anova is one-way only. Thus the reported significances in initial presentation of the data do not involve the Newman-Keuls test.

The comparisons between the different treatments used in the guinea pig cultures were carried out using the differences between each treatment and the control values for that treatment. This approach was used because it eliminates the repeated use of the same figures for the controls, a situation which arises because the different treatments were often applied to cells cultured from the same animals and would therefore be referenced back to the same control values.

Immunohistochemistry

The immunohistochemical staining used in this work employed the peroxidase anti-peroxidase (PAP) system. The principles of the system are that a primary antiserum, in this case raised in rabbits and directed against interleukin-8, binds to the antigen in the sections and is then itself the target of a second antiserum (here raised in swine and directed against rabbit immunoglobulins). The second antiserum also reacts with the peroxidase antiserum complex and a substrate for the peroxidase is exposed to the enzyme revealing a brown colour if the primary antiserum is bound to its target.

Material from pregnant and nonpregnant women was fixed in 4% formaldehyde for 24 hrs then processed and embedded in wax for sectioning. Wax embedded tissues were cut into 4μm sections using an American Optical Corp 820 Spencer microtome, mounted on glass slides and baked at 30°C overnight to attach them to the slides.

The slides were placed in xylene to dewax the tissue (10 minutes) then in pure ethanol for 60 seconds, followed by 95% ethanol. The sections were then placed in a methanol/hydrogen peroxide solution for 30 minutes to block endogenous peroxidase activity. Next the slides were immersed in running water for 5 minutes followed by 10 minutes in TBS with one change of the buffer after 5 minutes (the dishes used for the immersion of slides were agitated by gentle motion on a rocking table).

The next few steps were all carried out in a slide box, designed to maintain a moist atmosphere to prevent the tissue from drying out. The buffer was drained off the slides and any excess fluid carefully removed by capillary action through placing absorbent paper adjacent

to the sections. Normal swine serum (1 part serum, 3 parts TBS) was pipetted onto the sections and incubated at room temperature for 30 minutes. The NSS was then drained off and any excess removed. The primary antibodies (raised in a rabbit and directed against IL-8) and control immunoglobulins (normal rabbit serum. Both primary and control sera used at a dilution of 1/25) were pipetted onto the sections and incubated at room temperature for 30 minutes. The control immunoglobulins were used to highlight any non specific binding of rabbit immunoprotein to the tissue. The slides were then rinsed in a stream of TBS (from a wash bottle) and immersed in TBS as above (2 x 5 minutes) then any excess fluid removed. The second antiserum was applied in the same way as the NSS and primary antiserum and again incubated at room temperature for 30 minutes (second antibody used at a dilution of 1/40). Rinsing in a stream of TBS and two, 5 minute washes in TBS followed. The Peroxidase Anti-Peroxidase (PAP. Used at a dilution of 1/100) complex was pipetted onto the slides and incubated for 30 minutes (room temperature), again followed by rinsing in a stream of TBS and two TBS washes. Excess fluid removed, the DAB substrate was applied to the sections and incubated in the dark for approximately 5 minutes, till the colour developed enough to be seen by eye. The sections were then rinsed in running water for several minutes and subsequently the nuclei counterstained with hematoxylin for 30 seconds. The excess hematoxylin was washed away in water and the slides placed in 1% acid alcohol to differentiate the cytoplasm and nuclei. The slides were washed in water then in Scott's water to blue up the nuclei, then again in tap water. The final stage was to return the sections to xylene

through increasing strengths of ethanol and mount coverslips on the sections using Histoclear fixative.

Chapter 5

Regulation of Prostaglandin Production by Guinea Pig Cervical Cells in Culture

Regulation of Prostaglandin Production by Guinea Pig Cervical Cells in Culture

The aim of this work was to examine prostaglandin production by the pregnant guinea pig cervix and to test the effects of several agents in order to develop a better understanding of the way in which prostaglandin production by cervical cells may be regulated during the process of cervical ripening.

The effect of treating the guinea pigs with the anti-progestin RU486 was investigated since its action in promoting cervical ripening may involve the prostaglandin pathways. The other experiments were aimed at testing,

1) The effect of progesterone or its absence in *vitro*, both in otherwise untreated cells and those challenged by the compounds listed below.
2) The *in vitro* effect of the G-protein activator LPS.
3) The *in vitro* effect of the protein kinase C stimulant PMA.
4) The *in vitro* effect of the phosphatase inhibitor okadaic acid.
5) The *in vitro* effect of increased intracellular calcium availability using the ionophore A23187.
6) The *in vitro* effect of the antiprogestin, RU486.
7) The *in vitro* effect of oestradiol.
8) Interactions which may have occurred between the above compounds.
9) The effect RU486 may have had on the actions of the above compounds.

Prostaglandin Production *de novo*

In order to confirm that the prostaglandins measured were being produced *de novo* by the guinea pig cells, cultures were performed in the presence of indomethacin which inhibits the synthesis of prostaglandins by blocking the action of cyclooxygenase. It was found that the level of prostaglandins in the medium dropped below detectable quantities when indomethacin was present at 5μM. Later in this thesis LPS will be shown to increase prostaglandin levels in the guinea pig cultures, however, when the same concentration of LPS was applied to cells in the presence of indomethacin it was unable to raise PG values above those observed in the presence of indomethacin alone.

In addition, samples were collected from some cultures after a second and third 24 hour culture period in the presence of the treatment media and there was still a significant output of prostaglandin, the correlation coefficient between the first and second harvests of 0.918 and a coefficient of 0.910 between the second and third medium collection, indicating that the cells in different treatments were maintaining their activity relative to each other.

These experiments indicated that prostaglandin synthesis was occurring *de novo* and that the cells remained viable, certainly in terms of their ability to produce prostaglandins, for several days in culture.

Eicosanoids In Unused Medium

Samples of medium that were incubated in the absence of cells were moxed, as described previously, and then tested for the presence of eicosanoids and it was found that $PGF_{2\alpha}$ and PGFM were

undetectable. PGE_2 was found but in very small quantities such that the results could not be accurately read from the standard curve (that is they were probably negligible) and PGEM was found to be present in slightly greater quantities (approximately 300pg/ml) which were again difficult to assess accurately from the standard curve and which approached the values obtained in the assays of medium that had been incubated with cells. Significant amounts of thromboxane (TxB_2) were found (around 2000pg/ml). Subsequent TxB_2 assays were not performed because this contaminating material was similar to the amount measured in samples of medium that had been incubated with cells. Cultured medium levels did not appear to show any response to the treatments that had been found to affect other prostaglandins, suggesting that the cells were not producing thromboxane. The presence in the medium of these eicosanoids was due to the fetal bovine serum used in the complete medium and since thromboxane is involved in platelet aggregation and blood clotting it was not unreasonable to find it in serum from dead bovine fetuses.

Fetal bovine serum was included in the culture system because in its absence the level of prostaglandins found in the cultures was at the lower end of the standard curve where the accuracy of the readings was reduced. This also did not allow for any inhibitory activity to be observed and indicated that while PG synthesis may have been occurring *de novo* when FBS was absent, it also had some requirement for an exogenous substrate supply.

Effect of DMF and Ethanol

Dimethylformamide (DMF) was the vehicle used for okadaic acid and ethanol was the vehicle for PMA, progesterone, RU486, oestradiol

and A23187. In other cases the vehicle used was saline or water. Neither DMF nor ethanol was found to affect the control culture values for PGE_2, $PGF_{2\alpha}$, PGEM or PGFM when used at the same concentrations as in the treatment media (0.05% DMF, 0.2% ethanol).

Effects of Progesterone and RU486 *in vivo*

Comparison of Basal Prostaglandin Output

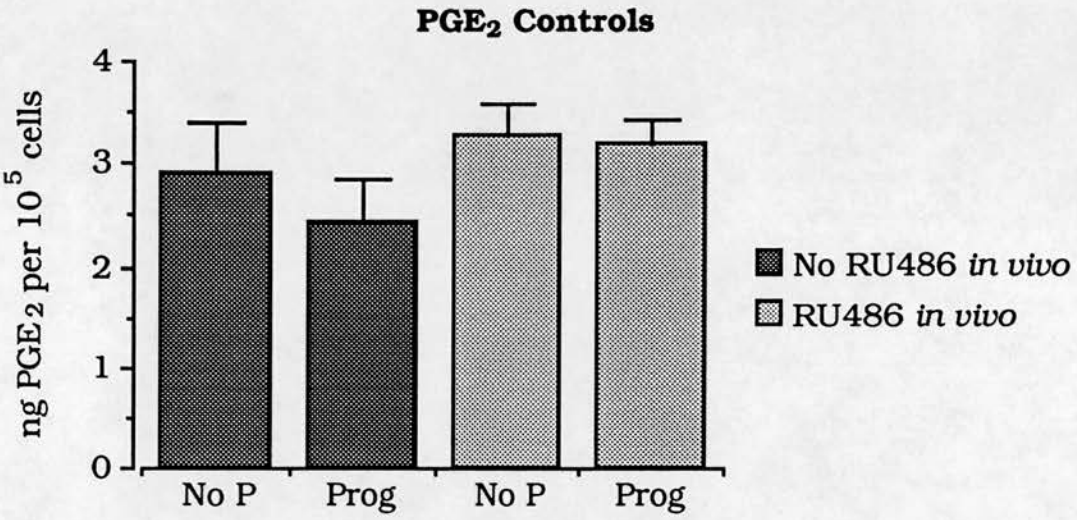
A principal hypothesis was that progesterone would suppress prostaglandin production, therefore progesterone was included in these experiments. In the natural situation the cervix would be in an environment where the progesterone concentration was high, although this would be very difficult to determine, justifying the use of 10^{-6}M progesterone. Because RU486 is considered to block receptors for progesterone, this made it relevant to examine any effect the two steroids may have had upon each other.

The figures in this section are divided into 4 blocks of results. The first two blocks are derived from control animals (no RU486 treatment *in vivo*) and the third and fourth blocks are derived from animals that were given RU486 *in vivo* . Thus, blocks one and three represent the data from control wells (no progesterone) and the second and fourth blocks represent data from progesterone containing control wells.

PGE_2 (Figure 5.1)

The control animals (those that did not receive RU486 *in vivo*) showed a reduction in PGE_2 output when their cells were cultured in the presence of progesterone. The overall effect of progesterone was significant (p=0.0092). Analysis of variance did not reveal a significant

difference between the control and RU486 *in vivo* animals. Although there appears to be a difference between the two groups of animals the interaction between progesterone and RU486 *in vivo* treatment did not reach significance (p=0.0526).



No P Progesterone free cultures

Prog Progesterone conditioned cultures

Figure 5.1 Comparison of basal PGE_2 production by guinea pig cervix cells in culture. (Mean ± sem, No RU486 *in vivo*, n=14, RU486 *in vivo*, n=9).

Progesterone effect p=0.0092

$PGF_{2\alpha}$ (Data not shown)

The effects of progesterone and RU486 *in vivo* on the output of $PGF_{2\alpha}$ were largely the same as for PGE_2. They followed a similar pattern suggesting an inhibitory effect of progesterone, however, there was no significant effect of progesterone or of RU486 *in vivo*.

$PGE_2/PGF_{2\alpha}$ (Data not shown)

No significant differences were detected between the production levels of the two prostaglandins at any level using analysis of variance.

PGEM and PGFM (Data not shown)

Metabolites of PGE_2 (PGEM) and $PGF_{2\alpha}$ (PGFM) were also examined, however, reproducibility of the assays for these was poor and did not allow comparison to be made between individual assays. For the PGEM assay the intra-assay variation was 18.2% and for PGFM it was 20%. The values obtained for these prostaglandin metabolites were highly variable because of the large error between assays (<100pg/ml to approximately 1000pg/ml) and were generally low which introduced another factor that would have reduced the accuracy of any analysis, since small changes in measured prostaglandin levels would have had a disproportionately large effect. Additionally, the medium itself was contaminated with PGEM before it even made contact with the cells. Therefore the results from the PGEM and PGFM assays were not suitable for analysis. If all the samples had been measured in one assay then valid comparisons could have been made between each treatment and each group of animals, however, this would have involved an assay of approximately 15,000 tubes and was thus impractical.

In an attempt to gain some insight into the effects on PGEM of RU486 *in vivo* and progesterone *in vitro* treatment, the following assay was performed. Samples of the medium from control wells, progesterone free and progesterone conditioned, from all but one of the guinea pigs (lack of medium available) were measured for the

prostaglandin metabolite. The results were compared but did not show any differences between the animals treated *in vivo* with RU486 and the controls, nor did they show any effect of progesterone on the level of PGEM.

Format of Data Presentation

The results which follow were obtained from 14 control animals, not treated with RU486, and from 9 animals given RU486 18-24 hours prior to being killed.

Each set of results (each figure) represents the information from 10 batches of cells treated with each substance or combination of substances (5 derived from control animals and 5 from animals given RU486 *in vivo*). In some instances there were fewer than 5 results for each treatment and they will be identified in the text. The graphs are divided into 4 blocks of results each relating to,

1) "Control"-the mean of the control values for the cultures concerned (i.e. culture medium only),
2) "Control (Prog)"-the mean of the progesterone conditioned cells (i.e. culture medium with progesterone added to act as a progesterone-alone control),
3) "Treated"-the mean of the values obtained with the particular treatment under examination (i.e. culture medium containing the additive(s) being studied) and
4) "Treated (Prog)"-the mean for the same treatment as in '3' but in the presence of progesterone (i.e. culture medium containing both progesterone and the agent being investigated).

Not all the data are represented graphically. However, they are presented numerically in the tables at the end of each section. Where there is no graph this will be marked by "Data not shown."

Effect of Lipopolysaccharide Alone and in Conjunction with Other Compounds on Prostaglandin Output by Cultured Guinea Pig Cervical Cells

LPS

LPS (lipopolysaccharide) is a product derived from the surface of gram negative bacteria. It is thought that in a proportion of cases of preterm delivery the cause may be related to the presence of bacterial infections in the upper genital tract (Taniguchi et al., 1991; Uldbjerg and Ulmsten, 1990; Romero et al., 1988; Lamont et al., 1990; Romero et al., 1989a; Romero et al., 1989b). In these experiments LPS was used to examine the likelihood that the cervix could produce prostaglandins under such circumstances. LPS has previously been shown to be an effective stimulant in cell culture systems at a similar concentration to that used here (Kelly et al., 1992; Strieter et al., 1989).

PGE_2 (Figure 5.2)

LPS at 4μg/ml had an obvious stimulatory effect on PGE_2 output. The increase observed was significant (p=0.0002) with no measurable effect of *in vivo* RU486 treatment or of progesterone.

$PGF_{2\alpha}$ (Data not shown)

LPS provoked a significant increase in $PGF_{2\alpha}$ production similarly to PGE_2 (p=0.0005). Comparisons indicated no overall effect of RU486 treatment or of progesterone.

$PGE_2/PGF_{2\alpha}$ (Data not shown)

Comparison of PGE_2 and $PGF_{2\alpha}$ showed that less $PGF_{2\alpha}$ than PGE_2 was produced (p=0.0027). The stimulatory effect of LPS was greater on PGE_2 synthesis (p=0.002). The two prostaglandins were not differentially affected by pretreatment of the guinea pigs with RU486 or the inclusion of progesterone in the culture medium.

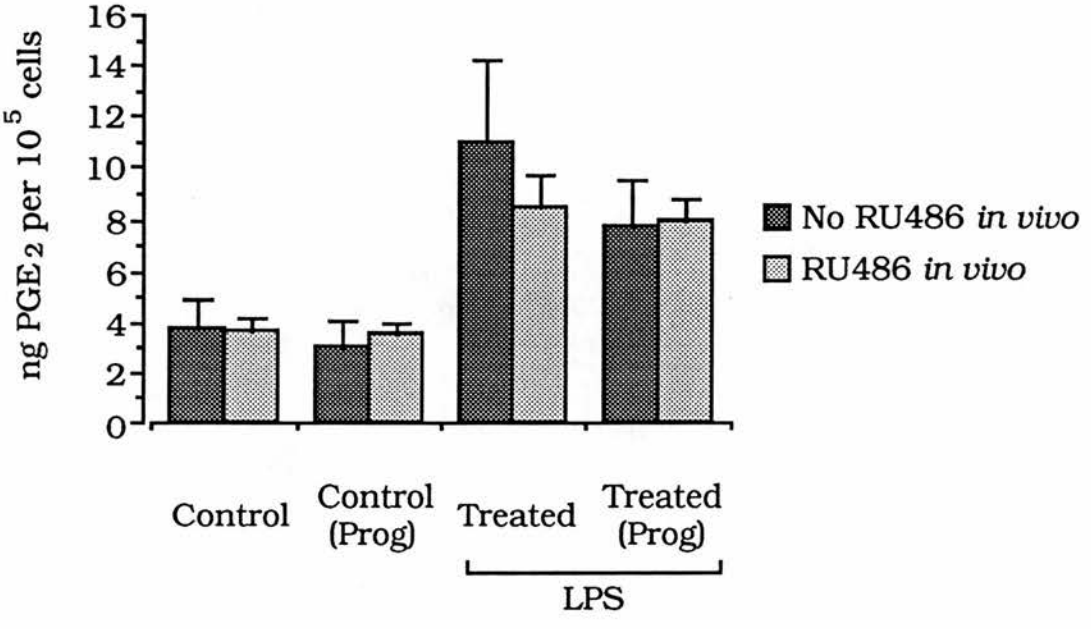


Figure 5.2 Effect of LPS (4μg/ml) on PGE_2 production (mean ± sem). LPS effect p=0.0002

LPS + RU486 (*in vitro*)

LPS (4μg/ml) and RU486 (1μM) were combined in one treatment medium and used to treat the cells. RU486 is known to promote cervical ripening in the guinea pig and was tested *in vitro* as well as *in vivo* in order to examine any role it may have in the production of prostaglandins in the late pregnant cervix. Once excised from the guinea pig the cervical tissue is no longer in contact with the antiprogestin and it was therefore included in the medium to determine whether or not this was relevant.

PGE_2 (Figure 5.3)

LPS still stimulated prostaglandin production when RU486 was added *in vitro*. As for LPS alone the only significant effect was that of the treatment medium, LPS + RU486 ($p<0.0001$).

$PGF_{2\alpha}$ (Data not shown)

LPS + RU486 *in vitro* resulted in a significant increase in $PGF_{2\alpha}$ ($p=0.0001$). Progesterone did not affect production nor did *in vivo* treatment with RU486.

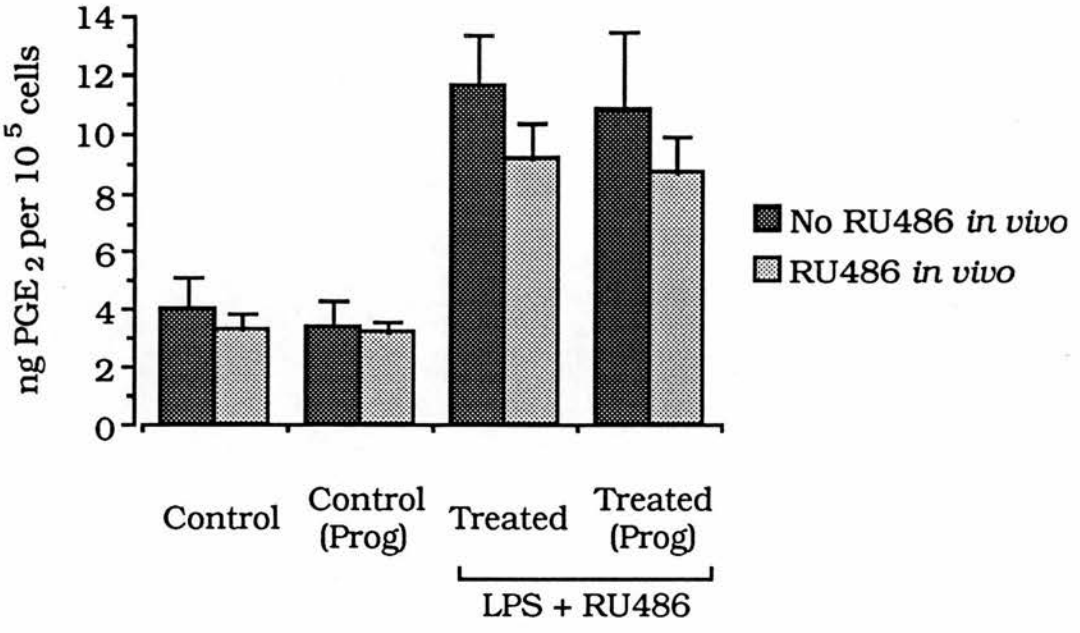


Figure 5.3 Effect of LPS (4μg/ml) + RU486 (*in vitro*, 1μM) on PGE_2 production (mean ± sem).

LPS + RU486 (*in vitro*) effect $p<0.0001$

PGE_2/$PGF_{2\alpha}$ (Data not shown)

Less $PGF_{2\alpha}$ than PGE_2 was produced ($p=0.0021$). The stimulatory effect of LPS + RU486 *in vitro* was greater on PGE_2 synthesis ($p=0.0009$). The two prostaglandins were not differentially affected by pretreatment of the guinea pigs with RU486 or the inclusion of progesterone in the culture medium.

LPS + A23187

A23187 is a calcium ionophore, thereby raising the intracellular calcium concentration. In the majority of reported cases it is a stimulant of prostaglandin synthesis (Hsu and Goetz, 1993; Ibe and Raj, 1992; Houmbard et al., 1991; Cabre et al., 1993). R Leask (Pers. Comm.) had observed an inhibitory effect of the ionophore and it was therefore of interest to examine its effects in the late pregnant guinea pig cervix to study the role of intracellular calcium in regulating prostaglandin production. A23187 was used at 2μM in conjunction with LPS (4μg/ml).

PGE_2 (Figure 5.4)

A23187 appeared to inhibit the stimulation of PGE_2 production by LPS. The p-value for the LPS + A23187 v Control was 0.0519, close to the accepted limit of significance, as is that for the interaction between RU486 *in vivo* and LPS + A23187 (*in vitro*), p=0.056. Although differences between the control and RU486 treated guinea pigs are identifiable when the data are analysed separately such analyses are more likely to produce spurious significance values as some of the power of the analysis is reduced. The only significant effect detected was an interaction between progesterone and the treatments applied to the cells (control or LPS + A23187), p=0.0244, indicating that progesterone affects the control wells differently to the LPS + A23187 wells. There appears to be a stimulatory effect of progesterone on the effect of LPS + A23187.

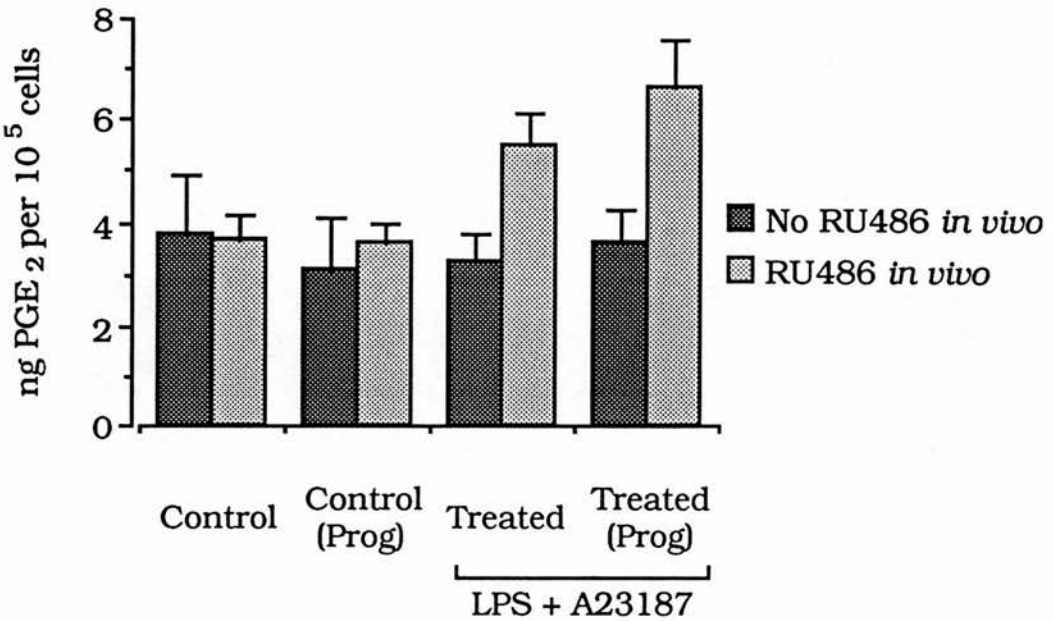


Figure 5.4 Effect of LPS (4μg/ml) + A23187 (2μM) on PGE_2 production (mean ± sem).

Progesterone x Treatment interaction p=0.0244

$PGF_{2\alpha}$ (Figure 5.5)

The results were similar to those for PGE2 in that there was no overall effect of RU486 treatment of the animals or of progesterone inclusion in the culture medium. Again the only detected interaction was that between progesterone and the treatments applied to the cells (p=0.0166), an apparent stimulation of prostaglandin production in the LPS + A23187 containing wells.

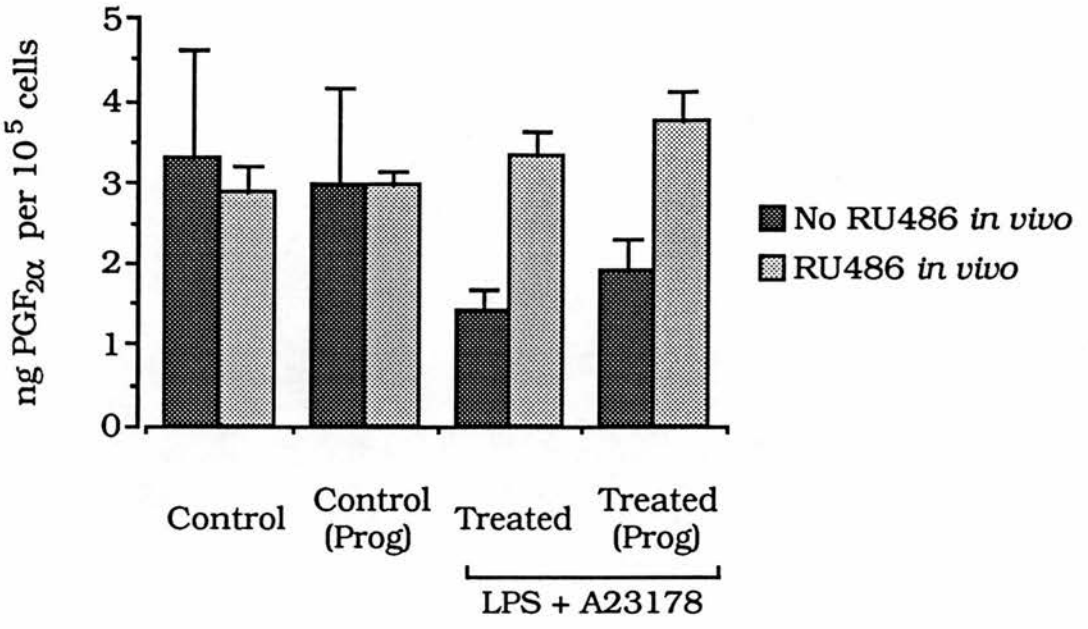


Figure 5.5 Effect of LPS (4μg/ml) + A23187 (2μM) on $PGF_{2\alpha}$ production (mean ± sem).

Progesterone x Treatment interaction p=0.0166

$PGE_2/PGF_{2\alpha}$ (Data not shown)

On the whole less $PGF_{2\alpha}$ than PGE2 was produced (p=0.001) The response of the cells to the LPS + A23187 treatment again differed in terms of PGE2 and $PGF_{2\alpha}$ production (less F than E, p=0.0004). The two prostaglandins were not differentially affected by pretreatment of the guinea pigs with RU486 or the inclusion of progesterone in the culture medium.

LPS + PMA

The phorbol ester PMA is an activator of protein kinase C. It was used to examine the involvement, if any, of the protein kinase C pathway in the production of prostaglandins by cultured cells of the guinea pig cervix derived from tissue excised in late pregnancy. It was also used in conjunction with other drugs in order to explore the independence/interdependence of the pathways involved. PMA was tested alone and the results of that experiment are presented later in this chapter (page 5.32)

PGE_2 (Figure 5.6)

LPS (4μg/ml) in combination with PMA (0.1μM) provoked an increase in PGE2 output (p=0.0002). The effect of combining LPS and PMA resulted in greater prostaglandin production than LPS alone but not significantly (see later). Neither RU486 *in vivo* nor progesterone treatment had any significant effects.

$PGF_{2\alpha}$ (Data not shown)

The stimulation evoked by the treatment used here was highly significant, p=0.0004. RU486 treatment *in vivo* and progesterone *in vitro* had no significant effect on the results.

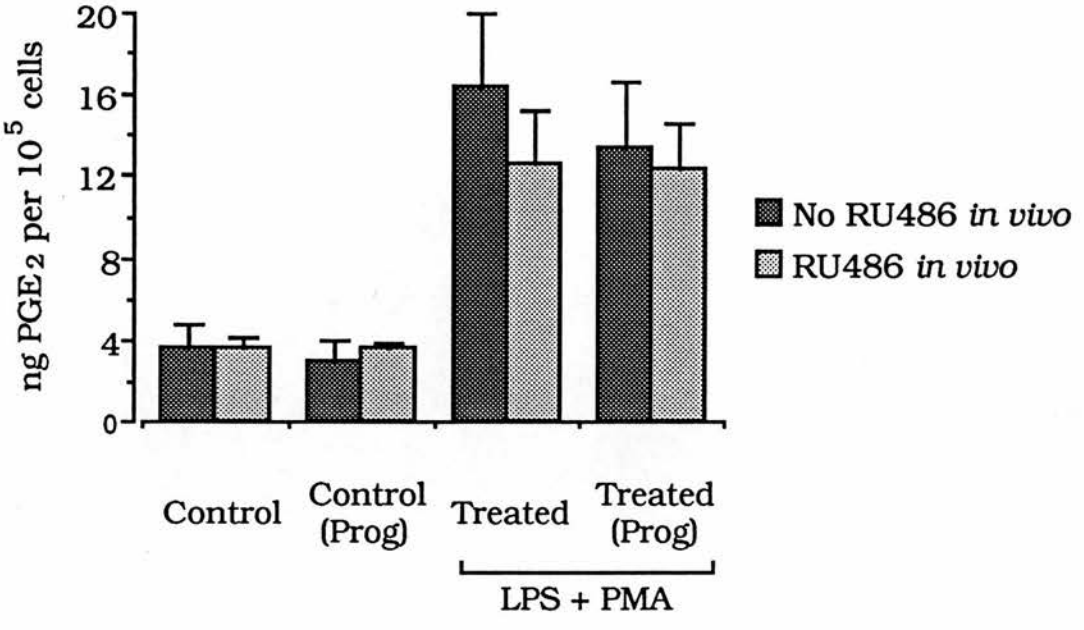


Figure 5.6 Effect of LPS (4μg/ml) + PMA (0.1μM) on PGE_2 production (mean ± sem).

LPS + PMA effect p=0.0002

$PGE_2/PGF_{2\alpha}$ (Data not shown)

The output of $PGF_{2\alpha}$ was less than that of PGE2 (p=0.001). The stimulatory effect of LPS + PMA was greater on PGE2 synthesis (p=0.005). The two prostaglandins were not differentially affected by pretreatment of the guinea pigs with RU486 or the inclusion of progesterone in the culture medium.

LPS + PMA + A23187

Since A23187 (2μM) appeared to have an unexpected inhibitory action in the experiments already described it was combined with LPS (4μg/ml) and PMA (0.1μM) to determine if it reduced prostaglandin synthesis in the presence of both stimulators. PMA was also tested with A23187 only and those results are presented later (page 5.36). A23187 had a less pronounced inhibitory action on PMA stimulated PGE_2 production than it did on LPS stimulated production, but it affect both PMA and LPS similarly with regard to $PGF_{2\alpha}$.

PGE_2 (Figure 5.7)

Despite the inclusion of A23187 in the medium there was still a significant stimulation of prostaglandin production, p=0.0013, similar to the effect observed with PMA + A23187 (see later). This was the first set of data to show a significant effect of the pretreatment of the guinea pigs with RU486, p=0.0432. The RU486 treatment *in vivo* resulted in a greater output of prostaglandin under the influence of LPS + PMA + A23187. This interaction between the treatment applied to the cells and the pretreatment of the animals with RU486 was significant, p=0.0176, the RU486 appearing to reduce the inhibitory effect of the A23187. From the figures in table 5.1-4 it is apparent that the RU486 maintains the stimulation at levels similar to those produced with LPS + PMA only.

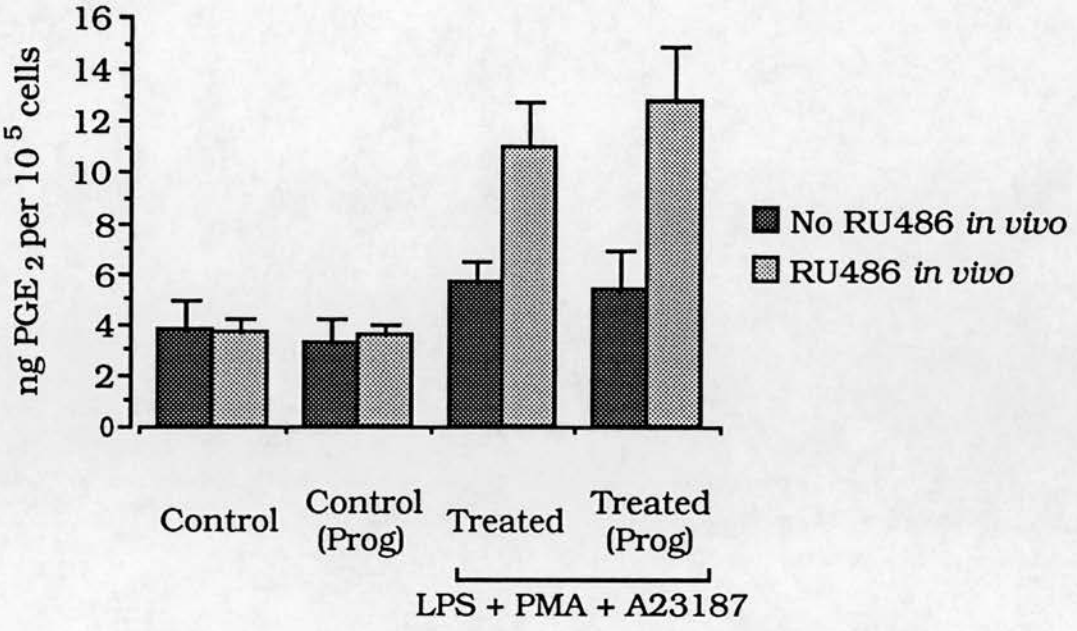


Figure 5.7 Effect of LPS (4μg/ml) + PMA (0.1μM) + A23187 (2μM) on PGE_2 production (mean ± sem).

RU486 *in vivo* effect p=0.0432

LPS + PMA + A23187 effect p=0.0013

RU486 *in vivo* x LPS + PMA + A23187 interaction p=0.0176

$PGF_{2\alpha}$ (Figure 5.8)

Analysis of variance indicated that LPS + PMA + A23187 did not significantly stimulate the production of $PGF_{2\alpha}$, and that RU486 *in vivo* and progesterone *in vitro* did not have any overall effect. However, significant interactions were detected 1) between the progesterone and RU486 treatments (p=0.0135), 2) between the LPS + PMA + A23187 and RU486 treatments (p=0.0422), and 3) between the LPS + PMA + A23187 and progesterone treatments (p=0.018). These interactions show 1) that in animals treated with RU486 the response of the cultures to progesterone was different to the control animals, the former showing greater $PGF_{2\alpha}$ production, 2) that in animals treated

with RU486 the response of the cultures to LPS + PMA + A23187 was different to the control animals, the former showing greater $PGF_{2\alpha}$ production, and 3) that the cultures treated with and without progesterone differed in their response to LPS + PMA + A23187, the presence of progesterone causing increased PG production.

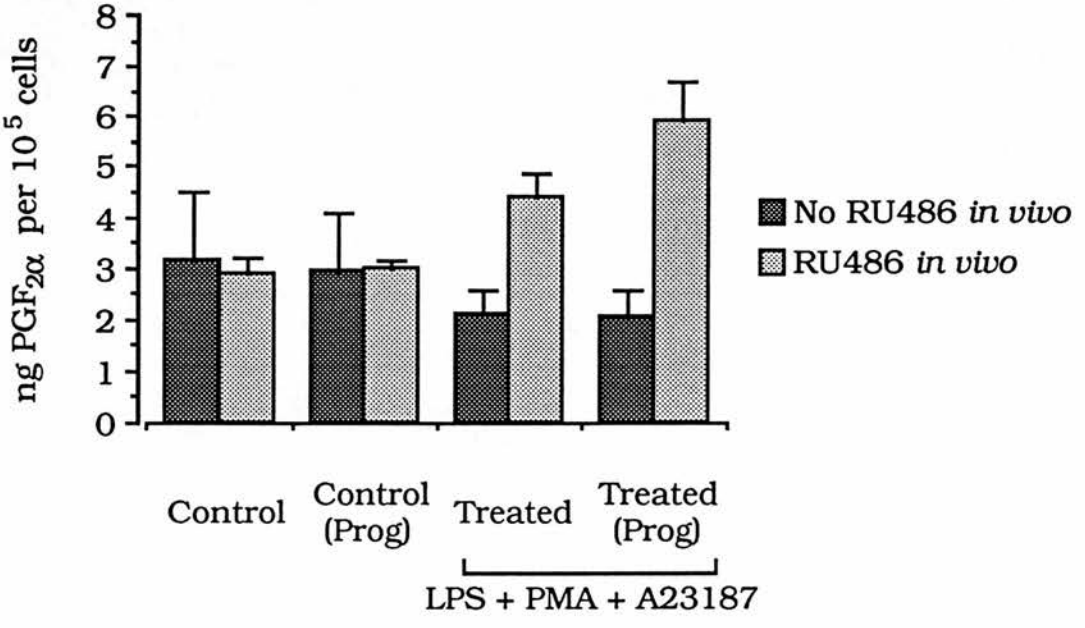


Figure 5.8 Effect of LPS (4μg/ml) + PMA (0.1μM) + A23187 (2μM) on $PGF_{2\alpha}$ production (mean ± sem).

RU486 *in vivo* x Progesterone interaction p=0.0135

LPS + PMA + A23187 x RU486 *in vivo* interaction p=0.0422

LPS + PMA + A23187 x Progesterone interaction p=0.018

$PGE_2/PGF_{2\alpha}$ (Data not shown)

Comparison of PGE_2 and $PGF_{2\alpha}$ showed that less $PGF_{2\alpha}$ than PGE_2 was produced ($p=0.0003$). The stimulatory effect of LPS + PMA + A23187 was greater on PGE_2 synthesis ($p=0.0003$). The two prostaglandins were not differentially affected by progesterone or by RU486 *in vivo*.

Comparisons of Results Obtained from Experiments Involving LPS

The data concerned are presented on pages 5.9 to 5.20. The differences between the treatments and their controls were compared using one way analysis of variance and the Newman-Keuls test to make pairwise comparisons.

PGE_2

Table 5.1 No RU486 *in vivo*, no progesterone

	PGE_2	A	B	C	D	E	Mn Diff (ng/10^5 cells)	sem
A	LPS	X	-	s	-	-	7.3	2.2
B	LPS+RU486	-	X	s	-	-	7.6	1.2
C	LPS+A23187	-	-	X	s	-	-0.5	1.1
D	LPS+PMA	-	-	s	X	s	12.6	2.7
E	LPS+PMA+A23187	-	-	-	s	X	1.8	1.3

'Mn Diff' Mean difference between treated cells and their controls.
Left, lower triangle $p \leq 0.01$, right, upper triangle $p \leq 0.05$
's' indicates a significant difference.

Table 5.2 No RU486 *in vivo*, progesterone

	PGE_2	A	B	C	D	E	Mn Diff (ng/10^5 cells)	sem
A	LPS	X	-	-	-	-	4.7	0.9
B	LPS+RU486	-	X	s	-	-	7.4	2.0
C	LPS+A23187	-	-	X	s	-	0.5	1.0
D	LPS+PMA	-	-	s	X	s	10.2	2.3
E	LPS+PMA+A23187	-	-	-	-	X	2.1	1.8

'Mn Diff' Mean difference between treated cells and their controls.
Left, lower triangle $p \leq 0.01$, right, upper triangle $p \leq 0.05$
's' indicates a significant difference.

Table 5.3 RU486 *in vivo*, no progesterone

	PGE_2	A	B	C	D	E	Mn Diff (ng/10^5 cells)	sem
A	LPS	X	-	-	-	-	4.8	0.8
B	LPS+RU486	-	X	-	-	-	5.8	0.7
C	LPS+A23187	-	-	X	s	s	1.8	0.2
D	LPS+PMA	-	-	s	X	-	8.9	2.1
E	LPS+PMA+A23187	-	-	-	-	X	7.3	1.3

'Mn Diff' Mean difference between treated cells and their controls.
Left, lower triangle $p \leq 0.01$, right, upper triangle $p \leq 0.05$
's' indicates a significant difference.

Table 5.4 RU486 *in vivo*, progesterone

	PGE_2	A	B	C	D	E	Mn Diff (ng/10^5 cells)	sem
A	LPS	X	-	-	-	-	4.3	0.5
B	LPS+RU486	-	X	-	-	-	5.5	0.8
C	LPS+A23187	-	-	X	s	s	3.0	0.7
D	LPS+PMA	-	-	-	X	-	8.6	2.0
E	LPS+PMA+A23187	-	-	-	-	X	9.2	1.8

'Mn Diff' Mean difference between treated cells and their controls.
Left, lower triangle $p \leq 0.01$, right, upper triangle $p \leq 0.05$
's' indicates a significant difference.

The four tables contain the data from the cells exposed to treatment media with and without progesterone from both groups of animals.

Summary of Key Findings

In the progesterone free cultures:

1) LPS + A23187 treatment of control animal cells was shown to result in significantly less PGE_2 production than the LPS alone, LPS + RU486 (*in vitro*) and LPS + PMA treatments.

2) LPS + PMA provoked greater stimulation than LPS + PMA + A23187.

3) In the RU486 pretreated animals both LPS + PMA and LPS + PMA + A23187 caused greater stimulation of PGE_2 output than LPS + A23187.

In the presence of progesterone:

1) LPS + A23187 treated cells from control animals produced less PGE_2 than LPS + RU486 (*in vitro*) and LPS + PMA treated cells.

2) LPS + PMA + A23187 caused a smaller increase than LPS + PMA.

In the RU486 pretreated animals both LPS + PMA and LPS + PMA + A23187 caused greater stimulation of PGE_2 output than LPS + A23187.

The pretreatment of the guinea pigs with RU486 appeared to block the effects of A23187 especially when used in conjunction with PMA.

$PGF_{2\alpha}$

Table 5.5 No RU486 *in vivo*, no progesterone

	$PGF_{2\alpha}$	A	B	C	D	E	Mn Diff (ng/10^5 cells)	sem
A	LPS	X	-	s	s	s	3.6	1.0
B	LPS+RU486	-	X	s	s	s	3.3	0.3
C	LPS+A23187	-	-	X	s	-	-1.9	1.2
D	LPS+PMA	-	-	s	X	s	8.3	1.8
E	LPS+PMA+A23187	-	-	-	s	X	-1.1	1.3

'Mn Diff' Mean difference between treated cells and their controls.
Left, lower triangle $p \leq 0.01$, right, upper triangle $p \leq 0.05$
's' indicates a significant difference.

Table 5.6 No RU486 *in vivo*, progesterone

	$PGF_{2\alpha}$	A	B	C	D	E	Mn Diff (ng/10^5 cells)	sem
A	LPS	X	-	-	-	-	3.1	1.1
B	LPS+RU486	-	X	-	-	-	5.1	2.2
C	LPS+A23187	-	-	X	s	-	-1.1	1.0
D	LPS+PMA	-	-	-	X	s	7.5	2.3
E	LPS+PMA+A23187	-	-	-	-	X	-0.9	1.1

'Mn Diff' Mean difference between treated cells and their controls.
Left, lower triangle $p \leq 0.01$, right, upper triangle $p \leq 0.05$
's' indicates a significant difference.

Table 5.7 RU486 *in vivo*, no progesterone

	$PGF_{2\alpha}$	A	B	C	D	E	Mn Diff (ng/10^5 cells)	sem
A	LPS	X	-	s	s	-	2.9	0.5
B	LPS+RU486	-	X	s	s	-	3.1	0.3
C	LPS+A23187	-	-	X	s	-	0.4	0.3
D	LPS+PMA	-	-	s	X	s	5.8	1.2
E	LPS+PMA+A23187	-	-	-	s	X	1.5	0.5

'Mn Diff' Mean difference between treated cells and their controls.
Left, lower triangle $p \leq 0.01$, right, upper triangle $p \leq 0.05$
's' indicates a significant difference.

Table 5.8 RU486 *in vivo*, progesterone

	$PGF_{2\alpha}$	A	B	C	D	E	Mn Diff (ng/10^5 cells)	sem
A	LPS	X	-	-	-	-	3.0	0.5
B	LPS+RU486	-	X	-	-	-	2.9	0.3
C	LPS+A23187	-	-	X	s	-	0.8	0.3
D	LPS+PMA	-	-	s	X	-	5.0	1.0
E	LPS+PMA+A23187	-	-	-	-	X	2.9	0.8

'Mn Diff' Mean difference between treated cells and their controls.
Left, lower triangle $p \leq 0.01$, right, upper triangle $p \leq 0.05$
's' indicates a significant difference.

Summary of Key Findings

In the absence of progesterone:

1) LPS + PMA was found to provoke greater stimulation of $PGF_{2\alpha}$ than the other four treatments for both the control and the RU486 *in vivo* animals.

2) The production of $PGF_{2\alpha}$ under the influence of LPS + A23187 was significantly less than LPS alone and LPS + RU486 (*in vitro*) for both the control and the RU486 *in vivo* animals.

3) The control animals (no RU486 *in vivo*) also showed lesser $PGF_{2\alpha}$ output than LPS alone and LPS + RU486 (*in vitro*).

In the presence of progesterone:

1) Both the control and the RU486 *in vivo* animals produced significantly less $PGF_{2\alpha}$ under the influence of LPS + A23187 than under the influence of LPS + PMA.

2) The control group (no RU486 *in vivo*) also showed a significant reduction in stimulation between the LPS + PMA and the LPS + PMA + A23187 treatments.

Summary of Data Involving LPS

The following tables summarise the statistically significant results already described.

Effects on PGE_2 Production

This table summarises how PGE_2 output was affected by the treatments applied to the animals and the cultures.

Table 5.9

Treatment Media	RU486 *in vivo*	Prog *in vitro*	Treatment	Interactions
LPS	NS	NS	Increase	NS
LPS+RU486	NS	NS	Increase	NS
LPS+A23187	NS	NS	NS	PxT
LPS+PMA	NS	NS	Increase	NS
LPS+PMA+A23187	Increase	NS	Increase	RxT

NS	No significant effect or interaction.
Increase	Significant increase in prostaglandin synthesis.
R	RU486 pretreatment of guinea pigs.
T	Treatment applied to cells.

Effects on $PGF_{2\alpha}$ Production

This table summarises how $PGF_{2\alpha}$ output was affected by the treatments applied to the animals and the cultures.

Table 5.10

Treatment Media	RU486 *in vivo*	Prog *in vitro*	Treatment	Interactions
LPS	NS	NS	Increase	NS
LPS+RU486	NS	NS	Increase	NS
LPS+A23187	NS	NS	NS	PxT
LPS+PMA	NS	NS	Increase	NS
LPS+PMA+A23187	NS	NS	NS	RxP, RxT, PxT

NS	No significant effect or interaction.
Increase	Significant increase in prostaglandin synthesis.
R	RU486 pretreatment of guinea pigs.
P	Progesterone applied to cells.
T	Treatment applied to cells.

Comparison of the Effects on $PGF_{2\alpha}$ and PGE_2 Production

This table summarises how $PGF_{2\alpha}$ and PGE_2 outputs were differentially affected by the treatments applied to the animals and the cultures.

Table 5.11

Treatment Media	RU486 *in vivo*	Prog *in vitro*	Treatment	Other Interactions
LPS	NS	NS	F<E	NS
LPS+RU486	NS	NS	F<E	NS
LPS+A23187	NS	NS	F<E	NS
LPS+PMA	NS	NS	F<E	NS
LPS+PMA+A23187	NS	NS	F<E	NS

NS No significant difference or interaction.

F<E Significantly less $PGF_{2\alpha}$ than PGE_2 produced.

Overview

The only direct effect of giving the guinea pigs RU486 *in vivo* was observed in the LPS + PMA + A23187 treatment set, where the antiprogestin produced an increase in PGE_2 output. Significant interactions were found between RU486 treatment *in vivo* and the LPS + PMA and the LPS + PMA + A23187 treatment sets, with regard to PGE_2 production, but only for the latter treatment set with regard to $PGF_{2\alpha}$. RU486 raised the production of prostaglandins above the levels observed in its absence.

For none of the *in vitro* treatments did progesterone produce any significant effects on the production of either prostaglandin. The only significant result of progesterone inclusion in the medium was an

interaction between it and the LPS + A23187 treatment and the LPS + PMA + A23187 treatment on $PGF_{2\alpha}$. In both cases progesterone appeared to have a stimulatory effect on the treated cells. In the latter treatment set progesterone also showed a significant interaction with RU486 pretreatment of the animals, the RU486 treated guinea pigs showing evidence of a stimulatory response.

Overall, each of the treatments resulted in an increase in PGE_2 production with the exception of the LPS + A23187 treatment. $PGF_{2\alpha}$ showed a similar response pattern. However, both media containing A23187 (i.e. LPS + A23187 and LPS + PMA + A23187) failed to produce a significant increase in this prostaglandin. Comparing the treatments with each other revealed an inhibitory effect of A23187. It also showed that combining LPS and PMA resulted in greater stimulation of $PGF_{2\alpha}$ production, but that this effect was not significant on PGE_2 production. This effect appeared to be lost or at least reduced when the guinea pigs were pretreated with RU486.

Comparison of the two prostaglandins revealed a trend of less stimulation of $PGF_{2\alpha}$ production than PGE_2 but no overall effect of progesterone. RU486 treatment of the guinea pigs caused a measurable effect in only one treatment set, LPS + PMA + A23187, where it had an inhibitory effect on PGF2a. This same comparison revealed an interaction between both *in vitro* progesterone and *in vivo* RU486 and the LPS + PMA + A23187. The RU486 and the progesterone appeared to permit the production of PGE_2 to be significantly greater than $PGF_{2\alpha}$.

Effect of Phorbol Myristate Acetate Alone and in Conjunction with Other Compounds on Prostaglandin Output by Cultured Guinea Pig Cervical Cells

PMA

The phorbol ester PMA is an activator of protein kinase C. It was used in the experiments described in this thesis to examine the involvement, if any, of the protein kinase C pathway in the production of prostaglandins by guinea pig cervix cells sustained in culture and derived from tissue removed towards the end of pregnancy. It was also used in conjunction with other drugs in order to explore the independence/interdependence of the pathways involved in prostaglandin production by those cells.

PGE_2 (Figure 5.9)

PMA at a concentration of 0.1μM stimulated PGE_2 production by guinea pig cervix cells from both the control and RU486 pretreated animals ($p=0.0001$). Progesterone in the culture medium had a small but significant inhibitory effect ($p=0.0201$). However, pretreatment with RU486 *in vivo* had no effect.

$PGF_{2\alpha}$ (Data not shown)

The response of $PGF_{2\alpha}$ to PMA was the same as PGE_2 ($P<0.0001$), but no effect of progesterone was observed or of RU486 pretreatment of the guinea pigs.

$PGE_2/PGF_{2\alpha}$ (Data not shown)

PGE_2 and $PGF_{2\alpha}$ were produced in similar quantities and were not differentially affected by RU486 *in vivo* or progesterone *in vitro*. The only significant effect was a greater inhibitory action of progesterone on PMA stimulated PGE_2 than on $PGF_{2\alpha}$, i.e. a prostaglandin x progesterone x treatment interaction (p=0.0409).

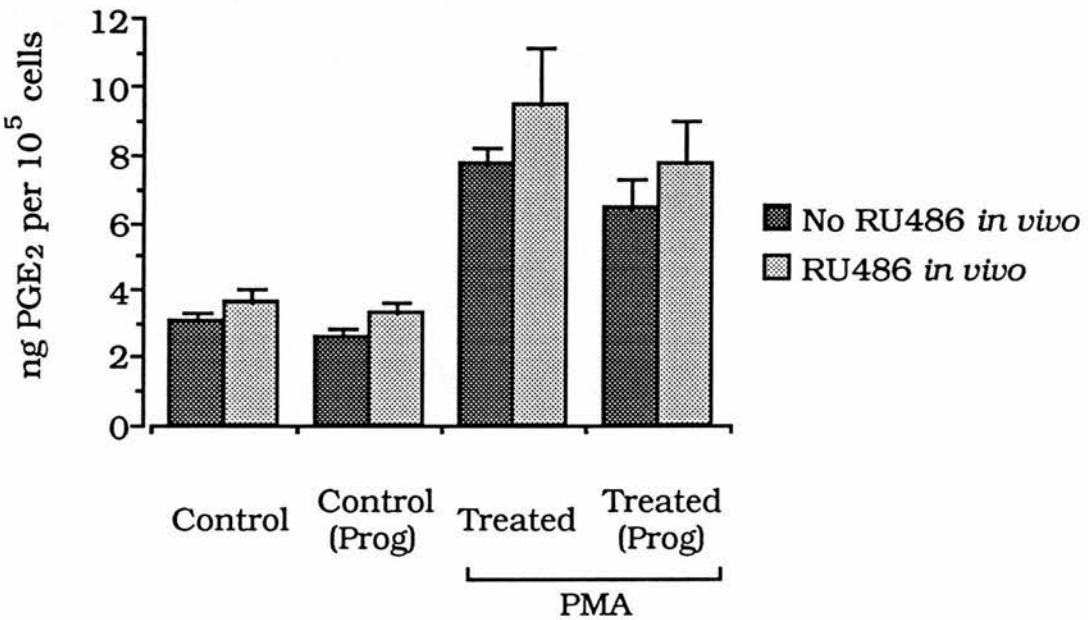


Figure 5.9 Effect of PMA (0.1μM) on PGE_2 production (mean ± sem).

PMA effect p=0.0001

Progesterone effect p=0.0201

PMA + RU486 (*in vitro*)

Since the excised tissue was no longer in an environment containing RU486 and this was one of the main objects of the experiments performed, the antiprogestin was included in the culture medium to study its effects on prostaglandin synthesis.

PGE_2 (Figure 5.10)

Cells were treated with PMA plus RU486 *in vitro*, at 0.1μM and 1μM respectively.

An obvious stimulation of PGE_2 output occurred with PMA + RU486 *in vitro* (p=0.0002). Progesterone did not affect PGE_2 nor did RU486 treatment *in vivo*.

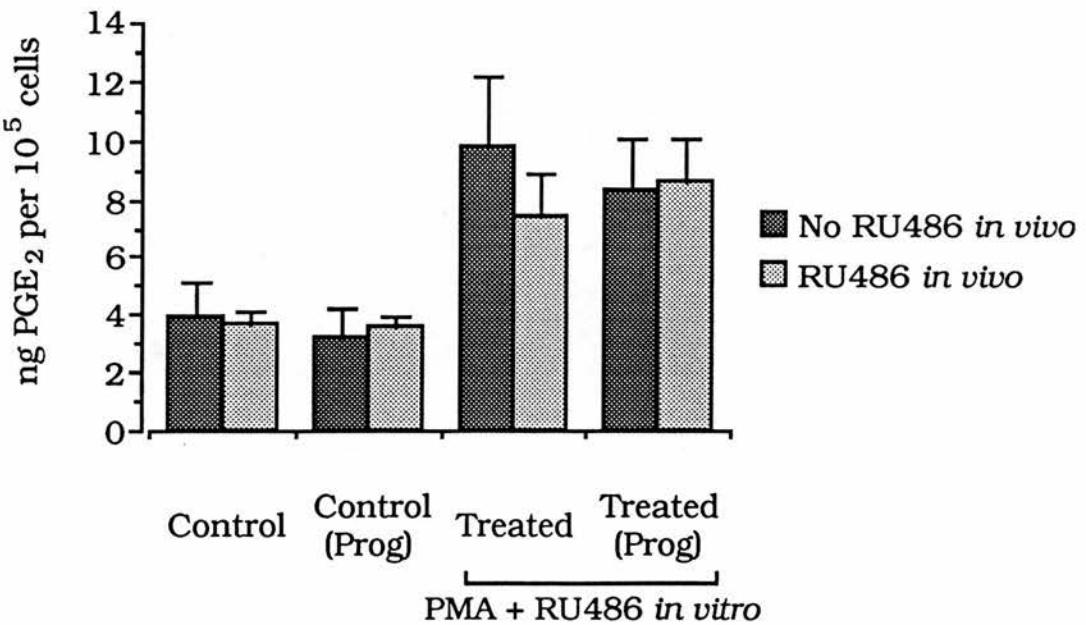


Figure 5.10 Effect of PMA (0.1μM) + RU486 (*in vitro*, 1μM) on PGE_2 production (mean ± sem).

PMA + RU486 *in vitro* effect p=0.0002

$PGF_{2\alpha}$ (Data not shown)

Again treatment with PMA + RU486 (*in vitro*) produced a significant increase in $PGF_{2\alpha}$ output (p=0.001), and progesterone and RU486 *in vivo* had no effect on the results.

$PGE_2/PGF_{2\alpha}$ (Data not shown)

Comparison of the two prostaglandins indicates that they were produced in similar quantities. No effect of progesterone or RU486 (*in vitro*) was found.

PMA + A23187

Since A23187 was found to inhibit the stimulation of prostaglandin production by LPS, its effects on PMA stimulation were examined. PMA activates protein kinase C and it has been shown to require calcium for this to take place. If A23187 blocked the action of PMA this would suggest an inhibitory action of increased free intracellular calcium downstream of the PMA interaction with the cell membrane and protein kinase C.

PGE_2 (Figure 5.11)

A23187 did not block the stimulatory effect of PMA. Thus, PMA (0.1μM) + A23187 (2μM) provoked an increase in PGE_2 output, (p=0.0011). The inclusion of A23187 did appear to reduce the effectiveness of the PMA but the reduction was not significant (see tables 5.12-14). Progesterone did not influence the results, nor did RU486 treatment of the guinea pigs.

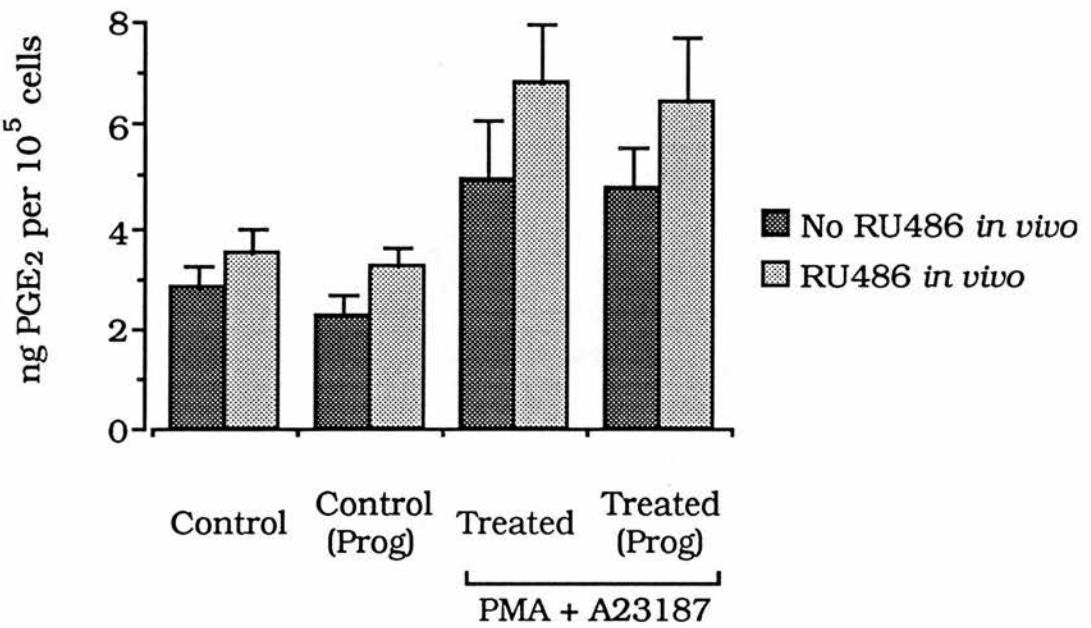


Figure 5.11 Effect of PMA (0.1μM) + A23187 (2μM) on PGE_2 production (mean ± sem).

PMA + A23187 effect p=0.0011

$PGF_{2\alpha}$ (Figure 5.12)

A23187 blocked the stimulatory effect of PMA (see tables 5.15-18), PMA + A23187 treatment not producing any significant change in $PGF_{2\alpha}$ production. No effect of progesterone or RU486 *in vivo* was found.

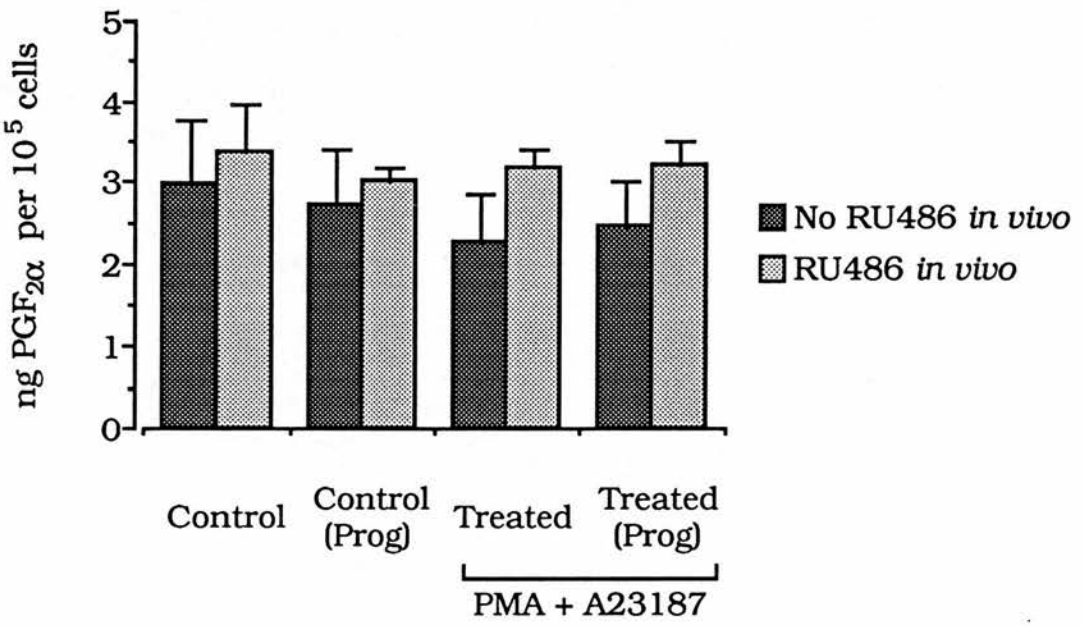


Figure 5.12 Effect of PMA (0.1μM) + A23187 (2μM) on $PGF_{2\alpha}$ production (mean ± sem).

$PGE_2/PGF_{2\alpha}$ (Data not shown)

The overall trend was for less $PGF_{2\alpha}$ production than PGE_2 ($p=0.0109$) and the difference in the effect of the PMA + A23187 treatment on the two prostaglandins was highly significant (less F than E, $p=0.001$). No effect of progesterone or RU486 *in vivo* was detected.

PMA + Okadaic Acid

Okadaic acid is an inhibitor of phosphatases and therefore prevents the dephosphorylation of intracellular proteins. It was examined to determine if such an action would increase the effect of the PKC activator by maintaining the phosphorylated state of intracellular proteins. Okadaic acid alone was found to stimulate prostaglandin production by guinea pig cervical cells in culture (see later page 5.51). Cells were treated *in vitro* with PMA (0.1μM) and okadaic acid (OA, 50nM).

PGE_2 (Figure 5.13)

PGE_2 synthesis was increased on exposure to PMA + OA, ($p=0.0192$). OA did not, however, potentiate the action of PMA (see tables 5.12-14). Neither progesterone in the culture medium nor RU486 *in vivo* had any significant effects on the outcome of the experiments.

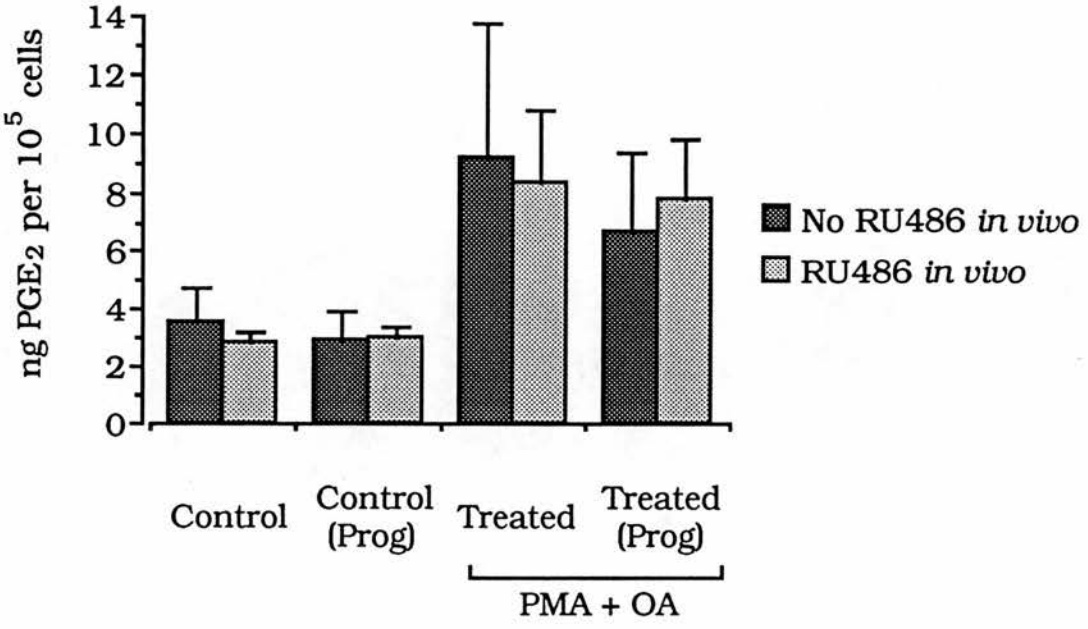


Figure 5.13 Effect of PMA (0.1μM) + Okadaic Acid (50nM) on PGE_2 production (mean ± sem).
PMA + OA effect p=0.0192

$PGF_{2\alpha}$ (Data not shown)

The response of $PGF_{2\alpha}$ was similar to that of PGE_2, the stimulatory action of PMA + OA producing the only significant change (p=0.0021). Again there was no significant difference between the PMA + OA and the PMA alone treatments (see tables 5.15-18).

$PGE_2/PGF_{2\alpha}$ (Data not shown)

Comparison of the two prostaglandins once again indicated that they were produced in similar quantities, and that both progesterone *in vitro* and pretreatment of the guinea pigs with RU486 did not influence prostaglandin synthesis.

PMA + LPS and PMA + LPS + A23187

These are the same results as in the LPS section under LPS + PMA and LPS + PMA + A23187 (Figures 5.6-8). They are included in the following analyses because they include data from experiments where PMA was used.

Comparisons of Results Obtained from Experiments Involving PMA

The data concerned are presented on pages 5.26 to 5.41.

PGE_2

Table 5.12 No RU486 *in vivo* , no progesterone

	PGE_2	A	B	C	D	E	F	Mn Diff (ng/10^5 cells)	sem
A	PMA	X	-	-	-	s	-	4.4	0.4
B	PMA+RU486	-	X	-	-	s	-	5.9	1.3
C	PMA+A23187	-	-	X	-	s	-	2.1	1.0
D	PMA+OA	-	-	-	X	s	-	5.6	3.4
E	PMA+LPS	-	-	s	-	X	s	12.6	2.7
F	PMA+LPS+A23187	-	-	-	-	s	X	1.8	1.4

'Mn Diff' Mean difference between treated cells and their controls.
Left, lower triangle $p \leq 0.01$, right, upper triangle $p \leq 0.05$
's' indicates a significant difference.

Table 5.13 No RU486 *in vivo* , progesterone

	PGE_2	A	B	C	D	E	F	Mn Diff (ng/10^5 cells)	sem
A	PMA	X	-	-	-	s	-	3.9	0.7
B	PMA+RU486	-	X	-	-	s	-	5.0	1.0
C	PMA+A23187	-	-	X	-	s	-	2.5	0.7
D	PMA+OA	-	-	-	X	s	-	3.8	1.7
E	PMA+LPS	-	-	-	-	X	s	10.2	2.3
F	PMA+LPS+A23187	-	-	-	-	s	X	2.1	1.8

'Mn Diff' Mean difference between treated cells and their controls.
Left, lower triangle $p \leq 0.01$, right, upper triangle $p \leq 0.05$
's' indicates a significant difference.

Table 5.14 RU486 *in vivo*

	PGE_2	No Progesterone Mn Diff (ng/10^5 cells)	sem	Progesterone Mn Diff (ng/10^5 cells)	sem
A	PMA	5.9	1.6	4.5	1.2
B	PMA + RU486	3.7	1.2	5.0	1.2
C	PMA + A23187	3.3	0.6	3.2	0.9
D	PMA + OA	5.5	2.5	4.7	2.0
E	PMA + LPS	8.9	2.1	8.6	2.0
F	PMA + LPS + A23187	7.3	1.3	9.2	1.8

'Mn Diff' Mean difference between treated cells and their controls.
No significant differences were detected.

Summary of Key Findings

In the presence and absence of progesterone the cells from control animals:

1) Produced significantly more PGE_2 in response to challenge with PMA + LPS than all the other PMA group treatments.

2) While A23187 inclusion was found to have a significant inhibitory effect on the PMA + LPS stimulation, its effect on PMA alone did not reach significance.

3) Okadaic acid did not potentiate the effect of PMA.

In the group of animals pretreated with RU486 there were no statistically significant differences between the various PMA containing treatments with regard to PGE_2 synthesis. The previously observed inhibitory effect of A23187 on PMA + LPS was lost.

$PGF_{2\alpha}$

Table 5.15 No RU486 *in vivo* , no progesterone

	$PGF_{2\alpha}$	A	B	C	D	E	F	Mn Diff (ng/10^5 cells)	sem
A	PMA	X	-	s	-	-	s	4.7	0.8
B	PMA+RU486	-	X	s	-	-	s	5.0	1.2
C	PMA+A23187	-	-	X	s	s	-	-0.7	0.5
D	PMA+OA	-	-	-	X	-	s	3.8	1.5
E	PMA+LPS	-	-	s	-	X	s	8.3	1.8
F	PMA+LPS+A23187	-	-	-	-	s	X	-1.1	1.3

'Mn Diff' Mean difference between treated cells and their controls.
Left, lower triangle $p \leq 0.01$, right, upper triangle $p \leq 0.05$
's' indicates a significant difference.

Table 5.16 No RU486 *in vivo* , progesterone

	$PGF_{2\alpha}$	A	B	C	D	E	F	Mn Diff (ng/10^5 cells)	sem
A	PMA	X	-	-	-	-	-	4.5	0.6
B	PMA+RU486	-	X	-	-	-	-	4.8	1.9
C	PMA+A23187	-	-	X	-	s	-	-0.3	0.3
D	PMA+OA	-	-	-	X	-	-	3.5	1.2
E	PMA+LPS	-	-	s	-	X	s	7.5	2.3
F	PMA+LPS+A23187	-	-	-	-	s	X	-0.9	1.1

'Mn Diff' Mean difference between treated cells and their controls.
Left, lower triangle $p \leq 0.01$, right, upper triangle $p \leq 0.05$
's' indicates a significant difference.

Table 5.17 RU486 *in vivo* , no progesterone

	$PGF_{2\alpha}$	A	B	C	D	E	F	Mn Diff (ng/10^5 cells)	sem
A	PMA	X	-	s	-	-	-	4.0	1.1
B	PMA+RU486	-	X	s	-	-	-	4.5	1.0
C	PMA+A23187	-	-	X	s	s	-	-0.2	0.5
D	PMA+OA	-	-	-	X	-	-	4.4	1.4
E	PMA+LPS	-	-	s	-	X	s	5.8	1.2
F	PMA+LPS+A23187	-	-	-	-	-	X	1.5	0.5

'Mn Diff' Mean difference between treated cells and their controls.
Left, lower triangle $p \leq 0.01$, right, upper triangle $p \leq 0.05$
's' indicates a significant difference.

Table 5.18 RU486 *in vivo* , progesterone

	$PGF_{2\alpha}$	A	B	C	D	E	F	Mn Diff (ng/10^5 cells)	sem
A	PMA	X	-	s	-	-	-	3.7	0.4
B	PMA+RU486	-	X	s	-	-	-	4.0	0.9
C	PMA+A23187	-	-	X	s	s	s	0.2	0.2
D	PMA+OA	-	-	-	X	-	-	3.4	0.8
E	PMA+LPS	-	-	s	-	X	-	5.0	1.0
F	PMA+LPS+A23187	-	-	-	-	-	X	2.9	0.8

'Mn Diff' Mean difference between treated cells and their controls.
Left, lower triangle $p \leq 0.01$, right, upper triangle $p \leq 0.05$
's' indicates a significant difference.

Summary of Key Findings

In the control animal group (no RU486 *in vivo*):

1) In the absence of progesterone, A23187 inhibited the stimulation of $PGF_{2\alpha}$ production provoked by PMA alone and by PMA + LPS.

2) In the presence of progesterone the inhibitory effect of A23187 was only significant on the response to PMA + LPS, both PMA + LPS + A23187 and PMA + A23187 producing less prostaglandin than PMA + LPS. The difference between PMA and PMA + A23187 was not significant.

Cells from guinea pigs given RU486 *in vivo*, in the absence of progesterone:

1) Produced less $PGF_{2\alpha}$ under the influence of PMA + A23187 compared with the other treatments, except PMA + LPS + A23187.

2) PMA + LPS + A23187 was only significantly different to PMA + LPS, showing an inhibitory effect of A23187.

With progesterone present in the medium the inhibitory effect of A23187 was again evident. PMA + A23187 resulted in less stimulation of $PGF_{2\alpha}$ than all of the other treatments.

Summary of Data Involving PMA

The following tables summarise the statistically significant results already described.

Effects on PGE_2 Production

This table summarises how PGE_2 output was affected by the treatments applied to the animals and the cultures.

Table 5.19

Treatment Media	RU486 *in vivo*	Prog *in vitro*	Treatment	Interactions
PMA	NS	Decrease	Increase	NS
PMA+RU486	NS	NS	Increase	NS
PMA+A23187	NS	NS	Increase	NS
PMA+OA	NS	NS	Increase	NS
PMA+LPS	NS	NS	Increase	NS
PMA+LPS+A23187	Increase	NS	Increase	RxT

NS	No significant effect or interaction.
Increase	Significant increase in prostaglandin synthesis.
Decrease	Significant decrease in prostaglandin synthesis.
R	RU486 pretreatment of guinea pigs.
T	Treatment applied to cells.

Effects on $PGF_{2\alpha}$ Production

This table summarises how $PGF_{2\alpha}$ output was affected by the treatments applied to the animals and the cultures.

Table 5.20

Treatment Media	RU486 *in vivo*	Prog *in vitro*	Treatment	Interactions
PMA	NS	NS	Increase	NS
PMA+RU486	NS	NS	Increase	NS
PMA+A23187	NS	NS	NS	NS
PMA+OA	NS	NS	Increase	NS
PMA+LPS	NS	NS	Increase	NS
PMA+LPS+A23187	NS	NS	NS	RxP, RxT, PxT

NS	No significant effect or interaction.
Increase	Significant increase in prostaglandin synthesis.
R	RU486 pretreatment of guinea pigs.
P	Progesterone applied to cells.
T	Treatment applied to cells.

Comparison of the Effects on $PGF_{2\alpha}$ and PGE_2 Production

This table summarises how $PGF_{2\alpha}$ and PGE_2 outputs were differentially affected by the treatments applied to the animals and the cultures.

Table 5.21

Treatment Media	RU486 *in vivo*	Prog *in vitro*	Treatment	Other Interactions
PMA	NS	NS	NS	ExPxT
PMA+RU486	NS	NS	NS	NS
PMA+A23187	NS	NS	F<E	NS
PMA+OA	NS	NS	NS	NS
PMA+LPS	NS	NS	F<E	NS
PMA+LPS+A23187	NS	NS	F<E	NS

NS	No significant difference or interaction.
F<E	Significantly less $PGF_{2\alpha}$ than PGE_2 produced.
E	Prostaglandin E or F.
P	Progesterone applied to cells
T	Treatment applied to cells.

Overview

RU486 treatment *in vivo* only affected the PMA + LPS + A23187 conditioned cultures and there it resulted in increased prostaglandin synthesis. The results for LPS containing cultures will obviously be the same as highlighted in the previous section.

Progesterone produced an overall decrease in PGE_2 synthesis in the PMA alone treatment, but had no such effect on any of the other cultures or on $PGF_{2\alpha}$.

In general, both PGE_2 and $PGF_{2\alpha}$ were stimulated similarly by the PMA-containing media employed in this section. The exceptions were A23187 containing media which did not have any significant effect on $PGF_{2\alpha}$. Comparing the treatments with each other revealed an inhibitory effect of A23187. It also showed that combining LPS and PMA resulted in a significantly greater stimulation of prostaglandin production, particularly PGE_2. This effect appeared to be lost when the guinea pigs were pretreated with RU486.

Progesterone had no effect, nor did RU486 *in vivo*.

Effect of Okadaic Acid Alone and in Conjunction with Other Compounds on Prostaglandin Output by Cultured Guinea Pig Cervical Cells

Okadaic Acid

Okadaic acid is an inhibitor of protein phosphatases and therefore prevents the dephosphorylation of intracellular proteins. It was examined to determine the effect of maintaining the phosphorylated state of basally phosphorylated proteins in the late pregnant guinea pig cervix. Okadaic acid (OM) was used to treat cells at a concentration of 50nM.

PGE_2 (Figure 5.14)

OA increased PGE_2 synthesis ($p=0.0058$). There was no significant effect of RU486 *in vivo* or progesterone *in vitro*.

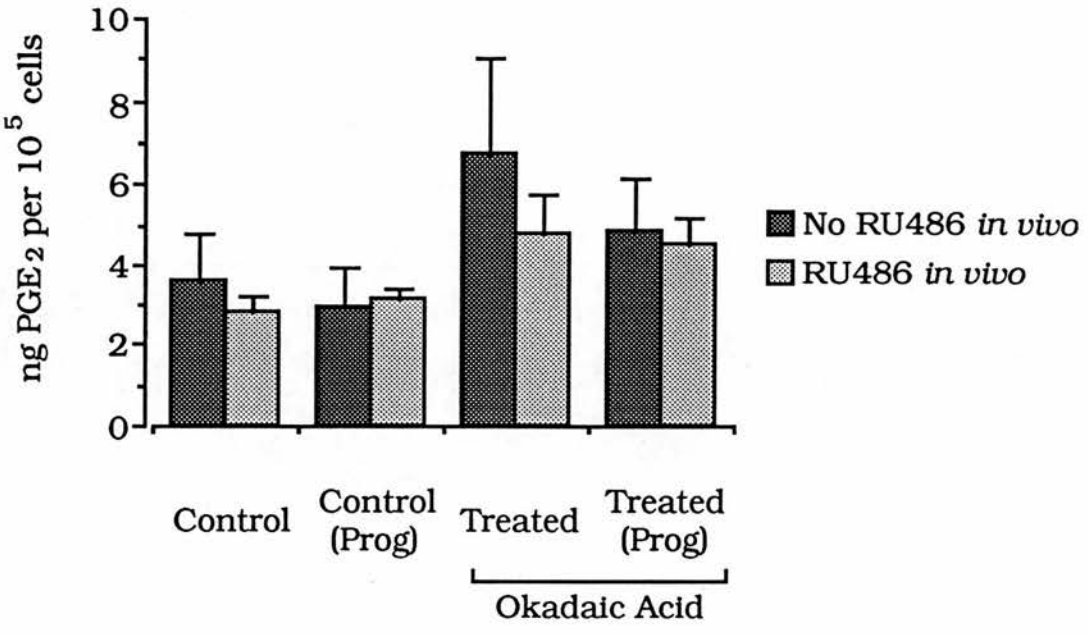


Figure 5.14 Effect of Okadaic Acid (50nM) on PGE_2 production (mean ± sem).

Okadaic Acid effect p=0.0058

$PGF_{2\alpha}$ (Figure 5.15)

OA treatment resulted in an increase in $PGF_{2\alpha}$ output (p=0.0048). Neither RU486 *in vivo* nor progesterone had an individual effect, however, there was a significant interaction between them (p=0.0241). This indicates that RU486 treatment of the guinea pigs blocked and possibly reversed a slight inhibition produced by progesterone.

$PGE_2/PGF_{2\alpha}$ (Data not shown)

The two prostaglandins were produced in similar quantities. Progesterone and RU486 *in vivo* had no significant effect.

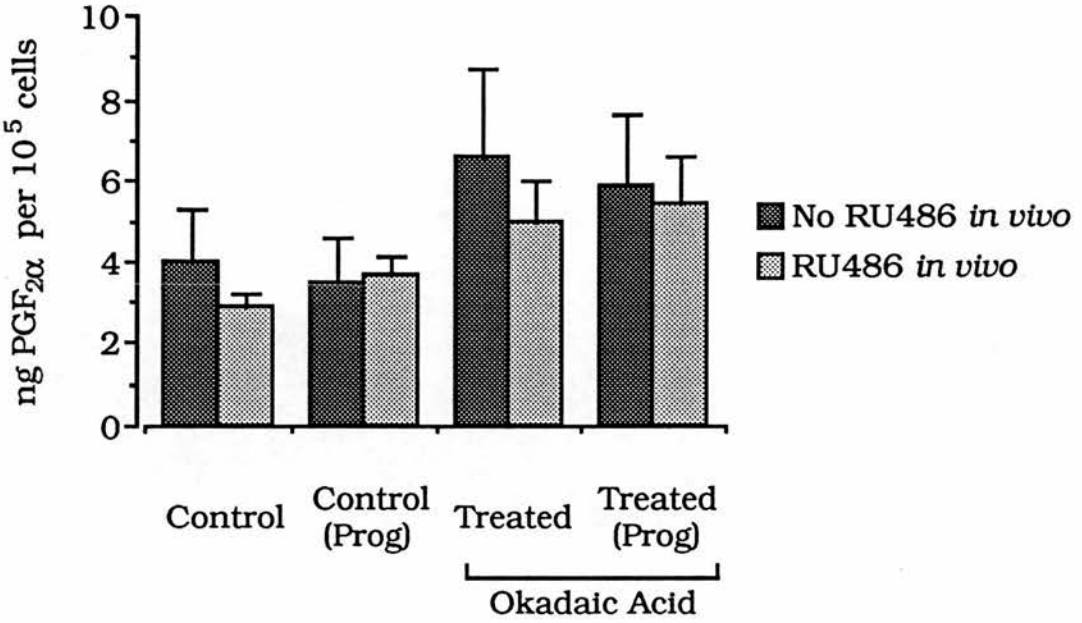


Figure 5.15 Effect of Okadaic Acid (50nM) on $PGF_{2\alpha}$ production (mean ± sem).

Okadaic Acid effect p=0.0048

RU486 *in vivo* x Progesterone interaction p=0.0241

Okadaic Acid + RU486 (*in vitro*)

Since the excised tissue was no longer in an environment containing RU486 and this was one of the main objects of the experiments performed, the antiprogestin was included in the culture medium to study its effects on prostaglandin synthesis.

PGE_2 (Figure 5.16)

Okadaic acid (50nM) and RU486 *in vitro* (1μM) combined stimulated PGE_2 (p=0.0012), however, there were no significant effects produced by RU486 *in vivo* or progesterone. The effect of okadaic acid + RU486 (*in vitro*) was not significantly different to that of okadaic acid alone (see table 5.22-23)

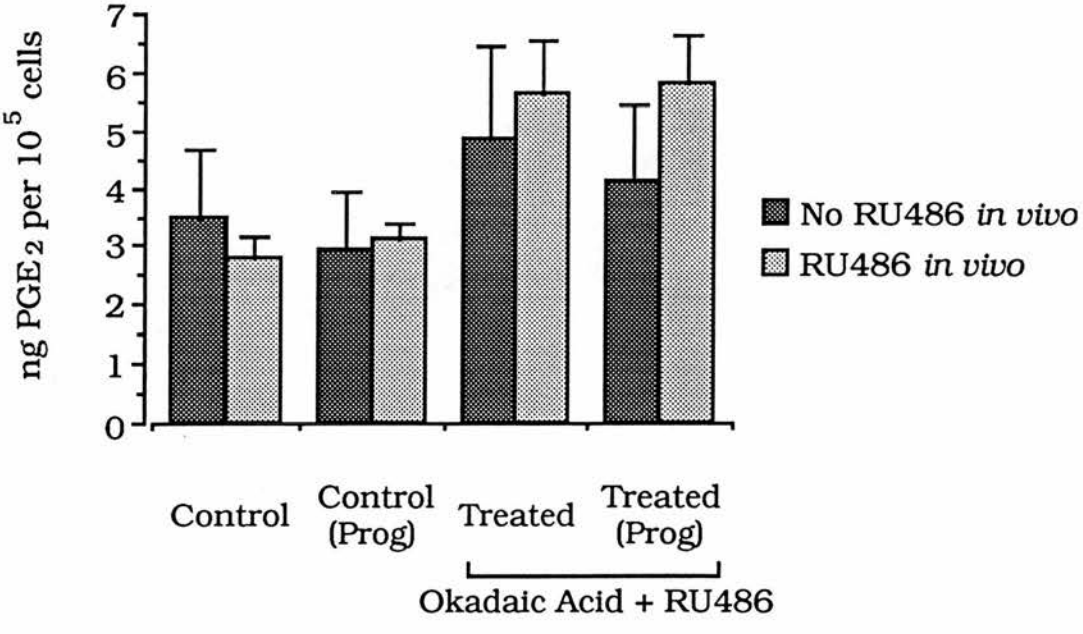


Figure 5.16 Effect of Okadaic Acid (50nM) + RU486 (*in vitro*, 1μM) on PGE_2 production (mean ± sem).

Okadaic Acid + RU486 (*in vitro*) p=0.0012

$PGF_{2\alpha}$ (Data not shown)

Significant stimulation of $PGF_{2\alpha}$ synthesis occurred under the influence of OA + RU486 in the culture medium (p=0.006). Treatment of the guinea pigs with RU486 did not result in any significant changes in $PGF_{2\alpha}$ synthesis, nor did progesterone in the culture medium.

$PGE_2/PGF_{2\alpha}$ (Data not shown)

PGE_2 and $PGF_{2\alpha}$ were produced in similar quantities and this was not significantly affected by progesterone or pretreatment of the guinea pigs with RU486 *in vivo*.

Okadaic Acid + A23187

Since A23187 was found to inhibit the stimulation of prostaglandin production by LPS, its effects on okadaic acid stimulation were also examined. Okadaic acid is a relatively new biochemical tool and it was also appropriate to examine the role calcium may play in its activity.

Cells were therefore exposed to okadaic acid (50nM) and the calcium ionophore (A23187, 2μM). This set of results contains information from only nine guinea pigs, five from control animals and four from those given RU486 *in vivo*.

PGE_2 (Figure 5.17)

A23187 appeared to block the stimulatory action of OA and statistically OA + A23187 did not induce any changes in PGE_2 synthesis. Despite this, comparison of the different treatments (see table 5.22-23) did not show the effect of OA alone to be significantly different to that of OA + A23187. Neither RU486 *in vivo* nor progesterone *in vitro* had significant effects on the results.

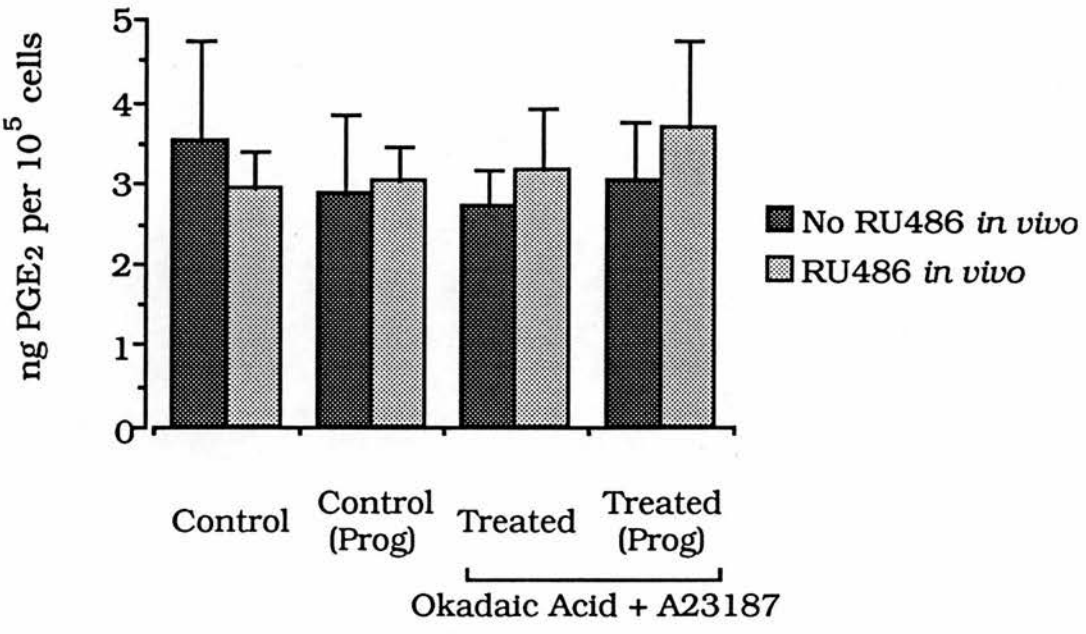


Figure 5.17 Effect of Okadaic Acid (50nM) + A23187 (2μM) on PGE_2 production (mean ± sem).

$PGF_{2\alpha}$ (Data not shown)

Again, A23187 appeared to have an inhibitory effect on OA stimulation and the OA + A23187 did not cause any significant change in prostaglandin synthesis when compared to the control. As observed with PGE_2 the difference between OA alone and OA + A23187 was not significant. RU486 *in vivo* and progesterone *in vitro* did not significantly affect the results.

$PGE_2/PGF_{2\alpha}$ (Data not shown)

Comparison of PGE_2 and $PGF_{2\alpha}$ showed they were produced in similar quantities. Progesterone in the culture medium and RU486 given to the animals had no significant effect.

Okadaic Acid + PMA

These are the same results as in the PMA section under PMA + Okadaic Acid (Figure 5.13). They are included in the following analyses because they contained Okadaic Acid.

Comparisons of Results Obtained from Experiments Involving Okadaic Acid

The data concerned are presented on pages 5.51 to 5.58.

PGE_2

Table 5.22 No RU486 *in vivo*

	PGE_2	No Progesterone Mn Diff (ng/10^5 cells)	sem	Progesterone Mn Diff (ng/10^5 cells)	sem
A	Okadaic acid	3.1	1.2	1.9	0.6
B	Okadaic acid + RU486	1.4	0.5	1.2	0.5
C	Okadaic acid + A23187	-0.8	1.0	0.2	0.6
D	Okadaic acid + PMA	5.6	3.4	3.8	1.7

'Mn Diff' Mean difference between treated cells and their controls.
No significant differences were detected.

Table 5.23 RU486 *in vivo*

	PGE_2	No Progesterone Mn Diff (ng/10^5 cells)	sem	Progesterone Mn Diff (ng/10^5 cells)	sem
A	Okadaic acid	2.0	0.9	1.4	0.7
B	Okadaic acid + RU486	2.8	0.7	2.7	0.7
C	Okadaic acid + A23187	0.2	0.3	0.6	0.6
D	Okadaic acid + PMA	5.5	2.5	4.7	2.0

'Mn Diff' Mean difference between treated cells and their controls.
No significant differences were detected.

Summary of Key Findings

Statistically no differences between PGE_2 synthesis in the presence of the various OA group treatments were significant. However, examination of the data suggests additional stimulation in the presence of PMA and once again an inhibitory action associated with A23187.

$PGF_{2\alpha}$

Table 5.24 No RU486 *in vivo*, no progesterone

	$PGF_{2\alpha}$	A	B	C	D	Mn Diff (ng/10^5 cells)	sem
A	Okadaic acid	X	-	-	-	2.6	0.9
B	Okadaic acid + RU486	-	X	-	-	1.7	0.8
C	Okadaic acid + A23187	-	-	X	s	-1.3	1.0
D	Okadaic acid + PMA	-	-	-	X	3.8	1.5

'Mn Diff' Mean difference between treated cells and their controls.
Left, lower triangle $p \leq 0.01$, right, upper triangle $p \leq 0.05$
's' indicates a significant difference.

Table 5.25 No RU486 *in vivo*, progesterone

	PGE_2	Mn Diff (ng/10^5 cells)	sem
A	Okadaic acid	2.4	0.8
B	Okadaic acid + RU486	2.0	1.2
C	Okadaic acid + A23187	-0.7	0.7
D	Okadaic acid + PMA	3.5	1.2

'Mn Diff' Mean difference between treated cells and their controls.
No significant differences were detected.

Table 5.26 RU486 *in vivo*, no progesterone

	$PGF_{2\alpha}$	A	B	C	D	Mn Diff (ng/10^5 cells)	sem
A	Okadaic acid	X	-	-	-	2.0	0.9
B	Okadaic acid + RU486	-	X	-	-	2.4	0.4
C	Okadaic acid + A23187	-	-	X	s	0.3	0.3
D	Okadaic acid + PMA	-	-	-	X	4.4	1.4

'Mn Diff' Mean difference between treated cells and their controls.
Left, lower triangle $p \leq 0.01$, right, upper triangle $p \leq 0.05$
's' indicates a significant difference.

Table 5.27 RU486 *in vivo*, progesterone

	PGE_2	Mn Diff (ng/10^5 cells)	sem
A	Okadaic acid	1.8	0.7
B	Okadaic acid + RU486	2.4	0.6
C	Okadaic acid + A23187	0.6	0.4
D	Okadaic acid + PMA	3.4	0.8

'Mn Diff' Mean difference between treated cells and their controls.
No significant differences were detected.

Summary of Key Findings

For $PGF_{2\alpha}$ the inhibitory effect of A23187 was significant but not on the effect of OA. While the influence of PMA did not produce a significant increase in prostaglandin synthesis over okadaic acid alone it did make the difference between OA + A23187 and OA + PMA significant. This latter effect was only seen in the absence of progesterone, but did occur in both groups of animals.

Summary of Data Involving Okadaic Acid

The following tables summarise the statistically significant results already described.

Effects on PGE_2 Production

This table summarises how PGE_2 output was affected by the treatments applied to the animals and the cultures.

Table 5.28

Treatment Media	RU486 *in vivo*	Prog *in vitro*	Treatment	Interactions
Okadaic acid	NS	NS	Increase	NS
Okadaic acid + RU486	NS	NS	Increase	NS
Okadaic acid + A23187	NS	NS	NS	NS
Okadaic acid + PMA	NS	NS	Increase	NS

NS No significant effect or interaction.

Increase Significant increase in prostaglandin synthesis.

Effects on $PGF_{2\alpha}$ Production

This table summarises how $PGF_{2\alpha}$ output was affected by the treatments applied to the animals and the cultures.

Table 5.29

Treatment Media	RU486 *in vivo*	Prog *in vitro*	Treatment	Interactions
Okadaic acid	NS	NS	Increase	RxP
Okadaic acid + RU486	NS	NS	Increase	NS
Okadaic acid + A23187	NS	NS	NS	NS
Okadaic acid + PMA	NS	NS	Increase	NS

NS	No significant effect or interaction.
Increase	Significant increase in prostaglandin synthesis.
R	RU486 pretreatment of guinea pigs.
P	Progesterone applied to cells.

Comparison of the Effects on $PGF_{2\alpha}$ and PGE_2 Production

This table summarises how $PGF_{2\alpha}$ and PGE_2 outputs were differentially affected by the treatments applied to the animals and the cultures.

Table 5.30

Treatment Media	RU486 *in vivo*	Prog *in vitro*	Treatment	Other Interactions
Okadaic acid	NS	NS	NS	NS
Okadaic acid + RU486	NS	NS	NS	NS
Okadaic acid + A23187	NS	NS	NS	NS
Okadaic acid + PMA	NS	NS	NS	NS

NS No significant difference or interaction.

Overview

RU486 treatment of the guinea pigs had no direct effect on the synthesis of either prostaglandin. Its significant effect was its interaction with progesterone on the set of cultures treated with okadaic acid alone, and then only affecting $PGF_{2\alpha}$.

The only detected effect of progesterone was its interaction with RU486 mentioned above.

Overall, PGE_2 and $PGF_{2\alpha}$ both showed a tendency to increase output over control in the presence of OA except for OA + A23187 where there was no significant change. A23187 appeared to have an inhibitory action. The comparison of treatments showed that they generally did not result in significantly different effects in the cultures. The only differences found were between OA + A23187 and OA + PMA, in the absence of progesterone and only on $PGF_{2\alpha}$.

Both prostaglandins were produced in similar quantities and were not differentially affected by the different treatments or RU486 *in vivo* or progesterone. The interaction between RU486 *in vivo* and progesterone detected on $PGF_{2\alpha}$ production under the influence of okadaic acid alone was strong enough to produce an interaction between the these two factors at this level also.

Effect of RU486, A23187 and Oestradiol-17β on Prostaglandin Output by Cultured Guinea Pig Cervical Cells

RU486 (1μM)

RU486 is know to promote cervical ripening in the guinea pig and was tested *in vitro* as well as *in vivo* in order to examine any role it may have in the production of prostaglandins in the late pregnant cervix. Once excised from the guinea pig the cervical tissue is no longer in contact with the antiprogestin and it was therefore included in the medium to determine whether or not any action that may have been attributable to it was dependent on its continued presence.

Cells were exposed to 1μM RU486 *in vitro.*

PGE_2 (Figure 5.18)

RU486 *in vitro* had a stimulatory effect on PGE_2 synthesis ($p=0.0030$), as did RU486 *in vivo* ($p=0.0389$). There was also an interaction between these two forms of RU486 treatment ($p=0.0188$), RU486 *in vivo* augmenting the effect of RU486 *in vitro.* Progesterone did not directly affect prostaglandin production but it did interact with RU486 *in vivo* ($p=0.0375$), the RU486 blocking and possibly reversing an inhibition of prostaglandin synthesis in the presence of progesterone.

$PGF_{2\alpha}$ (Data not shown)

The response of $PGF_{2\alpha}$ was largely the same as that for PGE_2. RU486 *in vitro* stimulated output ($p=0.0006$), RU486 *in vivo*

stimulated output (p=0.0044) and there was a similar interaction between RU486 *in vivo* and progesterone (p=0.0413).

$PGE_2/PGF_{2\alpha}$ (Data not shown)

Comparison of PGE_2 and $PGF_{2\alpha}$ showed that overall both were produced in similar quantities. There was a significant three way interaction with RU486 *in vivo*, RU486 *in vitro* and the two prostaglandins (p=0.0483). The RU486 *in vivo* produced an increase in prostaglandin production by control cells and RU486 *in vitro* treated cells but this effect was greater for $PGF_{2\alpha}$ than it was for PGE_2.

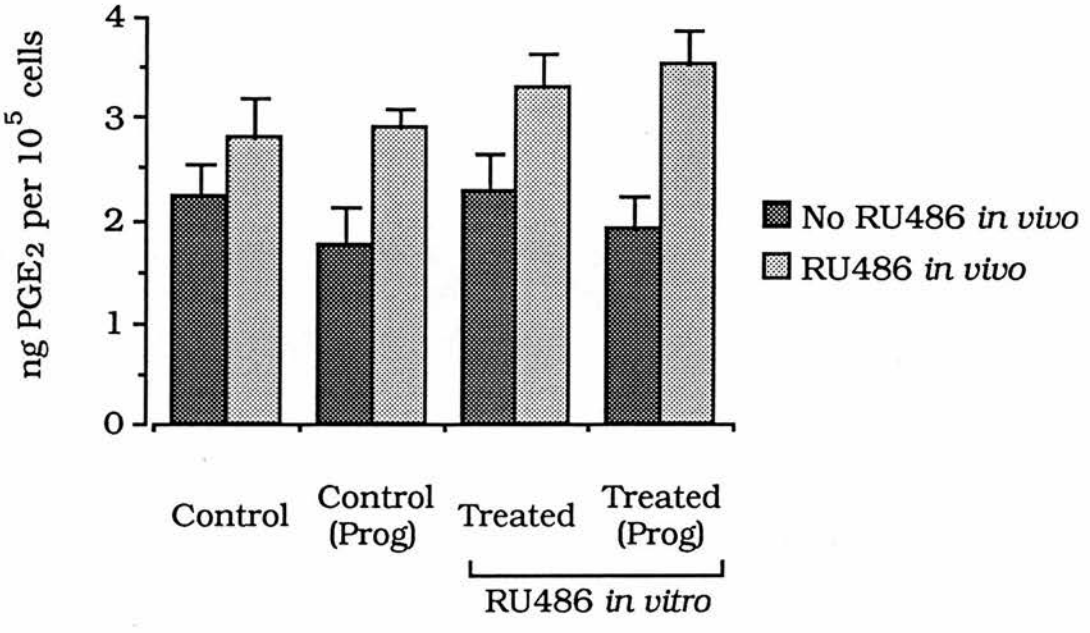


Figure 5.18 Effect of RU486 (*in vitro*, 1μM) on PGE_2 production (mean ± sem).

RU486 *in vitro* effect p=0.0030

RU486 *in vivo* effect p=0.0389

RU486 *in vivo* x RU486 *in vitro* interaction p=0.0188

RU486 *in vivo* x Progesterone interaction p=0.0375

RU486 *in vivo* x Progesterone interaction p=0.0375

RU486 (10μM, Data not shown)

RU486 was also used *in vitro* at a concentration of 10μM.

When the results from this treatment were compared with those from cells conditioned with RU486 *in vitro* at 1μM there were no significant differences found between the two concentrations of antiprogestin.

A23187

A23187 is a calcium ionophore, consequently raising the intracellular calcium concentration. In the majority of reported cases it is a stimulant of prostaglandin synthesis (Hsu and Goetz, 1993; Ibe and Raj, 1992; Houmbard et al., 1991; Cabre et al., 1993). R Leask (Pers. Comm.) had observed an inhibitory effect of the ionophore with human fetal membranes and it was therefore of interest to examine its effects in the late pregnant guinea pig cervix to study the role of intracellular calcium in regulating prostaglandin production. A23187 was used at 2μM.

PGE_2 (Figure 5.19)

A23187 did not significantly affect PGE_2 production. However, RU486 *in vivo* did result in an overall increase in output (p=0.0118). There was no overall effect of progesterone on PGE_2 synthesis, but the analysis did show an interaction with A23187 (p=0.0341), the progesterone blocking a mild inhibitory action of A23187 which was not large enough to be significant when analysed as an individual factor.

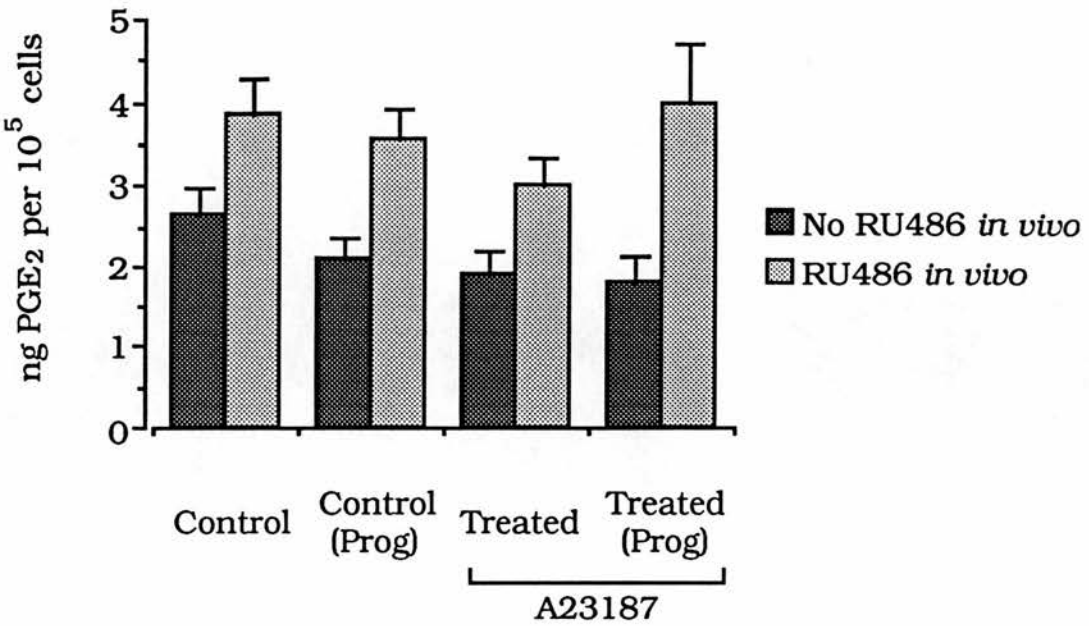


Figure 5.19 Effect of A23187 (2μM) on PGE_2 production (mean ± sem).

RU486 *in vivo* effect p=0.0118

Progesterone x A23187 interaction p=0.0341

$PGF_{2\alpha}$ (Figure 5.20)

A23187 had an inhibitory effect on $PGF_{2\alpha}$ synthesis (p=0.0010). RU486 treatment of the guinea pigs did not affect the synthesis of this prostaglandin, nor did progesterone. However, there was an interaction between progesterone and A23187 (p=0.0171), again evidence of blockade of the action of A23187 by progesterone.

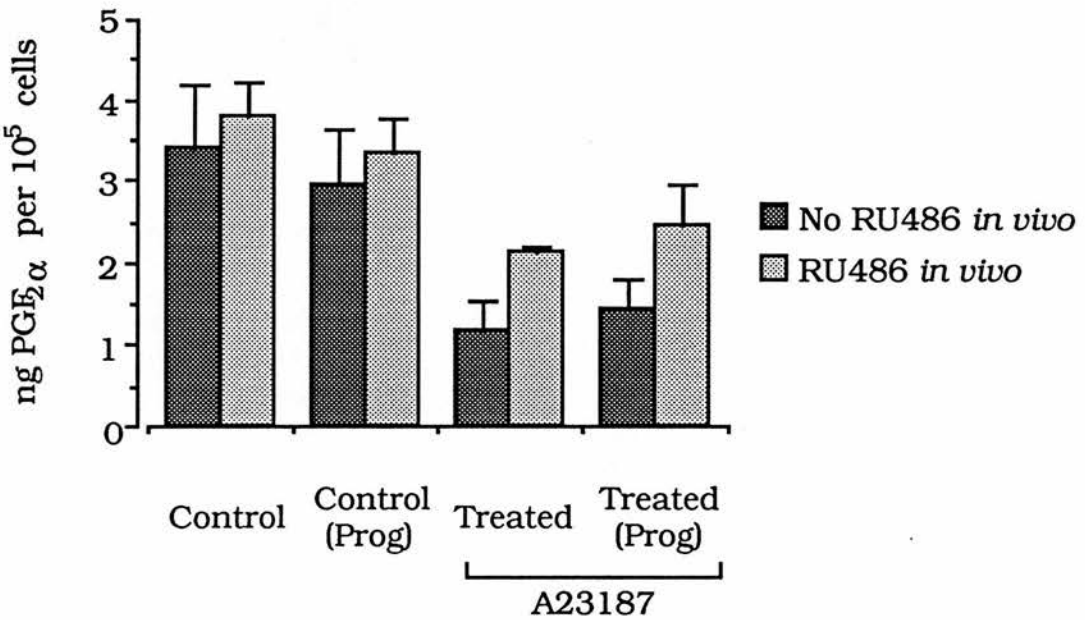


Figure 5.20 Effect of A23187 (2μM) on $PGF_{2\alpha}$ production (mean ± sem).

A23187 effect p=0.0010

Progesterone x A23187 interaction p=0.0171

$PGE_2/PGF_{2\alpha}$ (Data not shown)

Overall the analysis did not detect a difference in the production of the two prostaglandins. A significant interaction between A23187 and the two PGs (p=0.0130) showed that A23187 inhibited $PGF_{2\alpha}$ production to a greater extent than PGE_2.

Oestradiol-17β

Some authors have attributed cervical ripening effects to oestrogens, however, there are others have found no such effect. There are also reports of cultured fetal membranes producing increased amounts of prostaglandin when challenged by oestrogens. Thus, it was relevant to examine the effect oestradiol-17β would have on prostaglandin synthesis by cultured cells from the late pregnant guinea pig cervix.

Cells were cultured with oestradiol at a concentration of 10nM. This set of results was made up from five control animals and four given RU486 *in vivo*.

PGE_2 (Figure 5.21)

The small reduction caused by oestradiol was significant (p=0.0080). There were no significant overall effects of RU486 *in vivo* and progesterone *in vitro*. Progesterone and RU486 *in vivo* did show a significant interaction (p=0.0278), where the RU486 appeared to block and possibly reverse an inhibitory action attributable to progesterone.

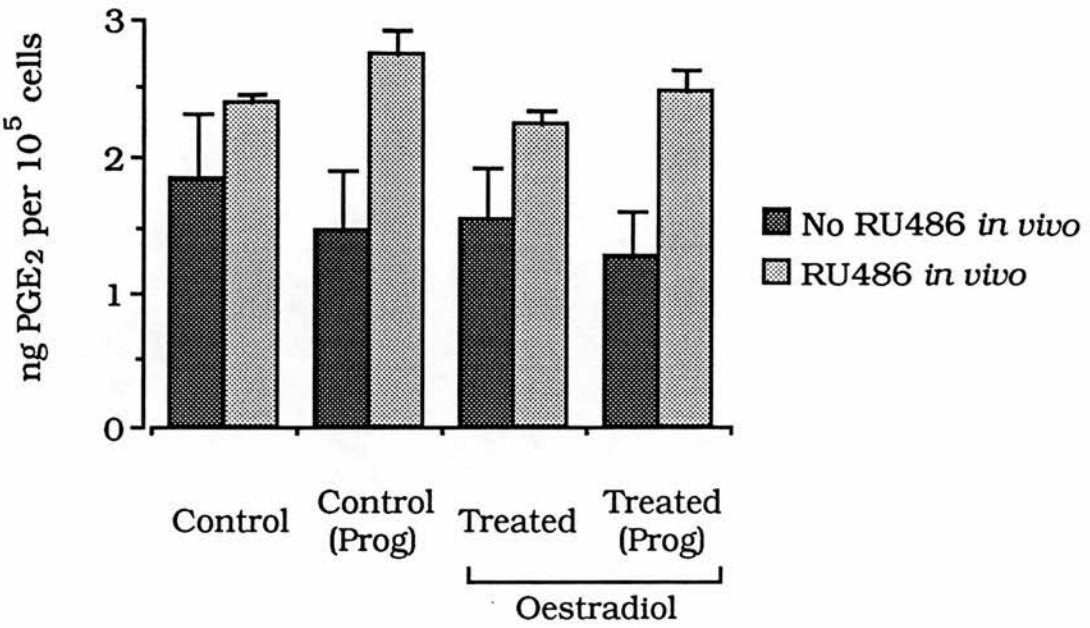


Figure 5.21 Effect of Oestradiol (10nM) on PGE_2 production (mean ± sem).

Oestradiol-17β effect p=0.0080

RU486 *in vivo* x Progesterone interaction p=0.0278

$PGF_{2\alpha}$ (Figure 5.22)

For this prostaglandin no significant effect of oestradiol was found. RU486 *in vivo* did, however, result in an increase in output (p=0.0104). Progesterone alone did not significantly alter the synthesis of $PGF_{2\alpha}$ but did interact with RU486 *in vivo* in a similar manner to that observed for PGE_2 (p=0.0080).

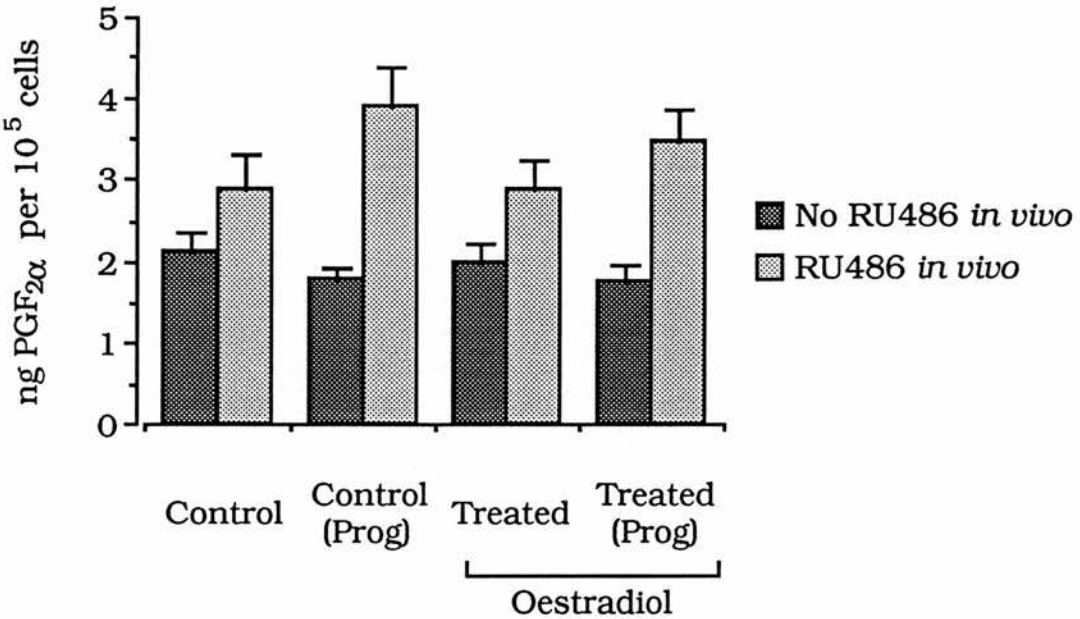


Figure 5.22 Effect of Oestradiol (10nM) on $PGF_{2\alpha}$ production (mean ± sem).

RU486 *in vivo* effect p=0.0104

RU486 *in vivo* x Progesterone interaction p=0.0080

PGE_2/$PGF_{2\alpha}$ (Data not shown)

Overall, PGE_2 and $PGF_{2\alpha}$ were produced in similar quantities. A significant interaction between the two PGs and progesterone (p=0.0477) indicated a stimulatory effect of progesterone on $PGF_{2\alpha}$ which was not detected on PGE_2 synthesis.

Medroxy Progesterone Acetate (MPA, 1μM, Data not shown)

MPA was included in the experiments as an analogue of progesterone. Four control animal cultures were treated but only two from the RU486 *in vivo* group.

Neither PGE_2 nor $PGF_{2\alpha}$ production showed any response to MPA.

The cultures from control animals produced less $PGF_{2\alpha}$ than PGE_2. The other results showed no difference but only two animals were involved in that analysis.

Serum Free Cultures (Data not shown)

Some cultures were carried out without FBS in the medium. As stated previously the basal production of prostaglandins in its absence lay at the lower end of the standard curve and this made the values obtained less reliable.

However, examination of the effects of LPS, PMA and OA indicated that they were all still capable of stimulating prostaglandin synthesis but to a much lesser degree than in the presence of FBS.

Summary of Data from RU486 *in vitro*, A23187 and Oestradiol

The following tables summarise the statistically significant results already described.

Effects on PGE_2 Production

This table summarises how PGE_2 output was affected by the treatments applied to the animals and the cultures.

Table 5.19

Treatment Media	RU486 *in vivo*	Prog *in vitro*	Treatment	Interactions
RU486 (1μM)	Increase	NS	Increase	RxP, RxT
A23187	Increase	NS	NS	PxT
Oestradiol	NS	NS	Decrease	RxP

NS	No significant effect or interaction.
Increase	Significant increase in prostaglandin synthesis.
Decrease	Significant decrease in prostaglandin synthesis.
R	RU486 pretreatment of guinea pigs.
P	Progesterone applied to cells.
T	Treatment applied to cells.

Effects on $PGF_{2\alpha}$ Production

This table summarises how $PGF_{2\alpha}$ output was affected by the treatments applied to the animals and the cultures.

Table 5.20

Treatment Media	RU486 *in vivo*	Prog *in vitro*	Treatment	Interactions
RU486 (1μM)	Increase	NS	Increase	RxP
A23187	NS	NS	Decrease	PxT
Oestradiol	Increase	NS	NS	RxP

NS	No significant effect or interaction.
Increase	Significant increase in prostaglandin synthesis.
Decrease	Significant decrease in prostaglandin synthesis.
R	RU486 pretreatment of guinea pigs.
P	Progesterone applied to cells.
T	Treatment applied to cells.

Comparison of the Effects on $PGF_{2\alpha}$ and PGE_2 Production

This table summarises how $PGF_{2\alpha}$ and PGE_2 outputs were differentially affected by the treatments applied to the animals and the cultures.

Table 5.21

Treatment Media	RU486 *in vivo*	Prog *in vitro*	Treatment	Other Interactions
RU486 (1μM)	NS	NS	NS	RxExT
A23187	NS	NS	F<E	NS
Oestradiol	NS	F>E	NS	NS

NS	No significant difference or interaction.
F<E	Significantly less $PGF_{2\alpha}$ than PGE_2 produced.
F>E	Significantly more $PGF_{2\alpha}$ than PGE_2 produced.
R	RU486 pretreatment of guinea pigs.
E	Prostaglandin E or F.
T	Treatment applied to cells.

Effect of Interleukin-1β and Tumour Necrosis Factor-α on Prostaglandin Output by Cultured Guinea Pig Cervical Cells

Interleukin-1 and tumour necrosis factor are important regulators of inflammatory responses. They can be produced by a variety of cell types, including macrophages/monocytes and polymorphs, and have been shown to stimulate fibroblasts to produce prostaglandins and collagenase. Since the main cell type in the cervix is the fibroblast it may be expected that these cytokines may be involved in the regulation of cellular functions, including the production of prostaglandins. As well as responding to IL-1, fibroblasts can produce it themselves and may therefore influence their own activity.

As mentioned earlier some experiments were carried out using serum free medium (culture medium with no fetal bovine serum added to it). PMA has already been shown to stimulate the production of prostaglandins in the guinea pig cultures used in this work where FBS was included. Other experiments with PMA using serum free medium were also performed and yielded similar results, a marked increase in prostaglandin synthesis being evident. As part of those experiments interleukin-1β (IL-1) and tumour necrosis factor-α (TNF) were also tested.

PGE_2 (Data not shown)

The results of this work relate only to cells derived from control animals.

IL-1 (up to 200units/ml, 23pM) had no effect on the production of PGE_2, nor did TNF (up to 200units/ml, 0.6nM) or a combination of the two cytokines.

No differences were detected between the three treatment conditions.

$PGF_{2\alpha}$ (Data not shown)

$PGF_{2\alpha}$ production was also found to be unaffected by treatment with IL-1, TNF or IL-1 + TNF. Again there were no differences between the three treatments.

$PGE_2/PGF_{2\alpha}$ (Data not shown)

Comparison of these two prostaglandins indicated that overall more $PGF_{2\alpha}$ was produced than PGE_2 in the cultures (IL-1 set $p=0.0249$, TNF set $p=0.0290$, IL-1 + TNF set $p=0.0295$) but that the IL-1 and TNF did not affect the comparison.

Problems Encountered with Interleukin-1 and TNF

IL-1

Interleukin-1 has been reported by several authors to stimulate prostaglandin production (Pfeilschifter et al., 1989; Elias, 1988; Elias et al., 1987), however, in the experimental work for this thesis no such effect was observed. Concurrent experiments using the same stock of IL-1 (British Biotechnology), also showed no response to the cytokine (I A Greer, Pers. Comm.). The IL-1 used in those experiments was dissolved in a protein free, physiological salt solution and used in sheep. Ovine and human IL-1β show 62% amino acid homology, ovine and murine IL-1β show 58% amino acid homology, all three show the same processing characteristics required of human IL-1β for biological activity (McInnes, 1993), and the ovine form has been shown to be similar enough to the mouse form that it can stimulate murine cells (Andrews et al., 1991). Thus it is probable that recombinant human IL-1 would be active in sheep.

The possibility that the IL-1 required the presence of other proteins to reduce the likelihood of it adhering to the storage tube was considered and a new supply of IL-1 was made up in 5% BSA/PBS solution or as before. The two stocks of IL-1 were then compared but once again no differences in prostaglandin output were observed.

Further experiments with IL-1 purchased from another supplier (Boehringer Mannheim) also failed to show any stimulatory, or inhibitory, effects of the cytokine, suggesting that human IL-1 may not be active in guinea pigs. However, Rajabi et al. have shown that procollagenase mRNA levels can be raised following conditioning of

guinea pig cervical cells with recombinant human IL-1 (Rajabi et al., 1991).

TNF

TNF has also been shown by other workers to stimulate prostaglandin synthesis (Pfeilschifter et al., 1989; Elias, 1988; Elias et al., 1987). The experiments carried out during this work did not find evidence of such in the guinea pig cell cultures but did uncover a mild stimulatory effect of the same stock of TNF on human cervical explants. This indicates that the TNF that was used was not biologically inactive or lost in handling as considered for IL-1.

IL-8 Production by Guinea Pig Cervical Cells in Culture

The cytokine interleukin-8 is known to be chemotactic for neutrophils and also to stimulate their degranulation. The presence of leucocytes in the ripe cervix may therefore be under the influence of IL-8, and for this reason the portion of guinea pig culture medium that was stored at -20°C (without methyloximation) was examined for its presence.

Samples of culture medium were selected on the basis of their response to stimulatory compounds. Medium from cells that showed an increase in prostaglandin production was assayed for IL-8 using antibodies directed against recombinant human IL-8.

There was no evidence of interleukin-8 production either in control medium or in that from stimulated cells. However, this may have been because the antibodies were directed against the human cytokine and not the guinea pig form.

Results Obtained Using the HeLa Cell Line

An experiment was carried out using the HeLa cell line as these cells were derived from a human cervix. This cell line was produced in the 1950s, was the first cell line developed and has been maintained ever since.

The response to challenge by LPS was examined. The cells were plated out at 10^5 cells per well and LPS included in the culture medium at 4μg/ml (as for the guinea pigs) and also at ten times that concentration (40μg/ml). In neither of the treatments was there any effect of LPS on the output of PGE_2.

HeLa cells are thought to originate from epithelial cells of the cervix and this suggested that the PGE_2 produced by the guinea pig cervices probably did not come from any epithelial cells in the cultures.

The effect of PMA on HeLa cells was examined at 0.1μM and at 0.01μM. Both concentrations caused stimulation of PGE_2 synthesis that was greater than the highest standard. This indicated that epithelial cells in the guinea pig cultures may, at least in part, have been the source of this prostaglandin when challenged by PMA.

The effect of OA on HeLa cells was examined at 50nM and caused stimulation of PGE_2 synthesis that was greater than the highest standard. This indicated that epithelial cells in the guinea pig cultures may, at least in part, have been the source of this prostaglandin produced in response to okadaic acid.

Chapter 6

Guinea Pig Study Discussion

Guinea Pig Study Discussion

The following pages examine and discuss the preceding results obtained from the guinea pig work.

The cultures used in this work were derived from and representative of the whole, late pregnant, guinea pig cervix. Therefore there would have been a variety of different cell types present, including fibroblasts, smooth muscle, epithelial and endothelial cells, constituent macrophages and mast cells, and possibly other leucocytic cells. The cervix at this stage of gestation would have undergone certain changes associated with pregnancy, such as replacement of older collagen with newer, less cross-linked collagen and alterations in the GAG composition. Thus, it would be different from the nonpregnant cervix and also be primed for the last stages of cervical ripening

Thus, the results obtained, while not identifying the cell type responsible, give an indication about the capabilities of the guinea pig cervix as an organ of mixed cellularity, and therefore resembles the *in vivo* situation.

There is of course the disadvantage that the blood supply is lost, which means that any infiltration or effect of leucocytes is not accounted for. However, this applies equally to most *in vitro* studies. There is also a loss of geometric distribution of the various cell types, those that may not normally be in close contact with each other lying together, and vice versa.

This technique of tissue digestion and the release of cells has the advantage over simply incubating small pieces of material in that the bathing fluid comes into intimate contact with each of the cells

and the individual wells will show greater homogeneity, since such small pieces of tissue may be from different parts of the cervix. The tissue can also be distributed more evenly, between greater numbers of wells and therefore treatments, by virtue of the fact that the dissection of any material by hand is relatively crude and produces a wide range of sizes. In addition, the use of cell culture can allow time for the cells to become accustomed to their new environment and the subsequent removal of any cell debris prior to the commencement of the experiment. With tissue culture the material is generally incubated as soon after excision as possible and any necrotic tissue, which may affect the results, cannot be removed.

Prostaglandins

Although prostaglandins are not considered to be stored within cells, except for some neurones, the prostaglandin synthesis inhibitor, indomethacin, was used to confirm the synthetic capabilities of the cells being studied and did so, its addition to medium containing LPS blocking the stimulatory effect of that agent. The cells were also able to maintain prostaglandin synthesis for several days, an unlikely situation if this was due to the release of prepackaged stores.

Because the medium contained FBS it was examined for the presence of endogenous prostaglandins. No $PGF_{2\alpha}$ or PGFM were detected and negligible amounts of PGE_2, however, there was slightly more PGEM and very high levels of thromboxane B_2. The latter component's presence may be explained in terms of the clotting that would undoubtedly have taken place in the fetal blood. TxA_2 is an integral part of the clotting process and platelet aggregation, and quickly breaks down to TxB_2. The reason for the presence of PGEM in

the FBS is less clear, and surprising, since the level of PGE_2 measured was negligible and it seems unlikely that all of it would have been converted to the metabolised form. It is possible that this was due to a contaminant that cross reacted with the antiserum, however, its identification would involve extensive separation and purification of the constituent parts of the FBS and testing of them in the assay.

The inclusion of the bovine serum in the medium was justified by the low basal output of prostaglandins in its absence, which would have meant the results were read from the lower end of the standard curve which was less sensitive and thus less accurate giving less reliable results. In addition, this would not have allowed for the detection of any inhibitory effects on prostaglandin synthesis.

PGE_2 and $PGF_{2\alpha}$ were selected for assay because they represent the prostaglandins consistently identified by others as the predominant prostanoids produced by cervical tissue and therefore the most likely to play a significant role in cervical biology. In addition, human clinical experience has shown that PGE_2 and $PGF_{2\alpha}$ are effective ripening agents, PGE_2 being the prostaglandin of choice.

The assay system used in this work relied on the conversion of the prostaglandins in the harvested medium to their methyloxime derivative. This process protects the PGs from dehydration and therefore makes them more stable. The development of antisera is improved because the methyloxime derivative of the prostaglandin in question persists *in vivo* for longer, presenting a greater antigenic challenge, and there are also no endogenous methyloximated prostaglandins to bind to the antibodies produced and thus result in the loss of those antibodies from the circulation (Kelly et al., 1989).

Treatments

RU486 is now available to clinicians in the UK for the termination of early pregnancy. It was the first drug of its type to reach clinical trials and is the only antiprogestin to be given a product license in Britain.

RU486 was shown to be effective in producing cervical ripening several years ago (Lefebvre et al., 1990). It is thought to act at the receptor level as an antagonist to progesterone which implies that progesterone may be important in inhibiting the process of cervical ripening. As progesterone may have inflammation suppressing properties (Stites and Siiteri, 1983; Siiteri et al., 1977) this antagonism of the steroid supports the hypothesis that the changes that occur in the cervix are the result of an inflammatory style reaction. RU486 has also been shown to interact with prostaglandins and to increase the sensitivity of the uterus to administered PGs, as well as allowing spontaneous contractions to develop (Bygdeman and Swahn, 1985; Swahn and Bygdeman, 1988).

This work on guinea pigs was carried out in an attempt to find out more about the effects of RU486 on the endogenous prostaglandins and how other agents' *in vitro* activity may be affected by the antiprogestin. The aim of these experiments was to improve understanding of how RU486 produces cervical ripening and how it may be interacting with the natural ripening mediators. The dosage of RU486 was set at 10mg because it has been found to be effective in previous work with the guinea pig (Elger et al., 1987a).

It was also hoped that the production of interleukin-8 could be examined in the same preparations, but unfortunately the cytokine was undetectable in the assay system used. This may have been

because there was none, or very little, produced or because the guinea pig form of IL-8 was not recognised by the antiserum. One possible solution was to freeze dry the samples and reconstitute them in a smaller volume. However, with only 500µl available and no IL-8 detected in one fifth of that volume, it is unlikely that the minor degree of concentration that could have been produced (approximately 4 to 5 fold) would have been sufficient. Even if IL-8 was detected, at such low levels the results would have been in the lower range of the assay where the curve is relatively insensitive.

The study of interleukin-8 was thus confined to the medium harvested from the human biopsy material (see chapter 7).

The vehicles used for the agents employed in this work *in vitro* were dimethylformamide (DMF) or ethanol, which were found not to affect the quantities of prostaglandin produced by cells incubated in control medium containing only the vehicle as an additional component. Water and saline were also used as solvents.

RU486

The comparison of the combined basal production figures from each group of animals, showed that RU486 treatment of the animals did not have a significant effect on the output of either PGE_2 or $PGF_{2\alpha}$.

Because of the large variation between each PGEM assay, a single assay was performed incorporating medium from all the controls and from all the progesterone conditioned controls in order to give some indication of what effect RU486 may have had on the basal levels of this metabolite, thus allowing a comparison of those with and without RU486 pretreatment and those with and without progesterone

conditioning. This showed that RU486 *in vivo* did not affect the generation of PGEM.

From the results obtained for PGE_2 and $PGF_{2\alpha}$ under the influence of the other *in vitro* treatments it was found that giving guinea pigs RU486 prior to sacrifice had no effect on the stimulation caused by LPS, PMA, OA or combinations of these agents. The most obvious effect was generally associated with the media that contained the calcium ionophore, A23187, in conjunction with LPS and PMA. In these cases the ionophore appeared to have an inhibitory effect on prostaglandin synthesis, but in the cultures from animals pretreated with RU486 the effect of the ionophore was reduced. PMA or OA combined with A23187 did not show any significant effect of such RU486 treatment, suggesting that the effect may have been on the action of LPS. However, there did appear, based on the figures, to be a small increase in the synthesis of both prostaglandins in the PMA + A23187 treated cells when RU486 pretreatment had been given.

The experiment with A23187 alone showed a stimulatory effect of the antiprogestin on PGE_2 production, but here both the control and ionophore treated cells produced more prostaglandin. The output of $PGF_{2\alpha}$ was similarly greater from cells treated with A23187, the effect on the control cells being less than for PGE_2. RU486 did not significantly affect the action of A23187 itself.

Results obtained with cells in the oestradiol incubation group indicated that some stimulation of $PGF_{2\alpha}$ had occurred as a consequence of pretreatment of the animals with RU486. The antiprogestin did not affect the action of oestradiol. The significant interaction between RU486 and progesterone observed in this set of data appears to show an inhibitory effect of progesterone in the

absence of RU486 and a stimulatory effect of progesterone in the presence of RU486, for both prostaglandins.

In the cultures where additional RU486 challenge of the cells was provided by *in vitro* antiprogestin, a general stimulatory effect of *in vivo* RU486 was found, similarly to the A23187 set of data. This effect was apparent for both prostaglandins. RU486 interacted with progesterone in the same way as it did with oestradiol, again for both prostaglandins, but it also produced a significant increase in the stimulatory effect of the RU486 *in vitro*.

RU486 combined *in vitro* with LPS or PMA or OA did not show any significant effects of pretreatment with RU486 *in vivo*.

On the whole, the effect of RU486 *in vivo* was limited. In a few instances it caused a general increase in prostaglandin synthesis and in particular when associated with LPS and A23187 resulted in the blockade of an inhibitory effect of the calcium ionophore. Why RU486 interacts in this way is not clear. The results suggest a calcium dependent factor in the action of RU486 on LPS, since RU486 did not affect the action of LPS in the absence of A23187. It is possible that RU486, or a product of its action, requires calcium to amplify the effects of LPS rather than block the action of A23187. If the action of RU486 was on A23187 itself then the effect would also have been observed in the experiment using PMA.

If RU486 induces cervical ripening, at least in part, through an action on prostaglandins then the effect observed with A23187 (alone and with PMA) suggests that calcium may not be a significant factor in any increase in PG synthesis that occurs following administration of the antiprogestin.

Progesterone

The effect of progesterone inclusion in the culture medium was examined. Progesterone receptor levels in the cervix have been shown to be unchanged during the menstrual cycle (Sanborn et al., 1976; Sanborn et al., 1978), which suggests that they may not be strongly influenced by progesterone or oestrogen levels. The concentration of progesterone in the cervical tissue *in vivo* is difficult, if not impossible, to determine. However, the production of this steroid in reproductive tissues during pregnancy is high and increases with gestation (Buster and Simon, 1989; Liggins, 1983; Csapo et al., 1972) which means that the local concentration may well be high and justifies the use of 10^{-6}M progesterone in the culture medium.

From the comparison of the combined control values for all the guinea pigs in both groups, it is apparent that progesterone caused a modest fall in the synthesis of PGE_2 by cells derived from control animals. The same pattern was seen for $PGF_{2\alpha}$, but the reduction was not significant. No significant effect of RU486 *in vivo* on progesterone action was detected for either prostaglandin. The PGEM assay of the control and progesterone controls indicated that progesterone did not affect the generation of PGEM. It appears that inclusion of progesterone in the culture medium throughout the culturing period did not have a strong influence on the production of prostaglandins.

The majority of the experiments carried out did not find evidence of a direct effect of progesterone. The only set of data that showed such an effect was that of PMA alone, where progesterone produced an overall reduction in PGE_2 output. The effect of progesterone was more evident when looking at its relationships with the treatments applied to the cells and the pretreatment of the guinea

pigs with RU486. The interactions with treatments (LPS + A23187, LPS + PMA + A23187 and A23187 experiments) appear to show a stimulatory effect of progesterone. The interactions with RU486 *in vivo* (LPS + PMA + A23187, Okadaic acid, RU486 and Oestradiol experiments) also suggest a stimulatory activity.

Overall the results suggest that progesterone probably played little or no part in the control of prostaglandin synthesis in the guinea pig cervix cultures but given the clinical evidence concerning the ripening effects of the antiprogestins it seems likely that progesterone is crucial in the process. Despite the lack of an anticipated inhibitory function for progesterone with regard to the synthesis of prostaglandins, this steroid may well be involved in the suppression of the action of other inflammatory mediators such as the cytokines, for example as seen with interleukin-8 (Kelly et al., 1992). It is also possible that progesterone may prevent or at least delay cervical ripening by inhibiting uterine activity and thereby the mechanical stresses placed upon the cervix, such activity being likely to induce mechanical disturbances in the cervix and thus promoting prostaglandin production.

The RU486 and progesterone results taken together show neither to have a significant role in the production of prostaglandins by the late pregnant guinea pig cervix. Since progesterone did not produce the inhibitory effect that was anticipated, an antagonistic effect of RU486 was unlikely, and the results indicate that RU486 did not stimulate PG synthesis in the absence of progesterone.

Comparisons Between PGE_2 and $PGF_{2\alpha}$ Production by the Same Cells

Comparing the basal production levels of the two prostaglandins revealed that similar quantities of each PG were synthesised by the cells from RU486 treated and control animals and that progesterone had no significant affect.

Most cases where a difference was found showed less $PGF_{2\alpha}$ than PGE_2 production in the presence of the treatment medium. Differences between PGE_2 and $PGF_{2\alpha}$ synthesis were not unexpected since the effect of each compound on the synthesis of each PG need not necessarily be identical. All the LPS containing media resulted in greater production of PGE_2 than $PGF_{2\alpha}$ as did PMA + A23187 and A23187 alone. The data from the PMA alone experiment showed that, while the overall trend was for both PGs to be produced in similar quantities, there was a greater inhibitory effect of progesterone on PMA stimulation of PGE_2 than of $PGF_{2\alpha}$. The RU486 *in vitro* experiment showed there was a greater stimulation of $PGF_{2\alpha}$ than of PGE_2, but again no overall difference in the output of the two prostaglandins. The only other detected difference between the PGs was in the oestradiol data set. Here progesterone stimulated $PGF_{2\alpha}$ synthesis under control and treated conditions but not PGE_2 synthesis. Okadaic acid treated cultures showed no differences between $PGF_{2\alpha}$ and PGE_2 production.

These figures suggest that okadaic acid stimulates the production of $PGF_{2\alpha}$ and PGE_2 equally but that PMA, LPS, A23187 and RU486 preferentially induce PGE_2 over $PGF_{2\alpha}$.

The conversion of arachidonic acid to PGH_2 is common to both PGE_2 and $PGF_{2\alpha}$ (Smith, 1986; Kulmacz, 1987; Coleman et al. 1990).

Therefore it is possible that the effect of okadaic acid on the conversion of PGH_2 to PGE_2 and $PGF_{2\alpha}$ results from similar activity towards the isomerase and reductase enzymes responsible for each PG respectively (Smith, 1986; Kulmacz, 1987; Coleman et al. 1990) and the other drugs result in greater stimulation of the PGE_2 pathway. The enzyme prostaglandin E 9-keto reductase has been shown to covert PGE_2 to $PGF_{2\alpha}$ *in vitro*, thus it is possible that a differential effect could result form inhibition of this enzyme producing a build up of PGE_2 and a deficit in $PGF_{2\alpha}$. However, the reaction kinetics suggest that this is unlikely to be operative at physiological prostaglandin levels and that the enzyme has another function *in vivo* (Kulmacz, 1987).

Effects of the Different Treatments on Prostaglandin Production

The response of the guinea pig cells to the different agents employed in this work were varied but more usually provided evidence of a stimulatory action on prostaglandin synthesis. Some did not appear to affect PG synthesis and there were some cases where the output of prostaglandins from cells exposed to the test substance was lower than the control level. In most cases $PGF_{2\alpha}$ and PGE_2 responded with similar increases or decreases in output although, as already mentioned, not necessarily to the same degree.

LPS was obviously capable of stimulating the cells in the cervix cultures to produce prostaglandins E_2 and $F_{2\alpha}$, as was PMA. Both stimulated in the presence and absence of fetal bovine serum. The oestradiol results on the other hand appeared to show a more selective effect, inhibiting PGE_2 production but not $PGF_{2\alpha}$. The calcium ionophore, A23187, also seemed to show a selective tendency

towards one of the PGs over the other, inhibiting $PGF_{2\alpha}$ but not PGE_2. The antiprogestin, when used *in vitro*, stimulated both PGE_2 and $PGF_{2\alpha}$. Medroxy progesterone acetate used *in vitro* with control animal cells did not have any effect on the output of either prostaglandin. In serum free cultures IL-1 and TNF were found to be inactive.

Various combinations of the different agents were tested and although they may individually have had a significant effect on prostaglandin synthesis, there were obvious changes associated with combined treatments that did not always reach a significant level when compared to each substance on its own.

LPS and PMA were both found to stimulate PGE_2 and $PGF_{2\alpha}$ synthesis but only $PGF_{2\alpha}$ production, in the absence of progesterone, showed a significant increase when the two stimulants were combined, despite other apparent increases. Also, okadaic acid alone acted as a stimulant but when combined with PMA failed to produce a significant further increase in PG production. Comparison of the degrees of LPS and PMA evoked stimulation revealed no differences between the two. However, comparisons amongst LPS, PMA and OA showed that LPS and PMA both stimulated PGE_2 to a greater extent than OA, but this effect was less apparent for $PGF_{2\alpha}$.

PMA + OA did not produce significantly more of either prostaglandin than either stimulant alone, despite apparent increases produced when compared with OA on its own.

The inclusion of A23187 with LPS, PMA or OA in the culture medium appeared to cause a general inhibition of prostaglandin synthesis. However, many of these apparent differences caused by the presence or absence of A23187 in those cultures were not shown to be

significant. The most consistent factor with regard to the significance of the reduction was the inclusion of LPS in the medium. The majority of the LPS group results showed significant reductions associated with the calcium ionophore and the effect appeared to more often significant for $PGF_{2\alpha}$ than for PGE_2. The PMA group, despite similarly showing apparent reductions in PG synthesis in response to co-culture with A23187, showed fewer significant falls and those that were significant were again more common for $PGF_{2\alpha}$. No significant differences were found between the cells treated with okadaic acid and those treated with okadaic acid + A23187, even though the former produced a significant increase in PG production and the latter did not. The selective effect of A23187 on $PGF_{2\alpha}$ concurs with the previously mentioned activity of A23187 alone, where it was shown to inhibit $PGF_{2\alpha}$ but not PGE_2.

RU486 used on its own (*in vitro*) did produce an increase in PG synthesis but the presence of RU486 in the culture medium with LPS or with PMA did not show any additional stimulatory function of the antiprogestin.

Hela Cell Line Experiment

A minor side experiment with the HeLa cell line was performed with some of the agents used in the guinea pig cultures. This cell line was derived from a human, cervical tumour in the 1950s and is of epithelial origin. This experiment gave some qualitative data suggesting that the PMA and OA were activators of epithelial cells but implying that LPS was not, despite evidence that LPS can have stimulatory actions on human vascular endothelial cells (Strieter et al., 1989).

Action of the Substances Studied

G-Protein/Protein Kinase C/Phosphatase

The action of LPS is thought to involve G protein activation, which supports the presence of a receptor for LPS since G proteins appear to be associated with receptor mediated signal transduction (Stryer and Bourne, 1986). For cells other than those involved in the detection and removal of bacteria to possess such a receptor seems unlikely, unless LPS is merely utilising a receptor for some other endogenous molecule, such as that implied by the fact that it competes with acetylated LDL for the same binding site (Raetz, 1990). Such receptors for acetylated LDL were not found in significant quantities on cell types other than macrophages, and were absent from human fibroblasts (Brown and Goldstein, 1983). The effect of LPS observed in the guinea pig work shows that the cervix is capable of producing prostaglandins in response to the bacterium derived material. The HeLa result and the finding by Hampton et al. (Hampton et al., 1988) that fibroblasts may be deficient with regard to a binding site for LPS, and thus may not be able to respond to it, suggests that other cells of the cervix might be involved in LPS stimulated PG synthesis, the likely candidates being macrophages and possibly other resident white cells, or alternatively supporting the hypothesis that LPS can also intercalate into the membrane and affect intracellular activity in some way via that route.

Fibroblasts have been shown to produce prostaglandins (Elias et al., 1987) and are the major cellular component of the cervix. Both PMA and LPS were shown to be capable of producing similar increases in PG output from guinea pig cervix cultures and to exclude the

fibroblasts from the total cell population and their component of the prostaglandins synthesised, a significant difference would have been anticipated between the two stimulants, with LPS having far fewer cells which possess LPS receptors and thus in which it could provoke PG synthesis. This assumes that PMA would be active in any fibroblasts present, a situation which seems likely since PMA does not appear to act via a receptor in the cell membrane but by inserting itself into the membrane.

The activation of G protein in the cell membrane by LPS also suggests the involvement of a second messenger system. It has been shown that LPS can stimulate protein kinase C (Wightman and Raetz, 1984) and it is thought to inhibit adenylate cyclase (DeFranco, 1987).

The stimulatory action of PMA shows that the cells of the pregnant guinea pig cervix are able to respond to the phorbol ester, indicating that at least some of the cell types present can be induced to produce prostaglandins via protein kinase C, since PMA has a direct PKC binding and activating function. Thus the subsequent phosphorylation of intracellular proteins by PKC must be involved in PG synthesis and OA, which blocks the dephosphorylative action of phosphatases, would be expected to increase the stimulatory effect. However, the lack of any additional effect of OA over PMA alone may indicate that production of PGs in those cells stimulated through the PKC pathway had reached a plateau. Lipocortin is an endogenous inhibitor of prostaglandin production (Flower, 1988) and has been shown to be inhibited itself when phosphorylated (Hirata, 1981). Therefore, it is possible that PKC may promote prostaglandin synthesis, at least in part, through the removal of this endogenous homeostatic mechanism.

Although PMA and LPS independently stimulated both PGs the comparison between the two together and each alone showed mixed results, the majority of comparisons revealing no significant differences. This lack of a significant difference between the effect of PMA or LPS alone and the two agents in combination may suggest that they act via the same mechanism and this had reached a maximal level. However, the data appear to show that there was an increase in prostaglandin synthesis which was not detected statistically, except in a few cases, and this may indicate an additive effect through the same mechanism or alternatively through differential pathways.

Okadaic acid was shown to cause an increase in PGE_2 and $PGF_{2\alpha}$ synthesis. This shows that the basal production of PGs involves the phosphorylation and dephosphorylation of intermediate proteins/enzymes since OA inhibits the removal of phosphate groups, thereby increasing the level of phosphorylated proteins. This may result in the maintenance of an inactive state for molecular species that would normally be involved in prostaglandin degradation or the maintenance of an active state for other mediators which support prostaglandin production.

Okadaic acid was examined in conjunction with PMA. Since PMA acts through the activation of protein kinase C, and thereby the phosphorylation of intracellular mediators, it would be expected that inhibition of the natural reversal of that phosphorylation may increase the effects associated with PMA. This was not the observed effect. Okadaic acid was shown to raise the level of PG synthesis in its own right, presumably by altering the basal control mechanism, but not to affect the stimulation imposed by PMA. This may indicate that PMA

induced prostaglandin production is not dependent on the phosphorylative activity of PKC and that the effect of PMA is not produced merely through the modulation of the basal synthesis mechanism, or that PMA causes maximal, constant activation of phosphorylation thereby removing any role for okadaic acid.

Calcium/A23187

The importance of calcium in cellular activity is well recognised. The calcium ionophore, A23187, allows the concentration of calcium ions in the cytosol to increase by swapping two H^+ ions for each Ca^{2+} ion that flows down its concentration gradient (Alberts et al., 1983). In these experiments A23187 appeared to have a predominantly inhibitory effect on prostaglandin synthesis. The increase in Ca^{2+} seemingly having a greater effect on $PGF_{2\alpha}$ than PGE_2. This would suggest that the two PGs have different control mechanisms that differ in their sensitivity to calcium concentration. Calcium ions have been found to bind to lipocortin (Schlaepfer and Haigler, 1987), a protein which inhibits prostaglandin synthesis through the inhibition of phospholipases (Blackwell et al., 1980; Blackwell et al., 1982; Hirata, 1981; Hirata, 1983) and therefore a reduction in eicosanoid pathway substrate availability, thus A23187 may have produced this inhibitory action through an effect on lipocortin. It has been shown that calcium is required for the eicosanoid inhibiting activity of lipocortin (Cirino and Flower, 1987b; Cirino and Flower, 1987a) and that calcium can also permit lipocortin to bind to the phospholipase substrate molecules (e.g. phosphatidylethanolamine, phosphatidylserine) (Davidson et al., 1987; Aarsman et al., 1987; Rothhut et al., 1987). A reduction in substrate availability would have

been more likely to affect all downline processes to the same extent and this was not the observed effect.

The inhibition produced by A23187 was surprising. The bulk of the available evidence indicates that the calcium ionophore is a stimulant of prostaglandin production (Hsu and Goetz, 1993; Ibe and Raj, 1992; Houmbard et al., 1991; Cabre et al., 1993). It has been shown to increase PG output by the guinea pig uterus (Poyser and Ferguson, 1993), and also shown to stimulate PGE_2 production by cells in culture at a concentration of 1μM (Ljunggren et al., 1993). The concentration used in the experiments described in this thesis was 2μM. There is also evidence of a stimulatory effect of the ionophore on eicosanoid production at the higher concentration of 5μM (Ibe and Raj, 1992). A Japanese group have shown that A23187 stimulates the rabbit cervix to produce PGs (Okawa, 1990), and there is a report showing a greater degree of $PGF_{2\alpha}$ synthesis than PGE_2 under the influence of A23187 (Calderaro et al., 1991).The calcium ionophore has been shown to increase PG output through a stimulatory action on arachidonic acid release (Hsu and Goetz, 1993), and at the same time these authors showed that A23187 did not affect the conversion of arachidonic acid to prostaglandins. Potestio and Olson also observed increased arachidonic acid release by cells cultured with A23187, at concentrations as high as 5μM (Potestio and Olson, 1990). In the work presented here A23187 co-cultured with PMA resulted in apparent reductions in PG synthesis, although not always significantly, however, this is in complete contrast to the findings of Kellner and Van der Kraak (Kellner and Van der Kraak, 1992) who showed that the two act synergistically to increase prostaglandin

production. They also found that PGE was produced in greater quantities than PGF.

While there is most definitely a larger body of evidence to suggest that A23187 is a stimulant of prostaglandin synthesis there is, however, some opposing evidence that it may posses inhibitory actions. Gibb and Lavoie found that A23187 stimulated freshly isolated human amnion cells to produce PGs but had the opposite effect when co-incubated with dexamethasone, producing a greater degree of inhibition than the dexamethasone on its own (Gibb and Lavoie, 1990). A study on plasminogen activator showed that A23187 was inhibitory at concentrations up to 1μM. It also found that PMA could stimulate plasminogen activator production and that 0.05μM A23187 produced a synergistic increase in that stimulation. Interestingly they observed a blockade of the PMA induced stimulation when the A23187 concentration was raised to 0.5 and 1μM (Tilly and Johnson, 1990). Although the data presented in this thesis did not show a consistent, significant reduction in the action of PMA when co-incubated with A23187, they do appear to show a similar trend to the observations of Tilly and Johnson. The picture is similar with LPS, but there were more significant effects when both LPS and PMA were present.

A recent report has claimed that the addition of A23187 to gastric mucosa cells grown in culture caused cell death (Tepperman and Soper, 1994). The authors used more than six times as much (12.5μM) of the ionophore than the experiments in this thesis and two and a half times that used by Ibe and Raj (Ibe and Raj, 1992), who found that it increased PG production. Taken together with the observations in this thesis showing that A23187 did not produce the

same effects on PGE_2 synthesis and $PGF_{2\alpha}$ synthesis it would appear that cell death was not the reason for the fall in prostaglandin output, since a consistent effect would then have been produced.

Another interesting feature of the A23187 containing cultures was the fact that they appeared to permit *in vivo* RU486 treatment of the guinea pigs to produce an increase in prostaglandin synthesis when compared to the equivalent control animal cultures, especially the LPS + PMA + A23187 treated cells. This implies that PG stimulation by RU486 may be dependent on an increase in the concentration of calcium in the cytosol, despite the observation that the calcium ionophore appeared to have an inhibitory effect in general. This may reflect differing utilisation of available calcium when lipocortin stimulation is inhibited by the presence of RU486 in glucocorticoid receptors (Peers et al., 1988).

The action of A23187 (and calcium) on PG synthesis in the guinea pig cervix is obviously somewhat different to the often reported stimulatory effect. In order to examine this further it would be of interest to carry out similar experiments using calcium free media. However, this would also affect other calcium dependent systems and would probably have a deleterious effect on the adherence of the cells to the culture dish. The use of calcium channel blocking drugs would be of limited use since A23187 is considered to transport calcium across the membrane by complexing with the cation itself (Pressman et al., 1967).

Oestrogen

Oestrogen has been shown to promote prostaglandin production by decidua and amnion (Olson et al., 1983a; Kelly and Abel, 1980; Kelly and Abel, 1981). In the guinea pig cervix cultures oestradiol caused a reduction in PGE_2 but not $PGF_{2\alpha}$ synthesis. While the effect on PGE_2 was significant the magnitude of the change was small. The opposing effects of progesterone and oestrogen (Challis and Olson, 1988) would also have been expected to become apparent in such experiments, however no such interaction was detected. The lack of any consistent effects of progesterone and RU486, or of a major effect of oestradiol may be indicative of low numbers of steroid receptors. However, the fact that RU486 has been shown to cause cervical softening (Elger et al., 1987a) indicates that the cervix does contain receptors for this steroid, *in vivo*, and work by Rajabi et al. (Rajabi et al., 1991) showed that progesterone could influence collagenase output and RU486 was able to block progesterone's actions.

Thus, the experiments carried out for this thesis suggest that the steroid hormones do not play a significant role in the local control of prostaglandin synthesis in the late pregnant guinea pig cervix and may therefore not be involved in PG control in the latter stages of cervical ripening. It is possible that these hormones can influence the cervix by actions they have on surrounding tissues, most likely the uterus. Activity in this organ may result in paracrine interactions between the tissues, as they are physically a single entity.

Oestrogen's function in cervical ripening is not clear with several reports of a ripening effect of exogenously applied oestrogen (Gordon and Calder, 1977; Pinto et al., 1964; Pinto et al., 1965; Tromans et al., 1981; Gordon, 1981; Stewart et al., 1981), others

suggesting that it does not promote ripening (Thiery et al., 1978; Pedersen et al., 1981). There are also conflicting reports about the effects oestrogen may have on biochemical events, collagenase/collagenolytic activity having been found to both increase (Rajabi et al., 1991; Rajabi et al., 1991) and decrease (Woessner, 1979; Wallis and Hillier, 1981). The work in this thesis showed that oestradiol did not have any strikingly obvious effect on prostaglandin synthesis by guinea pig cervix. This was despite the fact that where oestrogen was found to produce an increase in procollagenase levels (Rajabi et al., 1991), the results were obtained using guinea pig cervical tissue and inhibition of cyclooxygenase resulted in the loss of that effect of the oestrogen, suggesting that prostaglandins may be involved in such an effect. Thus, the role of oestrogen remains open to question, but it is suggested that it may be involved in the regulation of gap junction formation in the myometrium (Bosc et al., 1987; Garfield et al., 1980; Puri and Garfield, 1982) so it may also have a similar effect in the cervix, allowing the individual cells to act in concert with each other to produce the rapid ripening observed in normal clinical situations.

Cytokines

Interleukin-1 and tumour necrosis factor have both been shown to stimulate prostaglandin synthesis (Dayer et al., 1985; Elias, 1988; Hammarstrom, 1989; Pfeilschifter et al., 1989). The lack of such an effect by IL-1 in these experiments was surprising and initially thought to be due to the preparation of the material but changes in the preparation and in the supplier failed to show any difference. Recombinant human IL-1, as used here, was found to stimulate

guinea pig cells to produce mRNA for procollagenase (Rajabi et al., 1991), which suggests that this human form of IL-1 can be active in the guinea pig. Thus it seems that IL-1 may not be a stimulant of cervical prostaglandin production. The biological inactivity of the TNF used was discounted by later experiments with human tissue but again introduces the question concerning the activity of recombinant human material being bioactive in other species.

The stimulant effect of LPS on prostaglandin production lends support to the proposals that the presence of intrauterine infection may be a factor in cases of preterm labour (Platz-Christensen et al., 1992; McGregor, 1988), possibly the result of the initiation of an inflammatory response directed against the invading microorganisms. However, this raises the issue of how the maternal immune system is able to respond to the infection while unable to recognise the fetally derived tissue. One possible solution to this problem lies in the fact that some microorganisms are able to produce prostaglandins themselves or produce enzymes, such as phospholipase A_2, which promote PG synthesis (McGregor, 1988; Lamont et al., 1990). The LPS results also support the suggestions that parturition is an inflammatory based function with cervical ripening and uterine contractions being stimulated and controlled, at least in part, through the actions of prostaglandins and cytokines. Even if the initiation of the inflammatory response is supplied by a non-maternal source, that is the fetus or an infection, or if these sources actually produce prostaglandins themselves, it may still be relevant to examine the ability of the late pregnant cervix to produce prostaglandins since endogenous PG production has been observed following stimulation with exogenous prostaglandin (Green et al., 1981).

Although the results obtained with guinea pig tissue did not provide evidence of an effect of the cytokines IL-1 and TNF on prostaglandin synthesis and this may have suggested that they have no function in the cervix, their central role in the process of inflammation implies that they are very likely to be involved in cervical ripening to some degree. The lack of response from the guinea pig cervix may have resulted from the absence of cell types capable of responding to those cytokines. However, the fact that human cervix did respond to TNF (chapter 7) suggests that this was probably not the reason as both tissue preparations would be expected to contain a similar range of cells, the main differences being that of species and the loss of any direct cell to cell interactions via gap junctions.

Summary

Cervical cells taken from late pregnant guinea pigs and maintained in culture are capable of producing prostaglandins E_2 and $F_{2\alpha}$. A variety of drugs were shown to alter the degree of prostaglandin output and the general trend was for greater amounts of PGE_2 to be produced than $PGF_{2\alpha}$. Production of PGs can be stimulated by activation of G-protein linked second messenger pathways involving protein kinase C and by preventing the dephosphorylation of intracellular proteins. An induced increase in cytosol calcium levels causes a reduction in prostaglandin synthesis. The antiprogestin RU486 does not have a significant effect on prostaglandin production by the late pregnant guinea pig cervix, nor do the endogenous sex steroids progesterone and oestrogen have a role. The cytokines IL-1 and TNF similarly do not influence PG synthesis in this culture system.

Chapter 7

Human Cervix and Interleukin-8 Production

Human Cervix and Interleukin-8 Production

The remodelling of the pregnant cervix is primarily dependent on the alteration of the connective tissue matrix. For this to occur the original connective tissue must be broken down and replaced. One of the enzymes crucial to this is collagenase, as it is one of only two enzymes capable of the initiation of collagen degradation (the other enzyme being elastase). It has been shown collagenase in the ripening cervix is of polymorphonuclear cell origin. The chemokine (a subdivision of the cytokines) interleukin-8 is a chemotactic factor for neutrophils, a PMN with granules containing collagenase (elastase is also found in neutrophils). Thus, the presence of IL-8 in the cervix would be an ideal method for inducing neutrophil migration to the cervical connective tissue, the subsequent degranulation and release of collagenase and ultimately the breakdown of the highly crosslinked collagen.

The initial aim of this section of work was to determine whether or not the human cervix was capable of producing interleukin-8, and if so to examine the influence of different *in vitro* treatments on that production.

It was also intended that the effects of pretreatment with the antiprogestin RU486 would be examined using biopsies from the cervices of women undergoing surgical termination of pregnancy.

The human cervical biopsies were derived from five separate groups of women. The difficulty in obtaining biopsies from RU486 treated women (group 5) meant that there were no results from this group.

The following data were produced using tissue obtained from the remaining four. Group 1 (P-Cgem) was from women given the prostaglandin E analogue Cervagem prior to surgical termination. These women were nulliparous. Group 2 (P-No Cgem) was from parous women not given cervagem pretreatment before surgical termination. Group 3 (NP) was from hysterectomy patients. These women were premenopausal and were not pregnant. They were at various stages of the menstrual cycle but there was insufficient information to permit the determination of any stage specific differences. Finally, group 4 (PM) samples were from the postmenopausal women being treated for unexplained uterine bleeding.

Production of IL-8 by the Human Cervix

Influence of PMA on Interleukin-8 Production (Figure 7.1)

PMA is an activator of protein kinase C (see chapter 3). It was used to examine the involvement, if any, of the protein kinase C pathway in the production of prostaglandins by human cervical tissue sustained in culture.

Interleukin-8 levels were determined in the medium of cultures from each group.

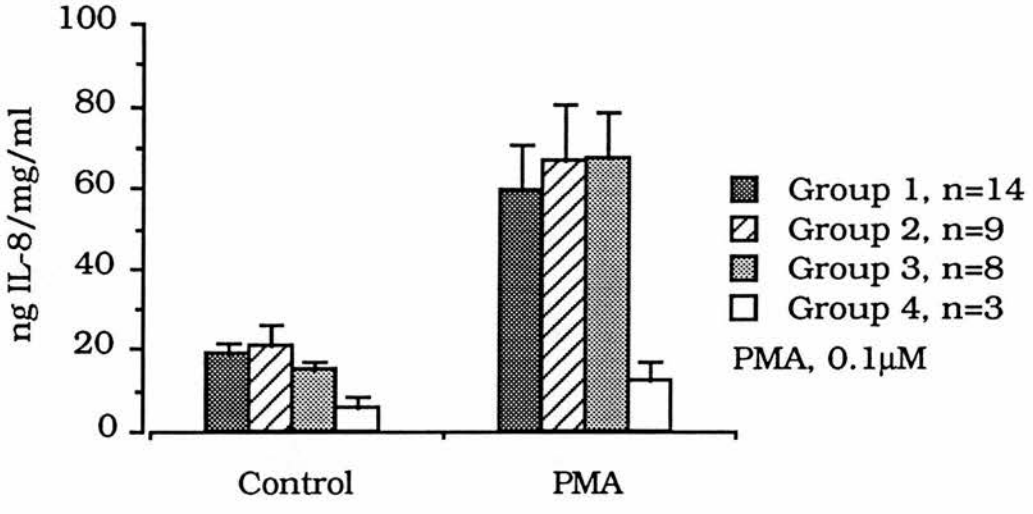


Figure 7.1 Effect of PMA (0.1μM) on IL-8 production by human cervical tissue explants (mean ± sem).

Group 1- Pregnant, cervagem treated

Group 2- Pregnant, no cervagem treatment

Group 3- Nonpregnant

Group 4- Post menopausal

Tissue group effect p=0.0115

PMA effect p=0.0001

The production of IL-8 by these four different tissues was not equal. There was an obvious reduction in output of IL-8 by the group 4 (postmenopausal) cervix, p=0.0115.

PMA was included in the culture medium at a concentration of 0.1μM. It caused a significant increase in the level of IL-8 in the medium, p=0.0001.

While there appeared to be an interaction between the tissue group and the treatment, that is that the group 4 tissues seemed to respond differently to the PMA than the other tissues, the relationship between group and treatment was not significant, p=0.1818. The lack of significance may be attributed to the small number of results in group 4 (only three samples).

Effect of Progesterone on Interleukin-8 Production (Figure 7.2)

Biopsies from nonpregnant patients (group 3) were larger than those from the other groups and more experiments could therefore be carried out with each one. This meant that four separate culture conditions could be set up with tissue from the same patient and thus allow direct comparison of the effect of progesterone on the basal and PMA stimulated levels of IL-8.

Complete medium with and without progesterone at a concentration of 1μM, and PMA containing medium (0.1μM PMA), again with and without progesterone at 1μM, were examined for their effect on IL-8 output.

Once more, PMA caused a significant increase in the output of IL-8, p=0,0132. Progesterone had no effect on the output of interleukin-8. Despite the apparently lesser degree of stimulation of

IL-8 production in the cultures containing progesterone the difference did not reach significance, p=0.0745.

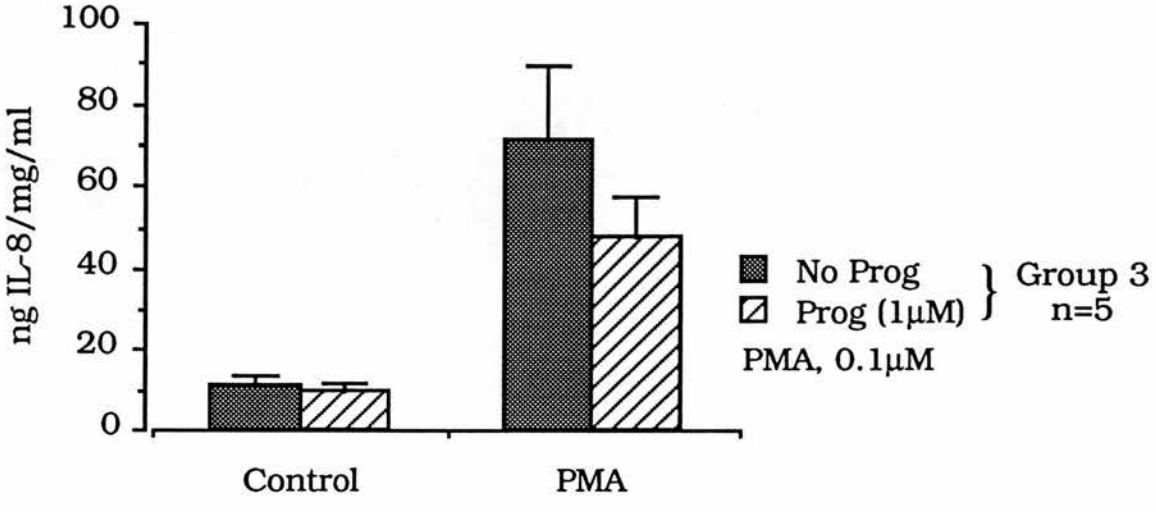


Figure 7.2 Effect of progesterone (1μM) on IL-8 production by human cervical tissue explants (mean ± sem).

Group 3- Nonpregnant

PMA effect p=0.0132

Effect of Okadaic Acid on Interleukin-8 Production (Figure 7.3)

Okadaic acid is an inhibitor of protein phosphatases and therefore prevents the dephosphorylation of intracellular proteins (see chapter 3). It was examined to determine the effect of maintaining the phosphorylated state of basally phosphorylated proteins in the human cervix.

Cultures were incubated with medium containing okadaic acid at 50nM. For this treatment, group 1 (P-Cgem) and group 3 (NP) tissue biopsies were available.

The two groups of tissue showed no significant difference in their production of IL-8, p=0.1442, and although there does appear to have been some inhibitory effect of okadaic acid on the nonpregnant cervix any effect of okadaic acid was not significant, p=0.6042.

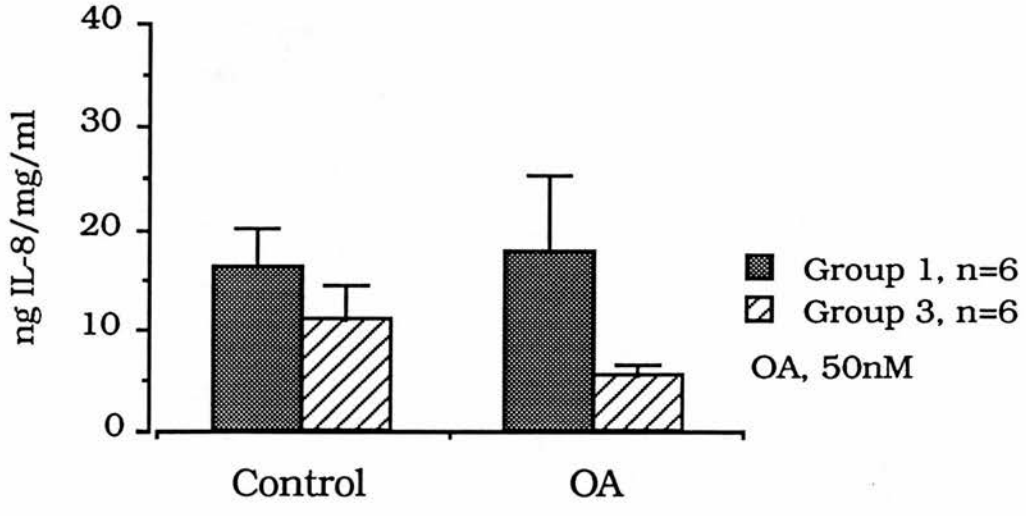


Figure 7.3 Effect of okadaic acid (50nM) on IL-8 production by human cervical tissue explants (mean ± sem).

Group 1- Pregnant, cervagem treated

Group 3- Nonpregnant

No significant differences

Effect of LPS on Interleukin-8 Production (Figure 7.4)

LPS (lipopolysaccharide) is a product derived from the surface of gram negative bacteria and it is thought to activate G-proteins.(see chapter 3). It is thought that in a proportion of cases of preterm delivery the cause may be related to the presence of bacterial infections in the upper genital tract. In these experiments LPS was used to examine the likelihood that the human cervix could produce

prostaglandins under such circumstances. It was tested at a concentration of 4μg/ml on cultures of nonpregnant cervical explants.

LPS caused a significant increase in the output of interleukin-8 compared to the control, $p \leq 0.01$. This analysis of variance was a one-way analysis and showed that a significant stimulation had taken place, $p=0.0003$, thus permitting the follow up Newman-Keuls test.

Effect of TNF on Interleukin-8 Production (Figure 7.4)

TNF is a cytokine involved in the regulation of immune responses and inflammation. It acts via specific receptors in the cell membrane (see chapter 3). Cervical tissue from nonpregnant patients was exposed to TNF at 100units/ml.

Again, an increase in IL-8 production was detected when compared to the results from the control tissue, $p \leq 0.05$.

Effect of DMF on Interleukin-8 Production (Figure 7.4)

DMF, the solvent in which okadaic acid was reconstituted, was studied in tissue from nonpregnant women.

The results indicated that this vehicle, at 0.05%, did not significantly affect the production of interleukin-8.

All of the results presented thus far concerning material from nonpregnant patients and the effects of okadaic acid, DMF, LPS and TNF were obtained from the same set of tissues and are therefore displayed together in the same figure (Figure 7.4).

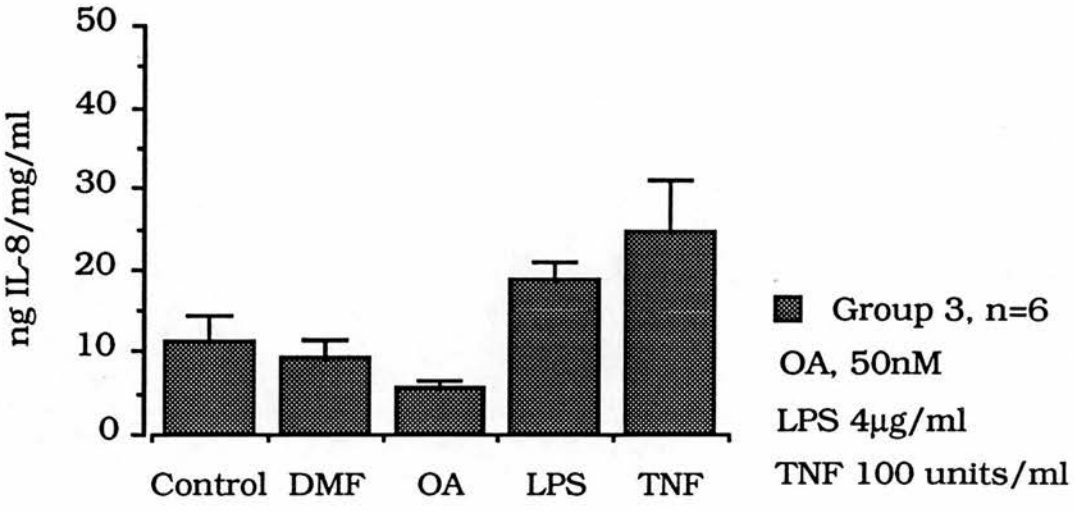


Figure 7.4 Effect of DMF, okadaic acid (50nM), LPS (4µg/ml) and TNF (100units/ml) on IL-8 production by human cervical tissue explants (mean ± sem).

Group 3- Nonpregnant

LPS greater than control, $p \leq 0.01$

TNF greater than control, $p \leq 0.05$

A further set of tissue derived from nonpregnant women was studied. These biopsies were exposed to PMA, PMA + A23187, TNF (at a higher concentration than previously) and ethanol, the vehicle used for progesterone, PMA and A23187.

Effect of Ethanol on Interleukin-8 Production (Figure 7.5)

Ethanol at a concentration of 0.2%, the maximum that would have been present in any of the cultures, had no discernible effect on IL-8 production.

Effect of PMA and PMA + A23187 on Interleukin-8 Production (Figure 7.5)

0.1μM PMA and 2μM A23187 were examined because early work on the guinea pig suggested that the calcium ionophore (A23187) may have had an inhibitory effect on prostaglandin synthesis (see chapter 5).

PMA alone was included as an appropriate control for the PMA + A23187 treatment. There were significant effects (using one-way anova $p=0.0002$). PMA treatment caused an obvious stimulation in IL-8 output, $p\leq0.01$. A23187 had a significant inhibitory effect on the action of PMA, $p\leq0.05$. The PMA treatment effect was also significantly greater than that of TNF at 500 units/ml, $p\leq0.05$. Although PMA + A23187 and TNF also raised IL-8 levels by apparently large amounts their effects were not found to be significant.

Effect of a Higher Concentration of TNF on Interleukin-8 Production (Figure 7.5)

TNF was tested at 500units/ml, as opposed to only 100units/ml previously.

The TNF appeared to promote the production of IL-8 by the nonpregnant cervical tissue but the increase over the control did not reach significance. A comparison of TNF at 100 units with TNF at 500 units did not detect a difference between them. The comparison was made using the difference between each TNF stimulated value and its respective control.

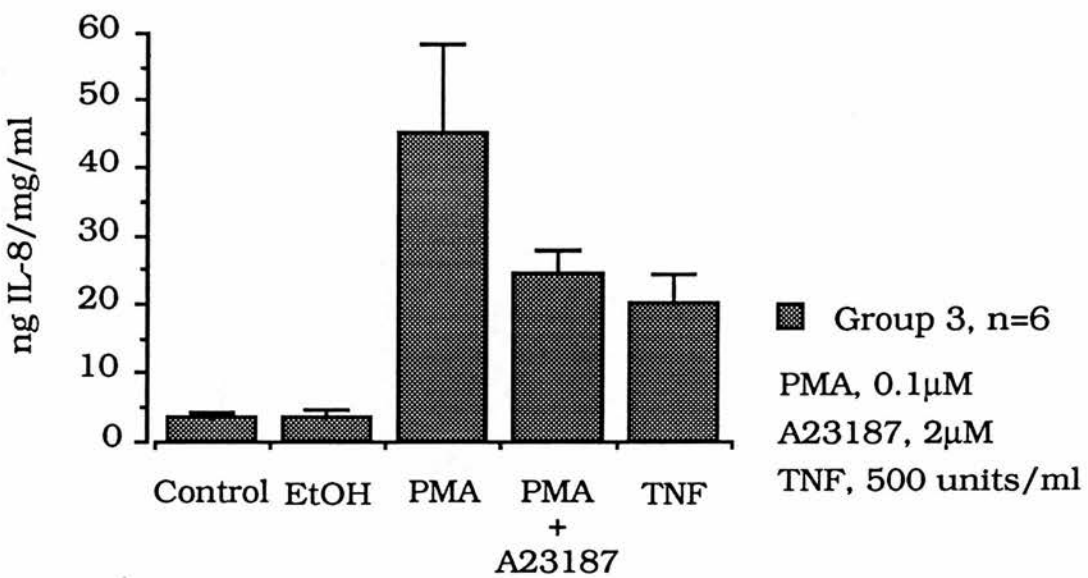


Figure 7.5 Effect of ethanol, PMA (0.1μM), PMA (0.1μM) + A23187 (2μM) and TNF (500units/ml) on IL-8 production by human cervical tissue explants (mean ± sem).

Group 3- Nonpregnant

PMA greater than control, $p \leq 0.01$

PMA greater than PMA + A23187 and TNF, $p \leq 0.05$

Maintenance of IL-8 Output

The data presented were derived from the medium harvested after the first 24 hr incubation period. As an indication that the tissue remained viable beyond this point, the cultures were maintained for a second 24 hrs. The correlation coefficient produced by comparing the results from each of the culture periods was 0.92 ($p \leq 0.001$). This strongly suggests that the tissue was still viable 48 hrs after excision from the patient.

A limited number of cultures were continued for a third period of 24 hrs. For those cultures the correlation between the first and second harvests had a coefficient of 0.94 ($p \leq 0.001$) and between the second and third harvests a coefficient of 0.98 ($p \leq 0.001$). While this does not give an indication of the level of cell viability, it does indicate that the tissue was behaving in a parallel manner on subsequent days.

Prostaglandin E_2

As an adjunct to the interleukin-8 study PGE_2 production by the excised human cervical tissue was also examined. The use of drugs employed in the guinea pig study may also show whether the guinea pig and human respond in a similar manner.

Production of PGE_2 by the Human Cervix

Prostaglandin levels were determined in the medium harvested from each group of tissues (figure 7.6).

For basal production no statistically significant differences were found between the four groups of tissue, p=0.2741.

Influence of PMA on PGE_2 Production (Figure 7.6)

PMA increased the level of PGE_2 synthesis, p=0.0358. As observed with IL-8 there was no significant interaction between the grouping and the treatment, p=0.7405, despite the apparent lack of response from the postmenopausal tissue.

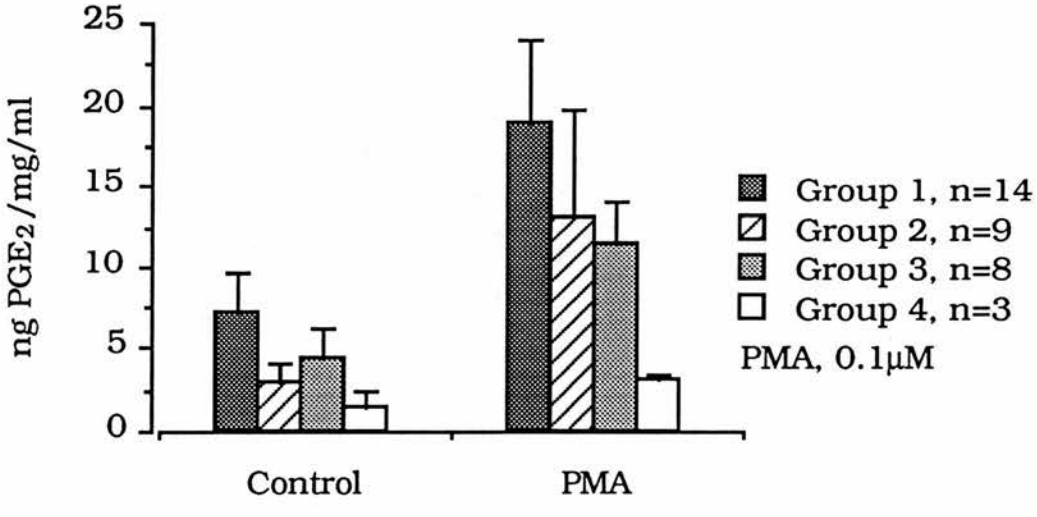


Figure 7.6 Effect of PMA (0.1μM) on PGE_2 production by human cervical tissue explants (mean ± sem).

Group 1- Pregnant, cervagem treated

Group 2- Pregnant, no cervagem treatment

Group 3- Nonpregnant

Group 4- Post menopausal

PMA effect p=0.0358

Effect of Progesterone on PGE_2 Production (Figure 7.7)

The cervix of nonpregnant women was cultured in complete medium and PMA containing medium (0.1μM PMA), in both cases with and without progesterone at a concentration of 10^{-6}M.

Again, PMA caused a significant increase in the output of the prostaglandin, p=0.0403. Progesterone had no significant overall effect on the output of PGE_2, p=0.1542. However, there was an interaction between the progesterone and the PMA treatment, p=0.0467, the progesterone reducing the stimulation of PGE_2 output by PMA.

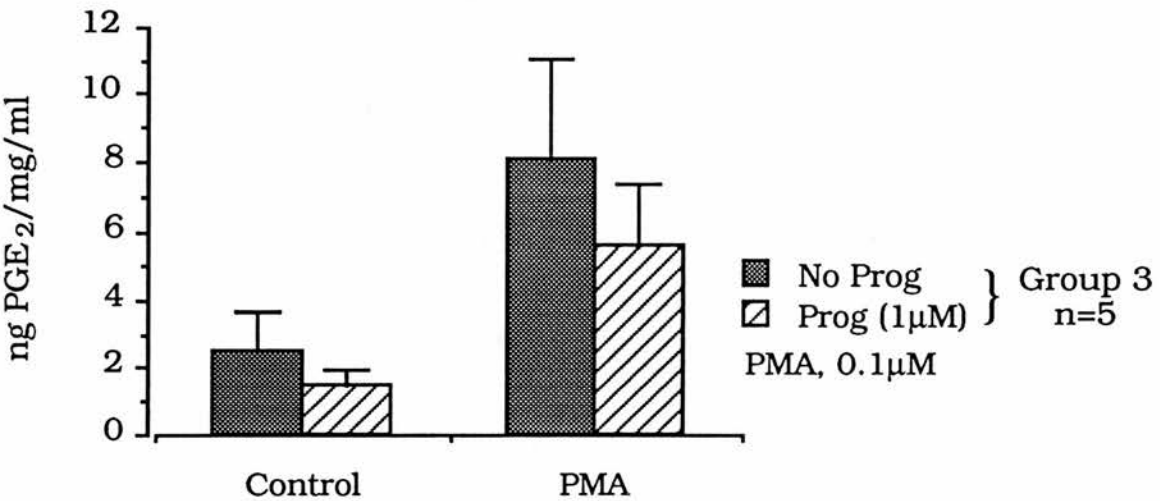


Figure 7.7 Effect of progesterone (1μM) on PGE_2 production by human cervical tissue explants (mean ± sem).

Group 3- Nonpregnant

PMA effect p=0.0403

Progesterone x PMA interaction p=0.0467

Effect of Okadaic Acid on PGE_2 Production (Figure 7.8)

The group 1 (P-Cgem) and group 3 (NP) tissue biopsies incubated with okadaic acid (50nM) showed a quite different response to that of interleukin-8.

The two groups of tissue responded in a similar manner to okadaic acid. Both produced the same basal quantities of PGE_2 and increased their output significantly and to the same degree when cultured in the test medium, p=0.0146. This effect of okadaic acid is distinctly different from the effect observed on IL-8 production. There it appeared to have an inhibitory action, although not significantly.

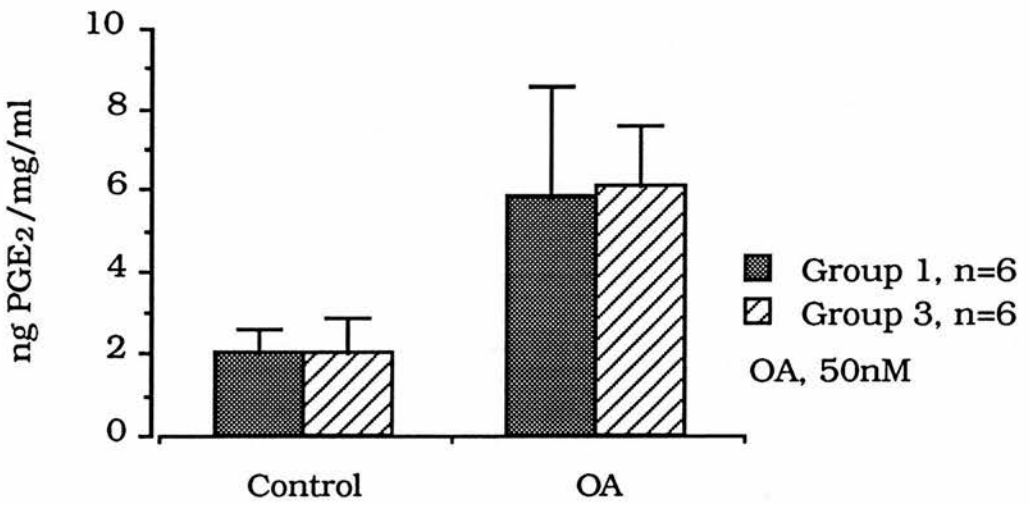


Figure 7.8 Effect of okadaic acid (50nM) on PGE_2 production by human cervical tissue explants (mean ± sem).

Group 1- Pregnant, cervagem treated

Group 3- Nonpregnant

Okadaic acid effect p=0.0146

The effects of okadaic acid, DMF, LPS and TNF on group 3 tissue (NP) are again presented in the same figure (figure 7.9) since they were all produced from common source material. The okadaic acid results will not be discussed again at this point.

Effect of LPS on PGE_2 Production (Figure 7.9)

The analysis of variance of these data obtained by studying tissue from nonpregnant women revealed a significant stimulation of PGE_2 production, $p \leq 0.0001$. The one-way anova was supplemented by the Newman-Keuls test. LPS (4μg/ml) conditioning resulted in significantly greater output of PGE_2 than the control medium, $p \leq 0.05$.

Effect of TNF on PGE_2 Production (Figure 7.9)

The effect of TNF (100units/ml) on PGE_2 was also examined on this cervical tissue from nonpregnant patients.

An increase in the production of the prostaglandin was detected in the presence of TNF at 100 units/ml, $p \leq 0.05$.

Effect of DMF on PGE_2 Production (Figure 7.9)

DMF did not affect the production of PGE_2 by the tissue from nonpregnant patients.

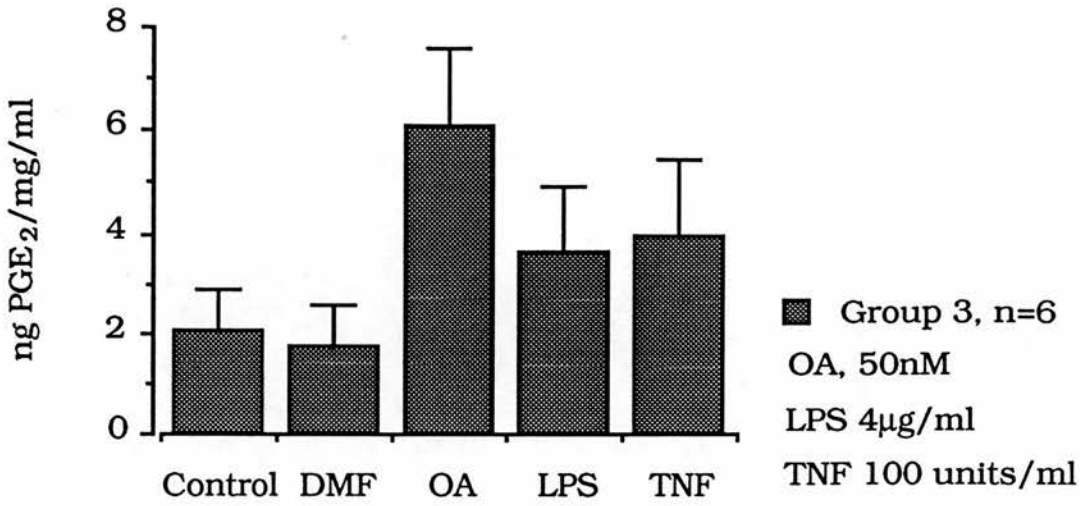


Figure 7.9 Effect of DMF, okadaic acid (50nM), LPS (4µg/ml) and TNF (100units/ml) on PGE_2 production by nonpregnant human cervical tissue explants (mean ± sem).

Group 3- Nonpregnant

LPS greater than control, $p \leq 0.05$

TNF greater than control, $p \leq 0.05$

The following results are from another set of tissues derived from nonpregnant women, as described in the previous section on interleukin-8.

Effect of Ethanol on PGE_2 Production (Figure 7.10)

Ethanol was tested as a vehicle control (0.2%). It had no discernible effect on PGE_2 production.

Effect of PMA and PMA + A23187 on PGE_2 Production (Figure 7.10)

0.1μM PMA and 2μM A23187 were examined because early work on the guinea pig suggested that the calcium ionophore (A23187) may have had an inhibitory effect on prostaglandin synthesis (see chapter 5).

The analysis of variance indicated that a significant increase in PGE_2 output had taken place, $p=0.0062$. PMA (0.1μM) treatment caused an obvious stimulation of PGE_2 output as did the combination of PMA + A23187, however, only the latter difference reached significance, $p\leq0.05$. Comparison of PMA treated cultures with PMA + A23187 treated cultures did not detect any difference between the two.

The quantity of PGE_2 produced by this tissue under the influence of these two treatments was higher than observed previously for PMA, the mean value approximately three times the mean from the data in first set of figures presented. There was no obvious reason for this and all of the data lay within three standard deviations of the mean.

Again, as found with okadaic acid, the observed interaction between PGE_2 and PMA + A23187 is in contrast to that between IL-8 and PMA + A23187.

Effect of a Higher Concentration of TNF on PGE_2 Production (Figure 7.10)

500units/ml TNF appeared to promote the production of PGE_2 by the nonpregnant cervical tissue but the difference between it and the control medium did not reach significance. Comparing TNF at 100 units with TNF at 500 units did not detect a difference between their effects.

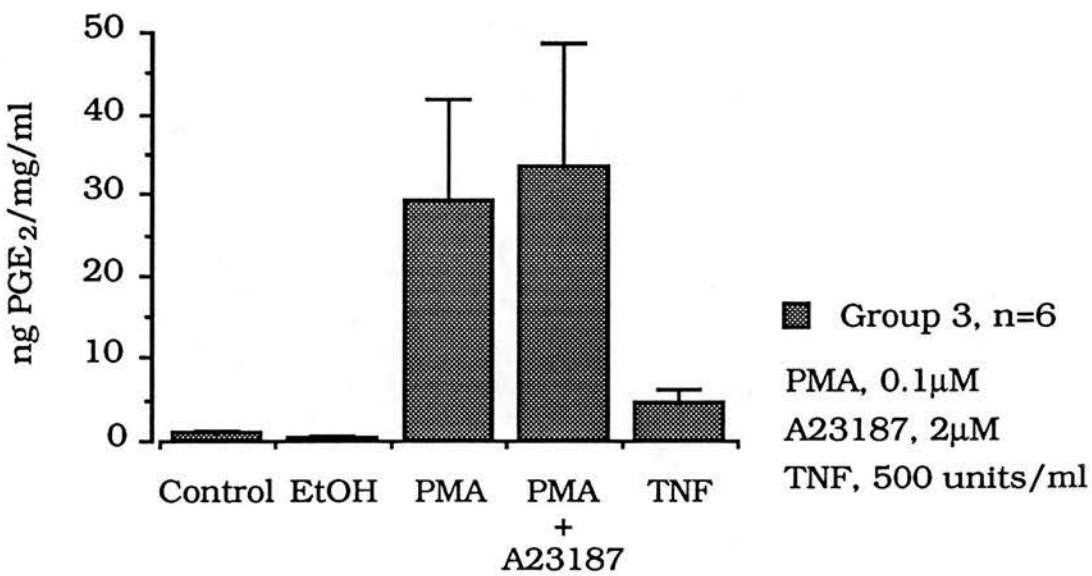


Figure 7.10 Effect of ethanol, PMA (0.1µM), PMA (0.1µM) + A23187 (2µM) and TNF (500units/ml) on PGE_2 production by human cervical tissue explants (mean ± sem).

Group 3- Nonpregnant

PMA + A23187 greater than control, $p \leq 0.05$

Maintenance of PGE_2 Output

The data presented were derived from the medium harvested after the first 24 hr incubation period. As an indication that the tissue remained viable beyond this point, the cultures were maintained for a second 24 hrs. The correlation coefficient produced by comparing the results from each of these two culture periods was 0.84 ($p \leq 0.001$). This tends to support the data obtained from the IL-8 results.

A limited number of cultures were continued for a third period of 24 hrs and the correlation between the first and second harvests for those cultures had a coefficient of 0.85 ($p \leq 0.001$) and between the second and third harvests a coefficient of 0.93 ($p \leq 0.001$).

Chapter 8

Human Cervix Discussion

Human Cervix Discussion

Interleukin-8

This work shows for the first time that the human cervix is capable of producing the neutrophil chemotactic and activating cytokine interleukin-8, albeit *in vitro*. The four groups of tissue from which results are presented produced IL-8 at similar basal levels, although, from the data it is apparent that the base lines were variable and suggests that the postmenopausal tissue produced less than the others. The figures obtained from early experiments appear to show generally higher basal output which may be related to inexperience with a new technique and a greater degree of trauma to the tissue. It seems possible that this may have provoked the initiation of an inflammatory reaction.

In figure 7.1 the response of tissue from groups 1, 2 and 3 appeared to be identical when challenged by PMA, with comparatively little effect on the post menopausal tissue (group 4). However, there was no significant interaction between the tissue group and the treatment and it is probable that this was due to the small number of samples that were made available from this group of women. The use of human cervix for experimental work is fraught with such problems, limited quantities being obtainable. The lack of homogeneity in the human population produces relatively large variation between patients which ultimately means that the statistically significant differences will be difficult to detect with small numbers of samples. It is nonetheless interesting that this tissue appeared not to respond to the PMA in the same manner as the tissue from the other groups of

women, and may indicate that there is an element of hormonal control in the production of IL-8. However, there were no apparent differences between the nonpregnant group and the two sets of tissue from pregnant women, the hormonal environments being quite different in the pregnant and nonpregnant groups but still under the influence of substantially more ovarian hormones than postmenopausal tissue.

Figure 7.2 shows the effect of progesterone *in vitro* which did not reveal any significant effect of the steroid despite the presence of a glucocorticoid/progestin response element in the IL-8 gene (Mukaida and Matsushima, 1992) and the inhibition of IL-8 production seen with chorio-decidual cells in culture (Kelly et al., 1992). The reduction in IL-8 levels seen in the PMA treated tissue co-incubated with progesterone may not have reached a significant level due again to the low number of patients involved, only five, and the fairly high variation between patients. In these experiments on the effect of progesterone, nonpregnant tissue was used because it allowed each of the four treatments to be applied to tissue from each of the patients, in the expectation that any differences would be more easily detected. Despite the difficulties associated with using human cervix the relevance of work on human tissue makes it a valuable tool, especially where it can be used to confirm more extensive studies in animal or cell culture models.

The similarity in the responses of the pregnant, cervagem treated, pregnant but not treated with cervagem and the nonpregnant tissues when cultured with PMA presents several interesting points. Firstly it suggests that exogenous prostaglandin treatment used to promote cervical softening does not do so by increasing the synthesis of IL-8, although it may reduce the threshold concentration of IL-8

that is required for it to exert its effects, as was seen in rabbit experiments (Colditz, 1990). Secondly, the similarities between tissue from pregnant and nonpregnant women may indicate that nonpregnant material could be used as a model for the cervix. This would be preferred since it is ethically more acceptable to obtain tissue from organs removed at hysterectomy rather than interfere with the intact cervices of women of reproductive age. However, the results obtained with okadaic acid (OA) suggest that the responses of the two tissues may not be the same. In addition, this work does not address the possible, or even probable, differences between the cervix of early pregnant or nonpregnant women and that obtained from term pregnant women.

The material from nonpregnant women was removed because of uterine dysfunction and the cervix not used if it appeared to be in any way abnormal. This was subsequently confirmed by routine histology. The stage at which these women were in their menstrual cycle was not always available because of the occurrence of almost constant bleeding or amenorrhoea, which meant that it was not possible to examine the results for any stage specific differences in IL-8 production that may have been present.

From the data obtained with PMA, LPS and TNF it is apparent that the human cervix has the capacity to up regulate its output of IL-8. The correlation between output in consecutive culture periods indicates that it can maintain those higher levels for two to three days at least. This latter point also implies that the IL-8 is not merely released from intracellular stores but is being synthesised *de novo*.

The effect of OA on IL-8 production remains open to question. In the pregnant tissue there was obviously no effect at the dose used,

however, the response from nonpregnant cervix was more ambiguous. The baseline was lower than that of the pregnant tissue in the first place (insignificantly), the level upon treatment with OA seemed to drop, but even then difference between the two groups of tissue when challenged by OA did not reach significance. This disparity between the groups of tissue may well be a real effect since PGE_2 measured in medium from the same cultures did not show the same pattern, indicating that the effect was not a blanket one on cellular function. Statistically no differences were detected between the two groups of tissue anyway.

Prostaglandin E_2

This study with human cervical tissue confirms its ability to produce PGE_2. Figure 7.6 shows the four groups of tissue and there were no significant differences between the outputs of each. Despite this, the pregnant, cervagem treated group does appear to produce slightly more than others, both basally and following PMA stimulation, which may indicate that the cervagem caused increased endogenous prostaglandin synthesis, as suggested by Uldbjerg and by Gréen (Uldbjerg and Ulmsten, 1990; Green et al., 1981). In a cross-reactivity test 1 and 10ng cervagem registered as 5 and 49pg PGE_2 respectively. Equating this to the assay of the human cervical tissue suggests that to give 1ng/mg/ml there would have to be contamination of the material at 0.2mg cervagem/mg tissue which seems unlikely since the pessary used contained only 1mg of cervagem and the PG analogue would be distributed in a relatively large volume of tissue, probably also being absorbed into the general circulation. The baseline PGE_2

levels from the cultures with OA were identical between nonpregnant and pregnant, cervagem treated tissue which suggests that cervagem did not contribute to any of the assayed prostaglandin.

Similarly to its effect on IL-8, PMA increased the synthesis of PGE_2 and the postmenopausal samples showed an apparently limited response to it. Once more the small number of women recruited from this group appeared to constrain the analysis, however, the data do suggest that the postmenopausal tissue may be less able to respond to PMA challenge.

There was generally a high degree of variability in the quantity of prostaglandin produced. The nature of the samples and the fact that it was human probably accounted for this. The biopsy material was often supplied in more than one piece. This was due to the plug of material coming apart when removed from the needle or because more than one attempt was made to take a biopsy, resulting in more than one piece of tissue. It was not possible to identify epithelial parts of the biopsies therefore the proportion of stromal and epithelial material in each well of the culture dish was variable. It is possible that this would have affected the production of IL-8 and PGE_2.

The prostaglandin response of nonpregnant group cervix to progesterone was similar to that of IL-8 except that progesterone interacted with the treatments differently, inhibiting the PMA stimulated cells more than the controls. An inhibitory effect of progesterone could be seen as contradictory to the situation with postmenopausal tissue where its absence *in vivo* may have been involved in the apparent lack of response to PMA.

An interesting result was obtained with okadaic acid. Both the group 1 (pregnant, cervagem) and group 3 (nonpregnant) tissues

responded the same in terms of PGE_2, showing the same baseline output and the same level upon exposure to OA, a significant increase. However, this contrasts with the effect on IL-8 where there was no change in the group 1 tissue but the suggestion of a reduction in output by the group 3 cervix. This indicates a probable difference in the mechanisms that control these two components.

LPS and TNF were shown to stimulate PGE_2 production, reinforcing the link with inflammation and its common mediators.

The last set of data from nonpregnant tissue was slightly unusual. The level of PGE_2 detected following PMA treatment was much higher than in previous experiments with nonpregnant group tissue but the IL-8 levels appeared to be similar to those observed earlier. This effect seemed to be confined to PMA conditioned tissue as the effect of TNF at 500 units/ml was comparable with TNF at 100 units/ml used in the other experiment with this tissue. The reason for this effect on PMA is unknown, one possibility is the incorrect dilution of the stock of PMA but this would presumably have affected the results for IL-8 also. Since IL-8 and PGE_2 seemed to respond in parallel to PMA in the other experiments it also seems unlikely that the tissue was in some way sensitised to the effects of PMA.

The calcium ionophore, A23187, seemed to inhibit the PMA stimulation of IL-8 synthesis but once again there was a difference between the IL-8 and PGE_2 response. A23187 did not affect the level of PGE_2.

A point worth noting is that the same TNF which failed to stimulate prostaglandin production by guinea pig cells in culture was active in these experiments, both for PGE_2 and for IL-8. This may be related to the fact that the cytokine was recombinant human TNF,

although it has been shown to stimulate rat cells (Pfeilschifter et al., 1989).

The correlation between prostaglandin synthesis on consecutive days supports similar results with IL-8 and the conclusion that tissue viability was maintained throughout the culture period.

General Discussion

As discussed in chapter 5, PMA activates protein kinase C and OA is a phosphatase inhibitor that does not inhibit protein kinase C, thus it is reasonable that they do not share the same spectrum of activity. The results suggest that activation of protein kinase C causes IL-8 production and the inhibition of protein dephosphorylation does not, and may in some circumstances be inhibitory.

The stimulation of IL-8 production by LPS and TNF agrees with the findings of others (Matsushima et al., 1992; Strieter et al., 1989).

The calcium ionophore, A23187, is presumed to cause an increase in intracellular calcium levels (Pressman, 1976; Reed and Lardy, 1972; Pfeiffer et al., 1974). In many cellular events raised intracellular calcium is associated with a stimulatory action. In the nonpregnant cervix there appeared to be an attenuation of the stimulation seen with PMA when A23187 was included in the culture medium. This combination was examined because early experiments with guinea pig material suggested it may have an inhibitory effect on prostaglandin synthesis, and because of similar findings by others (R Leask, Pers Comm.). There is disparity between the effect of A23187 on IL-8 and PGE_2 production in this human material which tends to support the notion that the observed effect is real and that

calcium inhibits the synthesis, or possible the release mechanism, of IL-8.

The maintained output of IL-8, as measured by the correlation coefficients, suggests that the tissue remained functional at least for the period of the experiment.

Although there is little doubt that the human cervix is able to produce interleukin-8 and to alter significantly that synthesis, the physiological mechanisms which control it are unknown. It is known, however, that in most cells examined there is a stimulatory effect of IL-1 and that this induces detectable increases in IL-8 mRNA within an hour of the initial challenge (Mukaida et al., 1994b). TNF has been shown to produce a significant increase in IL-8 mRNA expression as little as 30 minutes after initial contact (Kunkel et al., 1990). The increase in IL-8 mRNA has been shown to be independent of *de novo* protein synthesis (Mukaida et al., 1991; Mukaida et al., 1994b), and, in relation to IL-1 and TNF induced stimulation, at least partly dependent on the activation of transcription (Oliveira et al., 1992; Sica et al., 1990; Mukaida et al., 1990; Kasahara et al., 1991). IL-1 has also been shown to increase the stability of IL-8 mRNA (Kasahara et al., 1991). The NF-IL6, NF-κB and AP-1 bindings sites in the IL-8 gene are thought to be crucial in its activation (Mukaida et al., 1990; Mukaida et al., 1994a; Okamoto et al., 1994; Yasumoto et al., 1992; Mukaida et al., 1994b).

Once the IL-8 is synthesised, as a 99 amino acid protein (Schmid and Weissmann, 1987; Matsushima et al., 1988), it is cleaved by proteases to give variants of 69-77 amino acids in length (Yoshimura et al., 1989; Lindley et al., 1988; Van Damme et al., 1989; Baggiolini et al., 1994). There appears to be an increase in the

biological activity of IL-8 as the N-terminal is shortened (Walz and Baggiolini, 1990; Van Damme et al., 1990; Clark-Lewis et al., 1991). The influence of LPS and TNF, and similar results from other workers studying inflammatory mediators (IL-1, TNF, IL-3, IL-7, GM-CSF) (Matsushima et al., 1988; Larsen et al., 1989b; Strieter et al., 1989; Standiford et al., 1992; Takahashi et al., 1993), indicate that inflammatory processes may be crucial elements in the control of IL-8 production and therefore likely to be involved in IL-8 regulation in the ripening cervix.

These results may have significant clinical relevance. The failure of the cervix to ripen normally or the need to promote such changes in it before term have long been a problem for obstetricians. Similarly, the question of premature ripening has dogged obstetrical practice, neither situation being well understood or satisfactorily treated. It has been considered that infection may be an important cause of preterm cervical ripening and parturition (McGregor, 1988; Platz-Christensen et al., 1992) and prostaglandins, leukotrienes and interleukin-8 have been found in the amniotic fluid and cervical mucus in association with microbial colonisation (Cherouny et al., 1992; Romero et al., 1991; Platz-Christensen et al., 1992; Romero et al., 1987a). Thus the possibility that IL-8, and prostaglandins, could be involved in preterm delivery through the induction of an inflammatory reaction by microorganisms in the reproductive tract is attractive and supported by the response to the bacterium derived stimulant used in the experiments for this thesis, LPS.

Dealing clinically with an unripe cervix often relied on mechanical methods until more recently when prostaglandin and antiprogestin therapies became available. Treatment of the

incompetent cervix basically depends on the suturing of the organ in such a way as to close or narrow the opening in order to prolong the pregnancy and allow the fetus to mature for as long as possible. Thus, fuller knowledge of how and why the cervix softens and dilates would be a very useful addition to the obstetrician's repertoire.

Interleukin-8 may be an important part of the process of cervical ripening as it has the ability to attract cells (Yoshimura et al., 1987b; Peveri et al., 1988; Schröder et al., 1987; Larsen et al., 1989a) with the capacity to degrade the connective tissue matrix (Osmers et al., 1992), a seemingly integral part of cervical ripening as demonstrated many times by histological studies. Collagenase has been thought to be important in the degradation of the connective tissue of the cervix for some time, generally presumed to originate from the fibroblasts, the main cell type in the cervix. However, recent work has provided evidence that the ripening cervix may not rely on fibroblast derived collagenase but instead on leukocytic collagenase (Osmers et al., 1992). This fits in well with the theory that IL-8 production in the cervix attracts and activates neutrophils, resulting in tissue breakdown. It is also supported by the observations of leukocytic infiltration of the cervix at term (Junqueira et al., 1980; Rath et al., 1988b; Luque and Montes, 1989; Liggins, 1981).

This new work showing that the cervix can produce IL-8 and the proven effects of prostaglandins on cervical ripening, together with the ability of cytokines such as IL-1 and TNF to influence collagenase and prostaglandin production simply serves to reinforce the proposals published by Liggins (Liggins, 1981) that ripening of the cervix is based on an inflammatory type of response.

Further work in this area could pursue the effects of RU486. The attempts thereat for this thesis were unsuccessful because of problems associated with obtaining the biopsies. The patients in question were first trimester surgical termination cases who had agreed to participate, however, the Tru-Cut needle biopsy equipment proved to be unsuitable as the biopsies produced were inadequate for culturing. It seems likely that the cervical softening that resulted from RU486 treatment allowed the tissue to slide around the needle rather than enter it. Such work may then require the excision of a larger piece of cervix by more direct methods, similar to the technique of Uldbjerg (Uldbjerg et al., 1983b) who used a needle biopsy in early pregnant women but scissors and forceps in term and post delivery sampling. The fact that the cervix softened indicated an effect of RU486. The increased sensitivity of the uterus to prostaglandins following RU486 treatment leads to the consideration that the reported cooperation between PGE_2 and IL-8 (Colditz, 1990) may be further enhanced by the antiprogestin through the same or a similar mechanism to that which promotes prostaglandin responsiveness in the uterus, and thus partially explain its cervical ripening effects.

Other studies could examine the effects of the sex steroids. The work on postmenopausal material was very limited and should be continued and extended. Comparisons could be made between women taking hormone replacement therapy (HRT) and those not taking it. If the results of the preliminary experiments described in this thesis are confirmed it would of interest to know whether the cervix could be conditioned once again to produce large quantities of IL-8. It would also be prudent to examine the effects of other substances known to stimulate IL-8 and PGE_2 production by premenopausal cervix.

Molecular biological techniques could be employed to examine the tissue for evidence of activity at the IL-8 gene level, as already shown in other tissues (Mukaida and Matsushima, 1992). This would provide evidence of *de novo* synthesis, or not, of the cytokine.

The clinical use of IL-8 could be studied. The most probable routes of administration will be intracervical or intravaginal and either as a gel or pessary formulation although direct injection of the cytokine into the cervical tissue may be possible. The German company Schering are known to be involved in studies of IL-8 in the guinea pig. They have administered it to these animals and observed cervical ripening. If IL-8 does prove to be a useful adjunct to obstetric care in terms of cervical ripening, its use may supersede prostaglandins for this purpose since it is less likely to affect the myometrium, the initiation of uterine contractions induced once the cervix was in a favourable condition.

Conclusion

The human cervix from nonpregnant and early pregnant women is capable of producing interleukin-8 and prostaglandin E_2. There is some evidence that these two biological mediators are regulated by separate mechanisms. This was most obvious in terms of their response to the phosphatase inhibitor, okadaic acid, where PGE_2 showed an increase in output when challenged and IL-8 did not show any significant effect. The differential was also seen with PMA and PMA + A23187, where less IL-8 was detected in the presence of A23187 but the ionophore did not block the effect of PMA on PGE_2.

Comparison with the guinea pig study shows that the human cervix increased its output of PGE_2 when exposed to A23187 (in the presence of PMA) or to TNF, while the guinea pig reduced its production of the prostaglandin in response to the ionophore and did not show any response to TNF. The TNF effect may be attributable to the fact that the cytokine was a recombinant human peptide.

Chapter 9

Immunohistological Studies Using IL-8 Antiserum

Immunohistological Studies Using IL-8 Antiserum

Human Cervix

Human cervical tissue was also examined immunohistologically for evidence of interleukin-8, in an attempt to further examine its production, investigate the binding sites within the cervix for IL-8 and possibly show the cellular origins of IL-8 within the cervix.

In the presence of bound rabbit antibodies the tissue should take on a brown stain when the enzyme substrate (DAB) is applied (see methods, page 4.30). The nuclei were counterstained with hematoxylin (blue/purple).

Figure 9.1 shows brown staining of the cervical connective tissue matrix in the sections exposed to the IL-8 antiserum (upper panel) and the lack of any positive staining in the control Ig incubated sections (lower panel). Examination of the two photographs shows that they are of the same area of cervix and from consecutive, or very nearly consecutive, sections. These particular results were obtained with sections from nonpregnant women (group 3). However, similar observations were made in material from women in the first trimester of pregnancy. Figures 9.2 and 9.3 show sections of cervix from pregnant women, stained for IL-8 (upper panel), and the same area of the cervix in consecutive (or very close) sections but under control Ig conditions (lower panel). The photographs in Figure 9.2 are of tissue from women given cervagem (group 1), and those in figure 9.3 of tissue from women not given cervagem (group 2).

The general impression from these sections of all three tissue groups is of a nonspecific staining of the connective tissue matrix with

a distinct lack of strongly stained individual cells. The tissue sections in figures 9.1 and 9.3 contain large luminal structures, probably part of mucus glands or arterioles. However there is no evidence of blood in the lumen and in sections where red blood corpuscles (RBC) have been identified the vessel wall was much thinner. These "glandular" structures do not appear to stain positively for IL-8, nor does the epithelial area of tissue seen in figure 9.2.

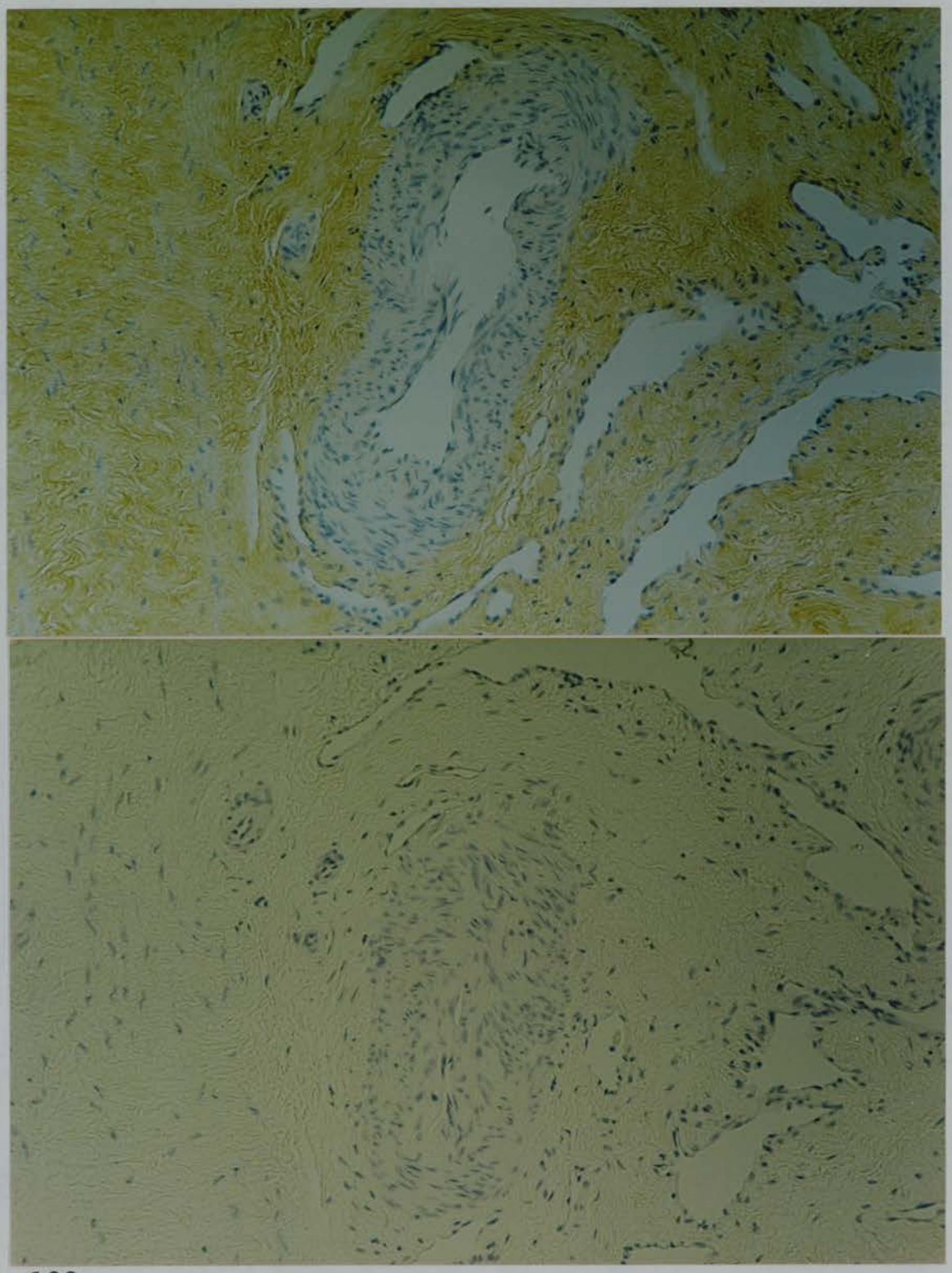


Figure 9.1 The same region of nonpregnant cervical tissue (group 3) exposed to the IL-8 antiserum (upper panel) and control Ig (lower panel).

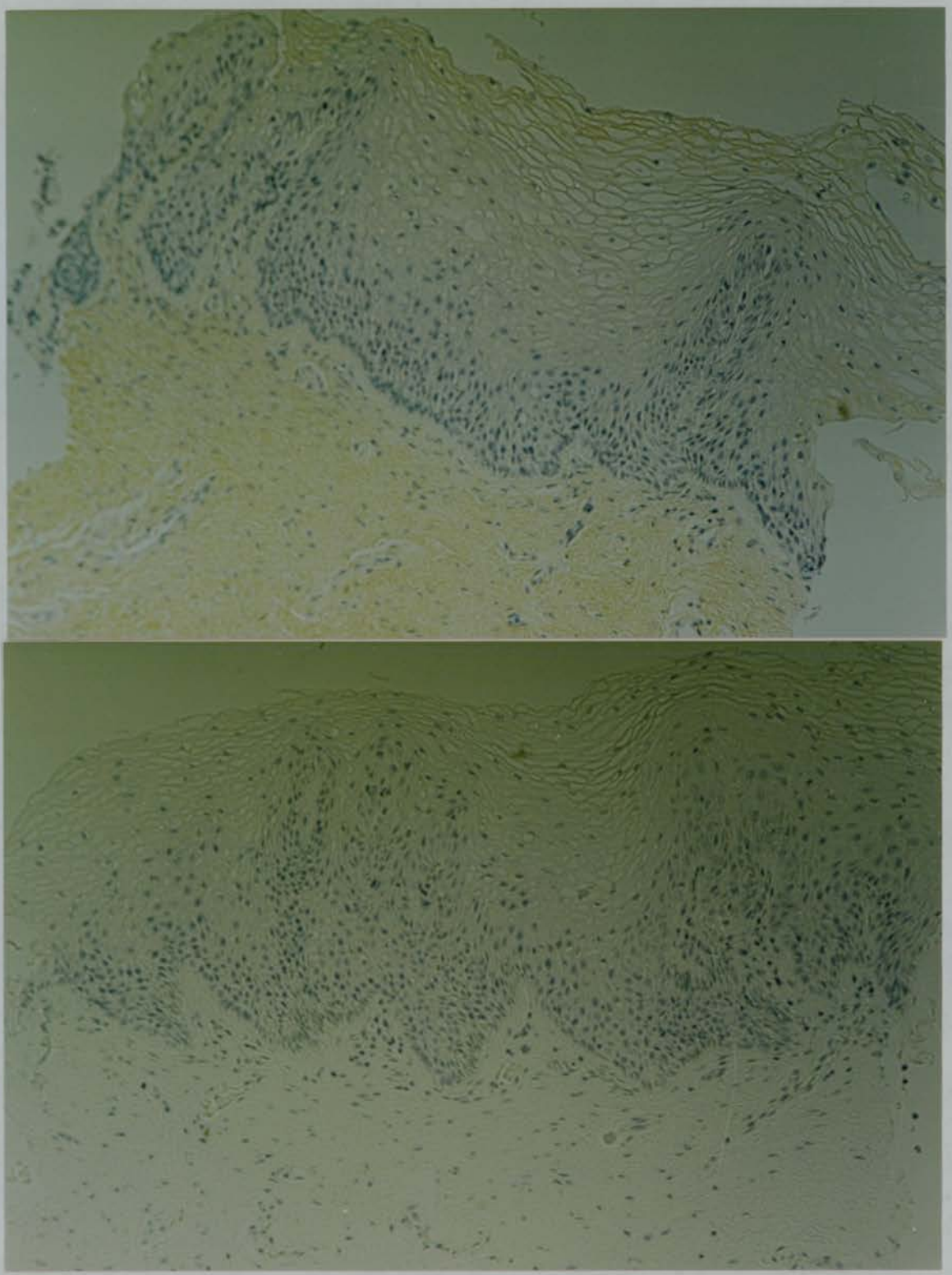


Figure 9.2 The same region of cervical tissue exposed to the IL-8 antiserum (upper panel) and control Ig (lower panel). This tissue was from a pregnant woman given cervagem (group 1).

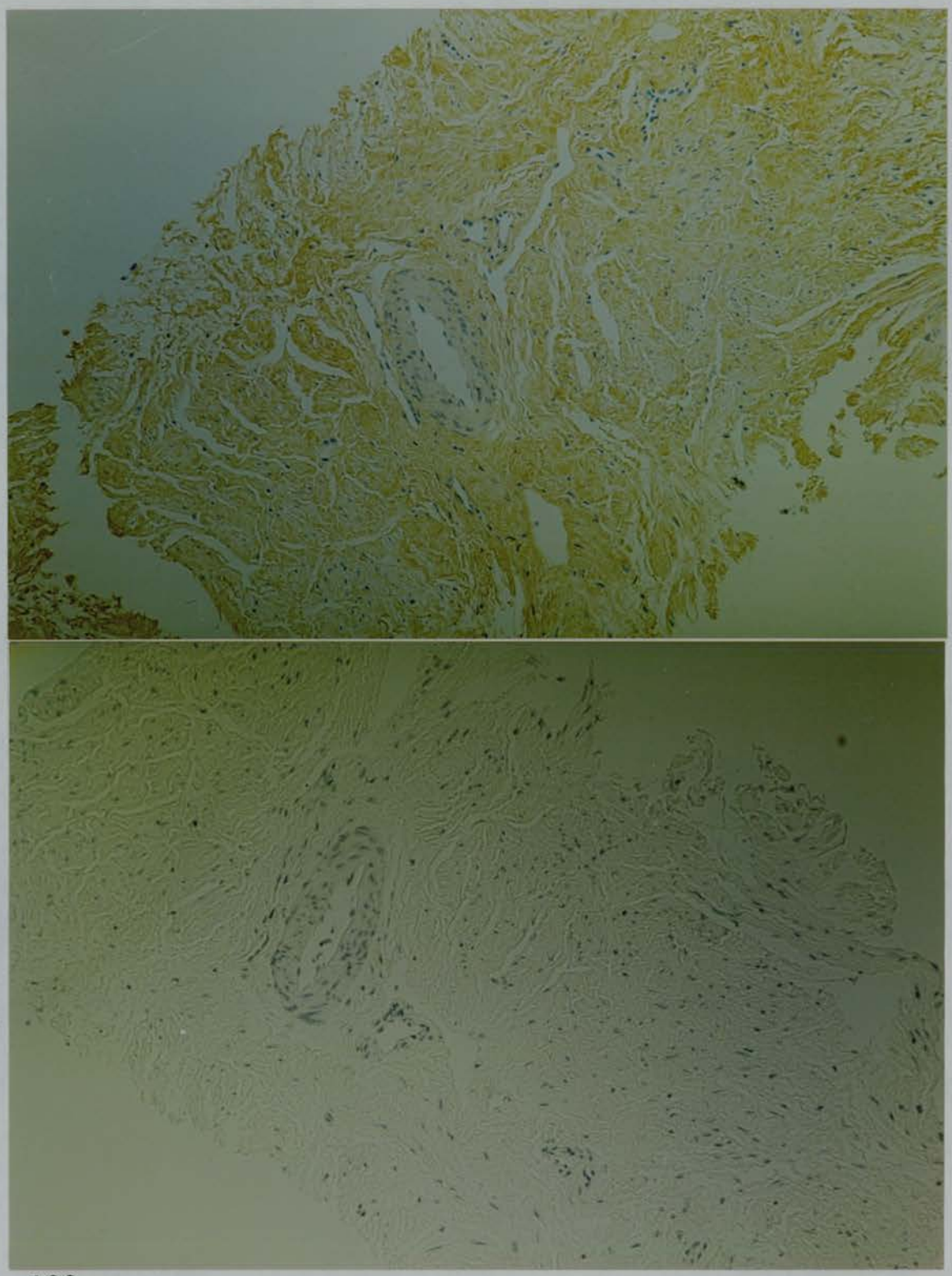


Figure 9.3 The same region of cervical tissue exposed to the IL-8 antiserum (upper panel) and control Ig (lower panel). This tissue was from a pregnant woman not given cervagem (group 2).

Red blood corpuscles have been claimed to bind IL-8 (Darbonne et al., 1991). Several sections were found to contain blood vessels with RBC in the lumen, easily identifiable by their concave surfaces. However, there was no evidence of IL-8 staining of these corpuscles despite positive staining of the tissue matrix. Figure 9.4 shows positively stained matrix in nonpregnant tissue but no brown colour associated with the RBC (upper panel). As a control for this positively stained section the lower panel shows another area of nonpregnant cervix containing RBC, but incubated with control Ig and exhibiting a lack of staining of the connective tissue matrix. Figure 9.5 is of a group 1 cervix displaying the same characteristic staining as the nonpregnant tissue (upper panel shows staining in presence of IL-8 antiserum, lower panel with control Ig).

The photographs in figure 9.6 are of cervix from a nonpregnant patient incubated with IL-8 antiserum (upper panel) and control Ig (lower panel). They have been presented here to illustrate the differences in cell density observed, but do not show consecutive sections of the same area of the tissue. Some areas were virtually acellular while others were much more densely populated.

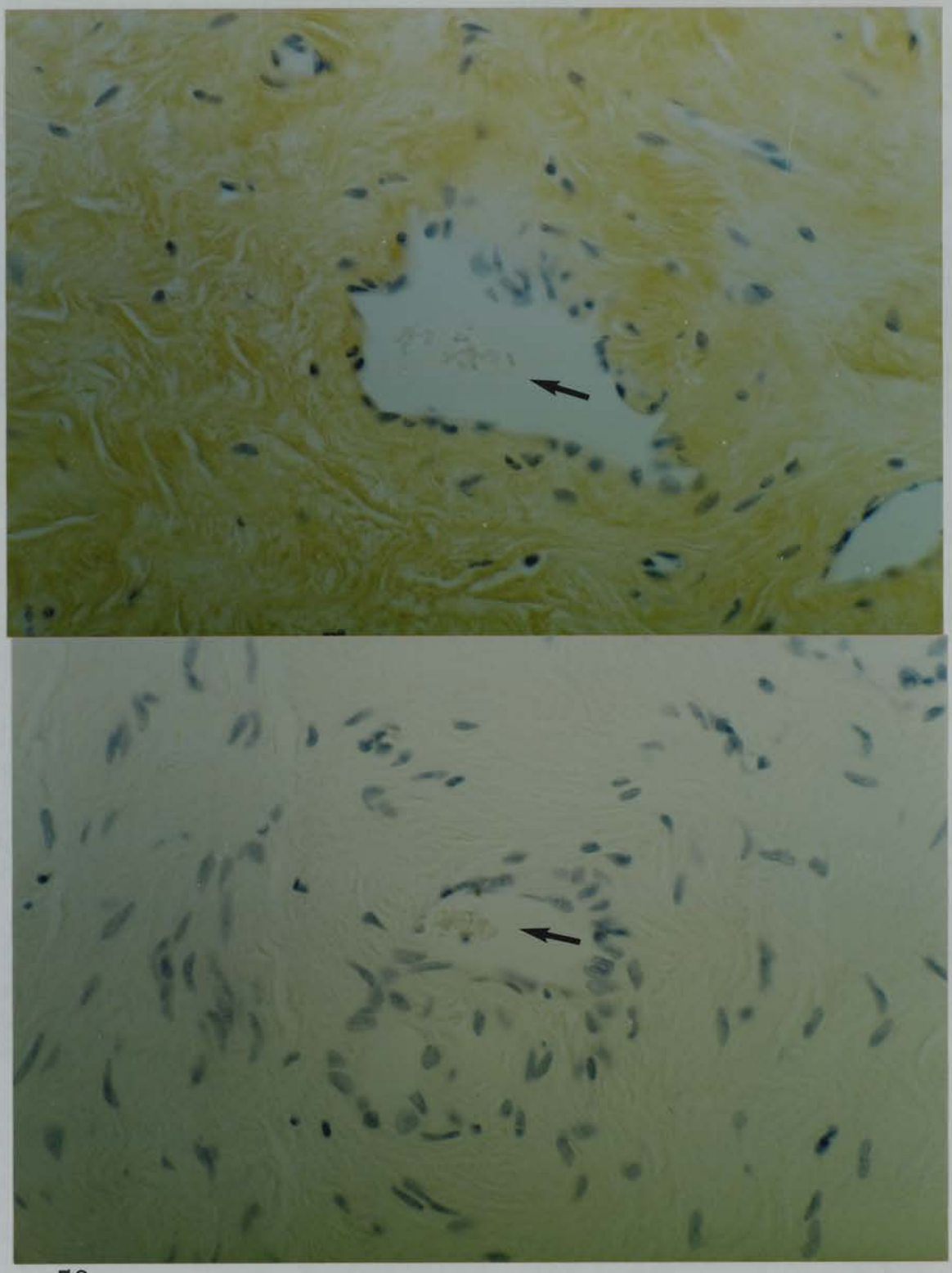


Figure 9.4 Red blood corpuscles (arrows) within nonpregnant cervical tissue (group 3) exposed to the IL-8 antiserum (upper panel) and control Ig (lower panel).

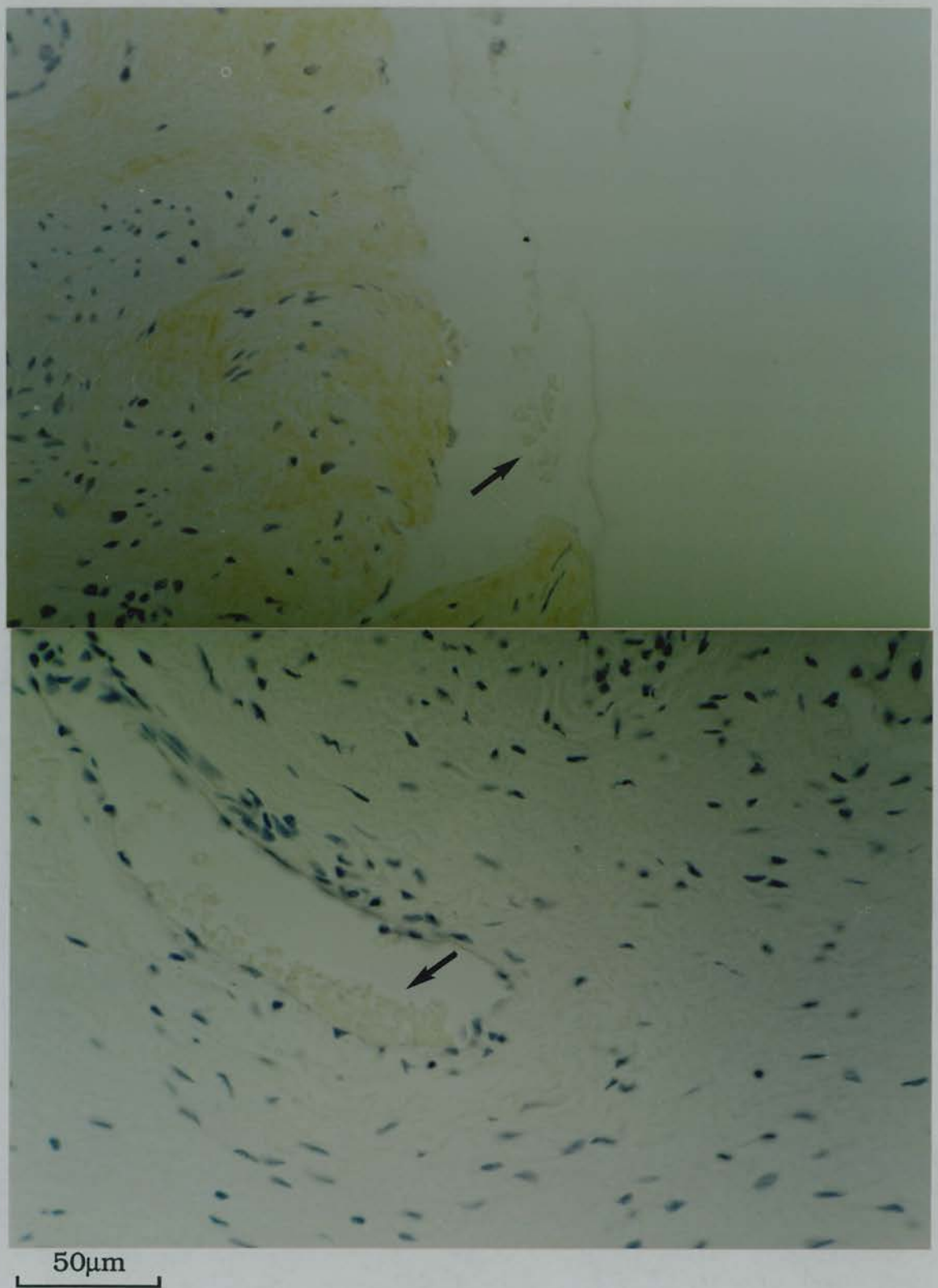


Figure 9.5 Red blood corpuscles (arrows) within cervical tissue exposed to the IL-8 antiserum (upper panel) and control Ig (lower panel). This tissue was from a pregnant woman (group 1).

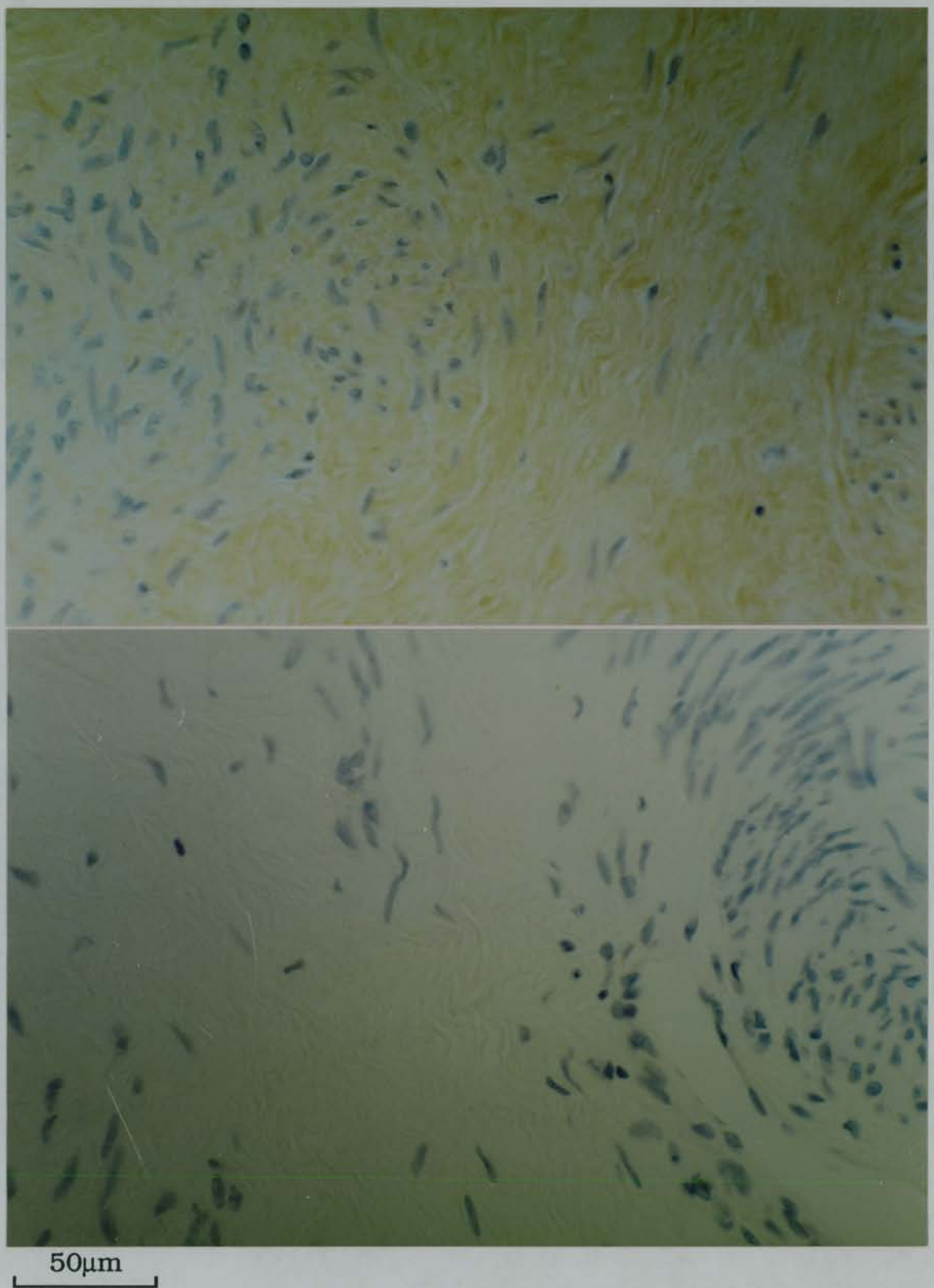


Figure 9.6 Nonpregnant cervical tissue (group 3) exposed to the IL-8 antiserum (upper panel) and control Ig (lower panel).

Marmoset Cervix

The cervix of a pregnant marmoset monkey became available, courtesy of Dr S F Lunn. The animal was 15 weeks pregnant (term is 21 weeks in this species) and had previously been used in a study of a vaccine against the oocyte antigen ZP3 approximately one year earlier. This monkey was sacrificed for immunohistological studies on the ovarian tissue.

The cervical tissue was fixed and embedded in wax and sectioned in the same way as the human material and similarly probed for immunoreactive interleukin-8.

Overall, the results were the same as those observed with the human tissue. The connective tissue stained positively when treated with IL-8 antiserum and displayed no staining with the control Ig. The glandular areas generally did not exhibit binding of the interleukin-8 antiserum (figure 9.7, upper panel shows staining in presence of IL-8 antiserum, lower panel with control Ig). However, where staining did occur it sometimes appeared to be more localised than it did in the human cervix (figure 9.8, upper panel with IL-8 antiserum, lower panel with control Ig). These photographs also show a generally higher cell density in the monkey tissue than observed in the human cervix.

Figure 9.9 shows the sections where positive staining occurred and where RBC were present. It revealed a distinct lack of reactivity between the IL-8 antiserum and the RBC.

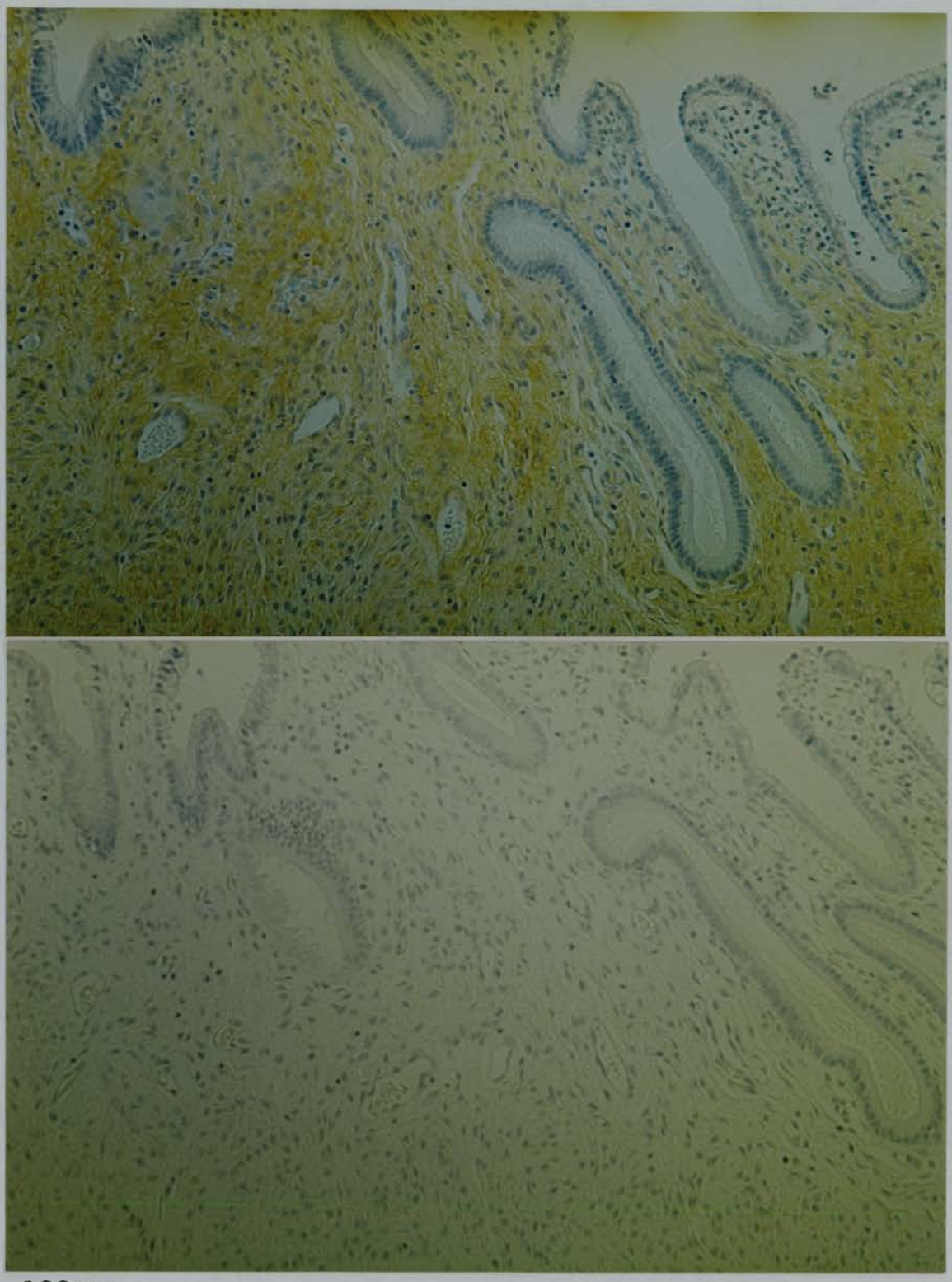


Figure 9.7 The same region of marmoset cervical tissue exposed to the IL-8 antiserum (upper panel) and control Ig (lower panel).

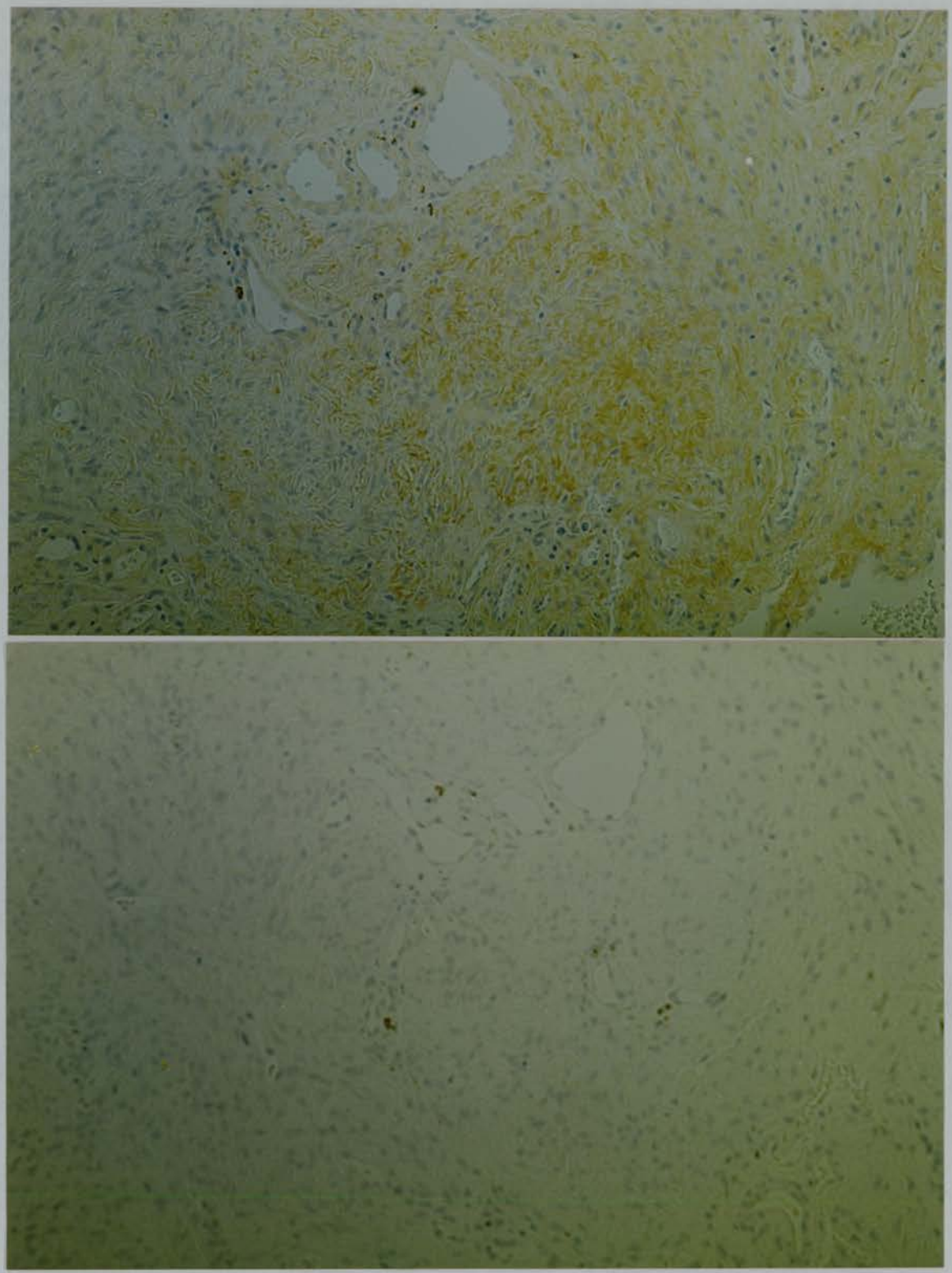


Figure 9.8 The same region of marmoset cervical tissue exposed to the IL-8 antiserum (upper panel) and control Ig (lower panel).

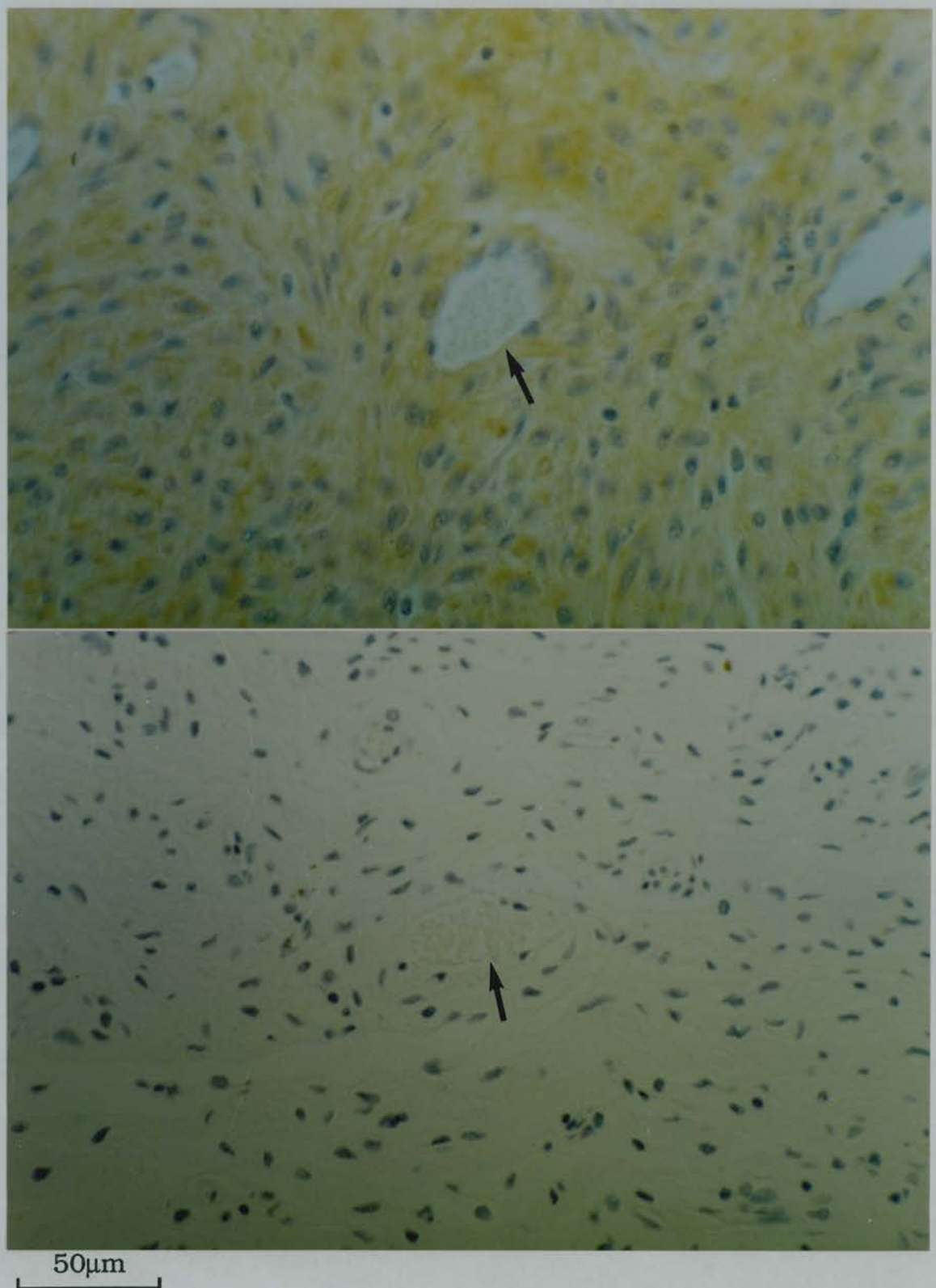


Figure 9.9 Red blood corpuscles (arrows) within marmoset cervical tissue exposed to the IL-8 antiserum (upper panel) and control Ig (lower panel).

The most interesting observation in the marmoset tissue was the presence of strongly positive individual cells in what seemed to be a deep, penetrating glandular structure. Figures 9.10 and 9.11 show a low and high power magnification of these cells following incubation with the IL-8 antiserum (upper panels). The control Ig incubations, (the lower panels of the same figures) show that in the absence of the IL-8 antibodies these same cells did not stain positively. The intense colour appeared to be specific for those cells and localised within well defined boundaries, taking on the semblance of cell membranes. There were very few of these positively stained cells and none were observed in the human cervical tissue. As can been seen, the majority of apparently similar cells in the same structure in the marmoset cervix did not exhibit any signs of immunoreactivity towards the IL-8 antiserum.

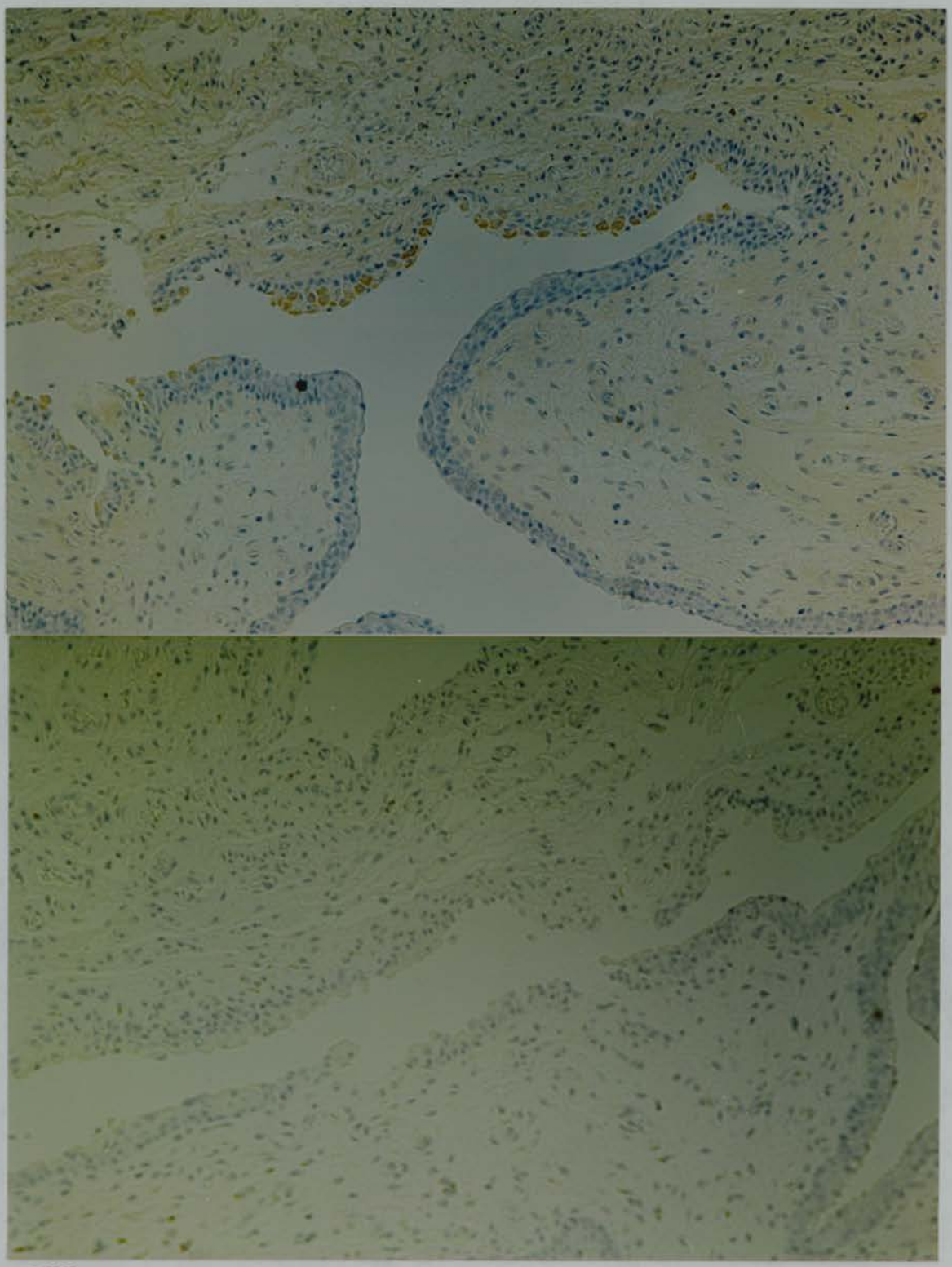


Figure 9.10 Low power magnification of the same region of marmoset cervical tissue exposed to the IL-8 antiserum (upper panel) and control Ig (lower panel).

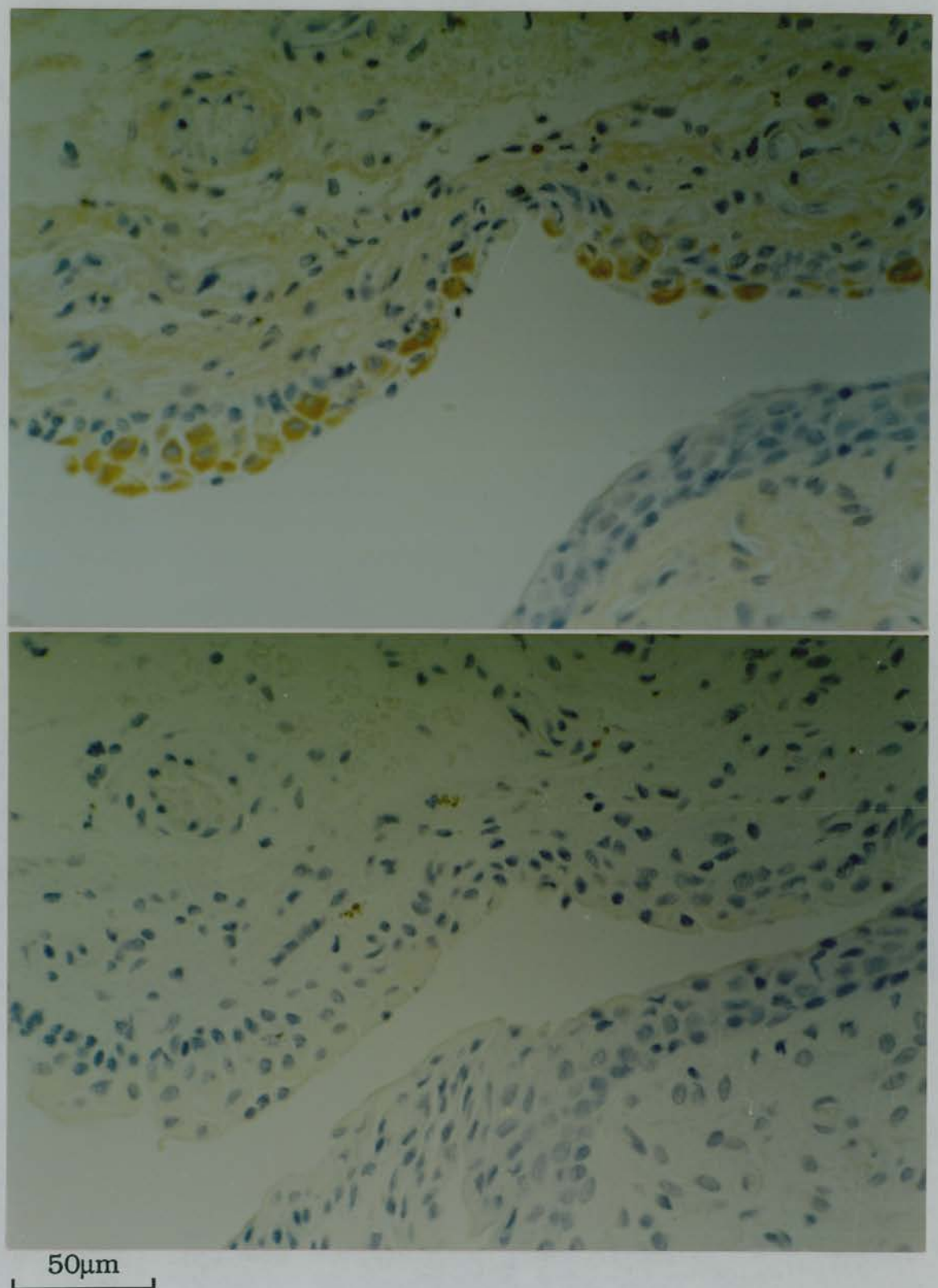


Figure 9.11 High power magnification of the same region of marmoset cervical tissue exposed to the IL-8 antiserum (upper panel) and control Ig (lower panel).

Nonspecific Binding of IL-8 to Connective Tissue

The immunohistological examination of the cervix, described previously, appeared to show binding of the interleukin-8 antiserum in an apparently nonspecific manner to the connective matrix. A simple experiment was carried out to investigate the possibility that the collagen in the connective tissue was coated with IL-8.

Insoluble collagen was incubated with some of the radiolabelled interleukin-8 used in the radioimmunoassay for the cytokine and the radioactivity that became associated with the collagen was determined.

The collagen (10mg) was incubated in a volume of 3ml of radiolabelled IL-8, diluted from stock with PBGS, for 1 hr at 37°C with gentle agitation. The collagen IL-8 mixture was then centrifuged twice at 10,000g (Biofuge) for two minutes each time. An aliquot of 500µl of the supernatant contained 34,683 cpm of radioactivity while the same volume of incubation fluid not in contact with collagen contained 43,611 cpm. The pellet produced by centrifugation was associated with approximately 40,000 cpm of radioactivity, a number which fell slightly upon washing the pellet in PBGS, possibly through the contamination of the supernatant with fibres of the insoluble collagen dislodged when the fluid phase was removed.

A further experiment was performed overnight under the same conditions. The results were essentially the same with a slight increase in the radioactivity associated with the collagen (approximately 50,000 cpm). Again, washing of the pellet caused the release of some activity and filtration of the wash supernatant, through a 0.2µm filter, confirmed that some of it was due to the

presence of fibres of collagen. Following a third wash in PBGS the supernatant contained only 6% of the activity measured in the collagen pellet at this stage compared with 39% after the first PBGS wash.

Discussion of Immunohistological Results

The immunohistological examination of the cervix showed staining of the connective tissue that was related to the application of antibodies directed against interleukin-8. The results suggested that the connective tissue matrix was either coated with IL-8 or the rabbit immunoglobulins were reacting with it in a nonspecific manner. However, the control sections which were incubated with normal rabbit serum did not show a positive stain, implying that rabbit proteins were not binding nonspecifically to the tissue.

All three groups of human tissue exhibited basically the same pattern of positive staining. The tissue culture study showed that the cervix, *in vitro* at least, produces IL-8 without provocation, although this may have been the result of the trauma of dissection. Thus, its presence in the cervical tissue would be expected and was apparently detected. However, there was a distinct lack of any focal production, except for a few cells in the marmoset tissue which lay outwith the connective tissue matrix. The general lack of positive staining of the glandular-like areas, especially in the human cervix, and the epithelial cells suggests that there was some degree of differential binding of the antiserum and the reaction was not entirely nonspecific.

The possibility that the connective tissue may bind IL-8 was investigated by looking for an interaction between collagen and a

radiolabelled form of the cytokine. This work suggested that the collagen and IL-8 may in fact bind together.

Of particular interest in this area is the recent proposal that a concentration gradient in a moving fluid phase would be impossible to sustain and that a chemoattractant would be more likely to be effective if immobilised (Rot, 1992; Tanaka et al., 1993).

The migration of leucocytes from the blood to the interstitium seems to be a multi-stage process whereby the cells become attached to the endothelium via molecules called selectins. The attachment of the cells at this stage is relatively weak and they are capable of 'rolling' along the blood vessel wall. A signalling event causes a change in the integrins found on the leucocytes, their adherence becomes stronger and they are no longer able to drift along the endothelium. This is followed by the change in shape and passage of these cells between those of the endothelial layer (Rot, 1992; MacKay and Imhof, 1993). IL-8 has been identified as a chemoattractant that can produce the change from 'rolling' attachment of neutrophils to the strong attachment via the integrins (Rot, 1992).

The manner in which IL-8 is immobilised has been suggested to involve proteoglycans protruding into the blood stream from the endothelial cells (Tanaka et al., 1993). These authors propose that IL-8 bound to the proteoglycans would effectively 'attract' the 'rolling' leucocytes and thus stimulate migration across the endothelial wall. Proteoglycan molecules attached to the lumenal surfaces of blood vessels are normally present and act in the prevention of inappropriate clotting of the blood and may be involved in lipid metabolism (Tanaka et al., 1993).

Thus, the apparent binding of IL-8 to the connective tissue in the cervical sections examined in this thesis may lend support to the theory that this molecule binds to proteoglycans. Such an action would presumably also permit the IL-8 to retain its activating properties, with regard to degranulation, as well as its chemoattractant properties.

Despite the indication that collagen and IL-8 can become associated with each other it is also possible that the IL-8 antiserum may contain antibodies which cross react with connective tissue. A more extensive immunohistological study of the cervix would be required to clarify the situation. The use of antiserum from several sources and the preincubation of the antisera with IL-8 prior to application to the tissue as alternative controls could be examined.

The few positively stained cells in the marmoset tissue appeared to be a real effect, as indicated by the intensity of the stain within apparent cytoplasmic boundaries (cell membranes) and the lack of any staining in the absence of the antiserum. This tissue was taken from a pregnant animal in the last third of her pregnancy. Examination of the human tissue did not provide evidence of similar focal production but the human material was all from first trimester or nonpregnant women. It is possible that the appearance of this staining is indicative of changes in cervical biology associated with increasing gestation. The collagenase found in cervical tissue at the end of pregnancy has been shown to be of polymorphonuclear origin. The activation of cells producing IL-8 in later gestation would provide a mechanism whereby neutrophils would be attracted into the cervix. The reason that few cells were observed in the marmoset tissue may be that this sample was taken in the early stages of stimulated IL-8

production, and that tissue collected at a later stage of the pregnancy would provide evidence of significantly more focal production of the chemokine.

It has been claimed that red blood corpuscles bind IL-8 and may remove it from the circulation in order to maintain the concentration gradient around its source and to limit the stimulation of neutrophils (Darbonne et al., 1991). This removal of IL-8 from the circulation may be of importance since it has been shown that when neutrophils have undergone shape change and shedding of selectin molecules before adhering to a vessel wall then they are subsequently unable to attach to the lumenal surface (Rot, 1992). This appears to be a fail-safe mechanism preventing generalised neutrophil activation and migration into the interstitium. However, none of the RBC observed in either the human or marmoset material reacted with the IL-8 antiserum despite its reactivity with the connective tissue. Since, as already mentioned, the cervix produces IL-8 at a basal level it would then be expected that any captive RBC in the tissue explant would bind the cytokine produced therein, unless of course it was internalised by them. The size of RBCs (2μm 'thick' and 7μm in diameter) would allow whole corpuscles to be present in the sectioned material since each section was 4μm thick.

The similarities between the three groups of human material coincides with their similar production of IL-8 observed in the tissue culture experiments. This evidence of IL-8 in the connective tissue, as shown in the preceding figures, and the production of IL-8 by the tissue in culture may raise questions concerning the source of the IL-8 measured in the culture medium. The possibility does exist that the treatments used in the experimental system caused the release of

IL-8 from the connective tissue. The apparent constitutive production by the cervix in culture may indicate dissociation of IL-8 from the connective tissue or that tissue necrosis is taking place and thereby releasing IL-8 into the medium. However, the consistent production over three days (see Chapter 7) indicates that the tissue remains viable and that significant necrosis is not occurring. This consistency of production also suggests that the IL-8 measured is produced by the tissue on stimulation rather than the release of 'stored' material.

Chapter 10

Main Findings and Suggestions for Further Work

Main Findings and Suggestions for Further Work

The following pages summarise the main findings of the work presented and contain suggestions for other studies in this area.

The antiprogestin RU486, given *in vivo*, did not appear to affect the production of PGE_2 or $PGF_{2\alpha}$ by pregnant guinea pig cervical cells in culture, nor did it influence the stimulatory properties of LPS, PMA or okadaic acid. The effect of LPS, PMA and okadaic acid supports the proposals that cervical ripening could be an inflammatory based process, showing that cervical production of prostaglandins can be modulated. The effect of LPS also provides further indications that microbial infections may induce preterm labour/cervical ripening by stimulating an inflammatory response. The most obvious effect associated with RU486 treatment of the guinea pigs was that seen with the calcium ionophore A23187. Most studies using A23187 report a stimulatory action of the ionophore, however, in experiments carried out for this thesis there was a distinct inhibitory response. The inhibitory effects of this material were attenuated in cultures derived from animals that were given the antiprogestin prior to sacrifice. This implies that RU486 may regulate calcium concentrations in its ripening action.

Additional RU486, introduced into the culture medium, again did not affect the actions of LPS, PMA, and okadaic acid, and only produced a slight increase in prostaglandin production by cells from RU486 pretreated animals.

The inclusion of progesterone in the culture medium seemed to have a slight inhibitory effect on basal prostaglandin synthesis, an effect that was blocked where the guinea pigs were given RU486.

Progesterone generally had no effect on the other treatments applied *in vitro* and in the instances where it did the responses were inconsistent and showed no particular pattern. Prostaglandins and inflammation are regarded as important regulators of the condition of the cervix. Progesterone and RU486 are considered to be important factors in cervical ripening, despite the apparent lack of any major effect on prostaglandins in these experiments. However, it is possible that progesterone could restrain cervical ripening through indirect mechanisms, such as maintenance of the quiescence of the uterus.

In the guinea pig experiments the cytokines IL-1 and TNF did not affect the production of prostaglandins.

The human study showed that cervical tissue from women can produce the cytokine interleukin-8 and that its synthesis can be upregulated by LPS, PMA and TNF. The tissue from postmenopausal women did not respond to PMA in the same way as the other tissues. The absence of an increase in IL-8 output from these samples suggesting some form of hormonal control may be operative. The production of this cytokine by the cervix may be significant with regard to its ability to direct its own remodelling during pregnancy, especially at term where rapid changes are crucial.

The pattern of PGE_2 production by human cervix was similar to that of IL-8, except when challenged by okadaic acid and by A23187. This suggests different control mechanisms for the two mediators. Again, there appeared to be some element of hormonal control evident by the lack of an effect of PMA on the postmenopausal tissue. The human tissue also responded to TNF by increasing its output of PGE_2.

The immunohistological work provided evidence of the presence of IL-8 associated with the connective tissue of the human cervix.

However, there was little indication for a cellular source of the cytokine.

With regard to further work, it would be of great interest to determine whether or not the results obtained when working with cervical material from postmenopausal women are repeatable. If this proved to be the case then the effects of hormone replacement therapy in such women should be studied.

It would also be useful to examine tissue from women at the end of their pregnancy and to probe for differences in the responses of tissue from the different groups of women, for example the term pregnant versus the early pregnant or the non pregnant.

Another study looking at the effect of RU486 on the production of IL-8 would be of interest. However, the sampling procedure would have to be modified.

The immunohistochemical study could be repeated, incorporating further controls to refine the results already presented, and to identify the source of the IL-8.

The use of IL-8 to produce cervical ripening has the potential to offer a major step forward in the induction of cervical maturation and clinical studies on its effects are required. Should it prove to be a significant factor in cervical ripening and in preterm cervical ripening, then the development of compounds able to block its effects may allow better management of the incompetent cervix.

Appendices

Appendix I

Reagents and Buffers

Tissue/Cell Culture and Assay Reagents/Buffers

BSA/tween Buffer

BSA-tween buffer was used in the interleukin-8 assay and was designed to block the adherence of IL-8 to the assay tubes and improve assay reliability. It contained the following (per litre),

12.1g	Tris(hydroxymethyl)methylamine
5.0g	BSA (Bovine Serum Albumin)
1.0g	Sodium Azide
0.02%	Tween 80

Tris, BSA and sodium azide were dissolved in 900ml purified water. Tween 80 is a very thick detergent and difficult to dispense in small volumes, therefore it was dissolved in warm water to give a 1% solution and 20ml of this (2ml 1% tween 80 per 100ml buffer) added to the main buffer volume. The pH was adjusted to 7.6 and the volume topped up to 1 litre with water. Sodium azide is included as an antimicrobial agent.

Complete Culture Medium

The medium used for culturing cells and tissue is described as Complete Culture Medium and contained the following,

500ml	RPMI1640
50ml	Fetal Bovine Serum
5ml	Penicillin-Streptomycin†
5ml	Glutamine††
200µl	Gentamicin§

Stock solutions:-
† 5000 international units penicillin/ml, 5000µg streptomycin/ml
†† 30mg glutamine/ml
§ 50mg gentamicin/ml

RPMI1640 is widely accepted as a medium capable of supporting growth of most mammalian cells and has been used successfully in this laboratory for some time with a variety of human and guinea pig culture systems.

Glutamine is an essential component of medium for the maintenance of tissue in culture. It is unstable above -10°C and was therefore stored at -20°C and added to the stock RPMI1640 (stored at 4°C) when complete medium was required.

Penicillin-streptomycin and gentamicin were included in the medium in order to reduce the risk of bacterial and mycoplasma growth during the culture period.

Digestion Medium

The collagenase-DNAse solution used for the main digestion process of the guinea pig cervix dispersal was identified as digestion medium and was composed of the following,

50ml	RPMI1640 (with glutamine, as above)
50mg	Collagenase†
5mg	DNAse††

Final concentrations:–
† 1mg collagenase/ml
†† 0.1mg DNAse/ml

Once the lyophilised enzymes had dissolved in warmed RPMI1640 it was sterilised by filtration through 0.22μm filters.

Collagenase initiated the digestion of the collagen matrix and because the collagenase preparation was impure and also contained other proteases, further, less specific, proteolytic degradation of the connective tissue took place. DNAse was present to breakdown any DNA that was inevitably released into the solution by dead or over digested cells and thereby prevent it from causing clumping of the dispersed cells.

Methyloximating Solution (MOX)

The antisera used in this work were raised against methyloximated prostaglandins and therefore the samples of culture medium required to be treated with methyloximating solution.

41g	Sodium Acetate
5g	Methoxylamine Hydrochloride
50ml	Ethanol
200ml	Purified water

These materials were combined and once the solids had dissolved the pH was adjusted to 5.8.

The methyloximation process (moxing) was carried out by adding half the sample volume of MOX (i.e. 2 parts culture medium + 1 part MOX) to the harvested medium. The two were mixed and left at room temperature overnight for the methyloximation to occur. This converts more than 95% of the PGs present to their methlyoxime derivatives (Kelly et al., 1986a). The addition of a methyloxime group improves the stability of the prostaglandins and allows their storage at 4°C.

Phosphate Buffered Gelatine Saline (PBGS)

Phosphate buffered gelatine saline (PBGS) was a general purpose buffer used in several preparations. It was produced in batches of 4 litres as follows,

36.0g	Sodium Chloride
34.4g	di-Sodium Hydrogen Orthophosphate Anhydrous
24.3g	Sodium di-Hydrogen Orthophosphate
4.0g	Sodium Azide
4.0g	Gelatine

The above compounds, except for the gelatine, were dissolved in approximately 3.75 litres of purified water. Gelatine is relatively insoluble in cold water and was dissolved in about 100-150ml warm water (less than 100°C). The gelatine was then added to the main volume of buffer and the pH adjusted to 7.8 with sodium hydroxide. Finally, the total volume was made up to 4 litres and the buffer stored at 4°C.

Purified Water

The water used in this work was purified using a RoStill™ apparatus which removed salts from the water supply by reverse osmosis.

Radiolabelling Using Chloramine T Oxidation

Chloramine T was used to oxidise sodium iodide to produce free iodine. The iodine (^{125}I) reacted with the tyrosine group of the Pro-Gly-Tyr peptide conjugated to the prostaglandins. To remove any free iodine, once the reaction had taken place between it and the peptide, sodium bisulphite was added to the mixture. This also removed any chloramine T present.

Standards Buffer (IL-8 assay)

The standards for the IL-8 assay were reconstituted and diluted with buffer composed of 50% BSA/tween buffer and 50% PBGS. This was to reduce the likelihood of the IL-8 adhering to the tubes.

Tissue Collection Buffer

The excised cervices were collected in sterile PBS plus 1% penicillin-streptomycin.

50.0ml	PBS
0.5ml	Penicillin-Streptomycin†

Stock solution:-
† 5000 international units penicillin/ml, 5000µg streptomycin/ml

The antibiotics were present to reduce the chances of microbial contamination.

Treatment Media

The media used to treat the cells in culture were prepared by aliquotting complete medium into 10ml lots and the appropriate volume of stock solution added to the vial using an accurate delivery, positive displacement pipette. The volumes added varied between 2 and 5µl per 10ml and the solvents used were PBS, ethanol or dimethylformamide. For the cells seeded in progesterone containing medium the treatment media were prepared using complete medium with 10^{-6}M progesterone. Progesterone stock was a 10mg/ml solution in ethanol, of which 3.15µl was added to 100ml medium.

Trypan Blue Exclusion

Cell viability assessment was performed by combining equal volumes of cell suspension and 0.4% trypan blue solution. The two were mixed and left for 2 minutes then the cells were examined using a haemocytometer and the proportions of viable and non viable cells determined.

Trypsin Pre-Digestion Solution

The trypsin pre-digestion solution used in the first stage of the guinea pig cervix digestion was made up as follows,

40ml	PBS
10ml	Trypsin (X10)
10mg	EDTA

The above solution was sterilised by filtration through 0.22µm filters.

The dispersal system used was derived from that of Rajabi et al (Rajabi et al., 1991) however, they do not indicate their reason for including trypsin. It may be the case that this enzyme was used to cleave any of the pro form of collagenase contained in the cervix (Cawston and Tyler, 1979) and possibly in an attempt to "loosen" the connective tissue matrix and allow easier dispersal of the individual

cells by degrading any other proteins that may be present in the cervix or between cells.

EDTA was present in order to sequester any calcium and magnesium and the PBS used did not contain either metal. Trypsin is more active in the absence of calcium and magnesium.

Trypsinisation Solution

Trypsinisation of cells already growing in culture dishes was carried out using X10 trypsin diluted by a factor of ten with PBS.

The medium supporting cell growth was removed and the cells washed at least once with PBS to flush away any fetal bovine serum and thus remove any enzyme inhibitors present. The trypsin was then added to the culture flask, sufficient to coat the surface of the vessel, and any excess decanted. The cells were then replaced in the incubator for 2-3 minutes to promote the action of the trypsin, followed by gentle agitation of the flask to dislodge the cells. Complete medium was added to the trypsinised cells to inhibit the enzyme and the mixture gently drawn up and down a pipette several times to disperse the cells.

For the assessment of cell numbers in cluster plate wells stronger trypsin solutions were used but in these cases there was no further use for the cells and therefore a more severe digestive treatment was not as unacceptable.

Wash Buffer

Prostaglandin assays incorporated a wash stage where the second antibody precipitate was resuspended in buffer to reduce the NSB values. This buffer was prepared as follows,

100ml	PBGS
1.25ml	1.25% triton X-100

Immunohistochemical Reagents/Buffers

Formaldehyde

Tissue fixation for immunohistochemical analysis was performed with 4% formaldehyde. The stock of 40% was diluted 1 in 10 with phosphate buffer, pH 7.6.

The tissues involved in this work were all small and 24 hours was adequate time for the fixation process.

Tris Buffered Saline (TBS)

The main buffer used in the immunohistological staining was tris buffered saline (TBS) which contained the following,

6.05g Tris(hydroxymethyl)methylamine
9.00g Sodium chloride

The tris and sodium chloride were dissolved in almost a litre of water, the pH adjusted to 7.6 and then the volume made up to one litre. Two litres was generally enough for the whole staining process.

Endogenous Peroxidase Blocking Solution

Mammalian tissues contain endogenous peroxidase activity and therefore this must be blocked before the substrate is applied to sections. This was done by treating the tissue with a solution of 3% hydrogen peroxide in methanol, made up as follows,

30ml 30% Hydrogen peroxide
270ml Methanol

Normal Swine Serum (NSS)

Normal swine serum (DAKO) was used to block any sites on the tissue that would non specifically bind the second antiserum, which was raised in swine, and therefore reduce the chances of false positive results. The normal swine serum was diluted with TBS, 1 part normal swine serum plus 4 parts TBS, to produce the reagent referred to as NSS

Primary Antiserum

The primary antiserum used in this study was directed against synthetically produced IL-8 (human amino acid sequence) and was raised by Dr R.W. Kelly in rabbits. It was diluted 1 in 25 with NSS.

Normal Rabbit Serum

Normal rabbit serum was used as a control for the rabbit primary antiserum and was diluted with NSS to the same concentration as the primary antiserum (1 in 25).

Swine Anti-Rabbit Second Antiserum

The second antiserum (DAKO) was raised in swine against rabbit immunoglobulins. It was diluted with NSS to 1 part in 40. Its function was to act as a bridge between the primary antiserum and the PAP complex.

Peroxidase Anti-Peroxidase Complex

The peroxidase anti-peroxidase (PAP) complex (DAKO) was a rabbit antiserum complexed with horseradish peroxidase and diluted to 1 part in 100 with NSS. The PAP complex bound to the swine anti rabbit second antiserum and acted as an amplification step in the staining procedure by virtue of the fact that each antibody was linked to more than one peroxidase molecule.

DAB Peroxidase Substrate

The peroxidase substrate was diaminobenzidine (DAB). It was dissolved in tris/hydrogen peroxide buffer as follows. The tris was made up similarly to the TBS but without sodium chloride. DAB (5mg) was dissolved in 10ml of the 0.05M tris buffer (pH 7.6) plus 3.3µl 30% hydrogen peroxide.

1% Acid Alcohol

A 1% solution of hydrochloric acid in 70% methanol was employed to differentiate the hematoxylin. This step removes the hematoxylin from the tissue but preferentially from the cytoplasm first leaving the nuclei stained.

Scott's Tap Water

Scott's tap water contained (per litre),

1g Potassium Hydrogen Carbonate
10g Magnesium Sulphate

The above were dissolved in water and the purpose of this solution was to, "blue up," the hematoxylin stained nuclei, to improve the contrast.

Appendix II

Materials and Suppliers

Materials	**Suppliers**
A23187 or Calcium Ionophore or Calcimycin	Sigma Chemical Co., Poole, Dorset, UK.
Bovine Serum Albumin (BSA)	Sigma Chemical Co., Poole, Dorset, UK.
Chloramine T	Sigma Chemical Co., Poole, Dorset, UK.
Carbon Dioxide	BOC, Edinburgh, UK.
Collagenase	Sigma Chemical Co., Poole, Dorset, UK.
Deoxyribonuclease 1 (DNAse 1)	Sigma Chemical Co., Poole, Dorset, UK.
3,3-DiAminoBenzidine (DAB)	Sigma Chemical Co., Poole, Dorset, UK.
N,N-Dimethylformamide	Rathburn Chemicals Ltd, Walkerburn, Scotland, UK.
Ethanol	BDH Chemicals, Poole, Dorset, UK.
Ethylene Diamine Tetraacetic Acid (EDTA)	Sigma Chemical Co., Poole, Dorset, UK.
Fetal Bovine Serum (FBS) (HEAT INACTIVATED)	Northumbria Biologicals, Cramlington, Northumberland, UK.
Formaldehyde (40% stock)	BDH Chemicals, Poole, Dorset, UK.
Gelatine	BDH Chemicals, Poole, Dorset, UK.
Gentamicin	Sigma Chemical Co., Poole, Dorset, UK.
HELA Cell Line	European Collection of Cell Cultures, Salisbury, Wiltshire, UK.

Hematoxylin	Sigma Chemical Co., Poole, Dorset, UK.
Histoclear	National Diagnostics, Aylesbury, Bucks, England, UK.
Hydrochloric Acid	BDH Chemicals, Poole, Dorset, UK.
Hydrogen Peroxide (30%)	BDH Chemicals, Poole, Dorset, UK.
Interleukin-1β	Sigma Chemical Co., Poole, Dorset, UK. Boehringer Mannheim, Lewes, Sussex, UK.
Interleukin-8	Collaborative Biomedical Products, Becton Dickinson Labware, Bedford, MA, USA.
Interleukin-8 Antiserum	ICN FLOW, ICN Biomedicals Ltd., High Wycombe, Bucks, England, UK.
Isopropyl Alcohol	BDH Chemicals, Poole, Dorset, UK.
L-Glutamine	Northumbria Biologicals, Cramlington, Northumberland, UK.
Lipopolysaccharide (LPS)	Sigma Chemical Co., Poole, Dorset, UK.
Medroxy Progesterone Acetate	Sigma Chemical Co., Poole, Dorset, UK.
Methanol	BDH Chemicals, Poole, Dorset, UK.
Methoxylamine Hydrochloride	Aldrich, Gillingham, Dorset, England, UK.
Normal Rabbit Serum	DAKO, High Wycombe, Bucks, England, UK.
Normal Swine Serum	DAKO, High Wycombe, Bucks, England, UK.

Oestradiol	Sigma Chemical Co., Poole, Dorset, UK.
Okadaic Acid	Sigma Chemical Co., Poole, Dorset, UK.
Penicillin-Streptomycin	Gibco, Paisley, Strathclyde, UK.
Peroxidase Anti-Peroxidase Complex (rabbit)	DAKO, High Wycombe, Bucks, England, UK.
Phorbol Myristate Acetate (PMA)	Sigma Chemical Co., Poole, Dorset, UK.
Phosphate Buffered Saline (PBS)	Northumbria Biologicals, Cramlington, Northumberland, UK.
Progesterone	Sigma Chemical Co., Poole, Dorset, UK.
Prostaglandins	Sigma Chemical Co., Poole, Dorset, UK.
RPMI1640 Dutch Modification	Northumbria Biologicals, Cramlington, Northumberland, UK.
RU486	Roussel Uclaf, Paris, France. (D Philibert)
Sep-Pak C-18 Cartridge	Millipore (UK) Ltd, Watford, Hertfordshire, UK.
Sodium Acetate Anhydrous	BDH Chemicals, Poole, Dorset, UK.
Sodium Azide	BDH Chemicals, Poole, Dorset, UK.
Sodium Bisulphite	Sigma Chemical Co., Poole, Dorset, UK.
Sodium Chloride	BDH Chemicals, Poole, Dorset, UK.
Sodium Iodide (^{125}I)	Amersham, Aylesbury, Bucks, England, UK.
Sodium Dihydrogen Orthophosphate	BDH Chemicals, Poole, Dorset, UK.

di-Sodium Hydrogen Orthophosphate	BDH Chemicals, Poole, Dorset, UK.
Sodium Hydroxide	BDH Chemicals, Poole, Dorset, UK.
Swine Anti Rabbit (Second Antiserum)	DAKO, High Wycombe, Bucks, England, UK.
Tris(hydroxymethyl)methylamine (Tris)	BDH Chemicals, Poole, Dorset, UK.
Triton X-100	Sigma Chemical Co., Poole, Dorset, UK.
Trypan Blue	Sigma Chemical Co., Poole, Dorset, UK.
Trypsin (X10)	Sigma Chemical Co., Poole, Dorset, UK.
Tumour Necrosis Factor Alpha (TNFα)	Sigma Chemical Co., Poole, Dorset, UK.
Tween 80 (Polyoxyethylene sorbitan monooleate)	Sigma Chemical Co., Poole, Dorset, UK.
Xylene	BDH Chemicals, Poole, Dorset, UK.
Cluster Plate (48 Well, With lid) (manufactured by Costar)	Northumbria Biologicals, Cramlington, Northumberland, UK.
Culture Flasks	Falcon, Becton Dickinson Labware, Bedford, MA, USA.
Filters (0.22μm)	Millipore (UK) Ltd, Watford, Hertfordshire, UK.
Glass Slides	BDH Chemicals, Poole, Dorset, UK.
Petri Dishes	Falcon, Becton Dickinson Labware, Bedford, MA, USA.
Scalpel Blades (Nº 11, Sterile)	Swann-Morton, Sheffield, England, UK.

Appendix III

Material Published as a Consequence of Work in this Thesis

leukin-8 production by the human cervix

G. Barclay, BSc,[a] Janet E. Brennand, MB, ChB,[a] Rodney W. Kelly, PhD,[b] and A. Calder, MD[a]

, Scotland

'ES: Our purpose was (1) to determine whether the human cervix is capable of producing -8 in vitro and to examine the possibility of stimulating an increase in any such output and (2) e the concomitant production of prostaglandins.

ESIGN: Cervical tissue was obtained from 48 women, 29 pregnant women undergoing surgical n of pregnancy (20 of whom were treated with the prostaglandin analog Cervagem), 14 ant, premenopausal women, and three postmenopausal women. Explants were cultured and m was assayed for interleukin-8 and prostaglandin E_2. Analysis of variance and Newman-Keuls ests were used.

: Significant quantities of interleukin-8 were produced by the tissue, and the data indicate that plants from pregnant and nonpregnant women behave in a similar way when challenged by yristate acetate but that the postmenopausal cervix loses its capacity for interleukin-8 n.

SIONS: Human cervix is capable of producing large amounts of interleukin-8 in vitro, and it may ced by the steroid hormones. Thus interleukin-8 could be an excellent candidate for a prime utrophil-mediated cervical ripening. (Am J Obstet Gynecol 1993;169:625-32.)

ds: Interleukin-8, cervical ripening, neutrophils

l ripening is the process by which the cervix from a rigid, closed structure designed to e uterine contents to one that becomes soft and ilate to accommodate the passage of the fetus. rvix consists mainly of connective tissue the of which, about 70%, is type I collagen.[1] muscle cells constitute about 10% of the cer- reas collagen-producing fibroblasts are the ular component.[3] Changes in the connective tissues occur throughout pregnancy,[1] but this remodeling of the collagen does not greatly alter the rigidity of the cervix. Only in the very last stages can its mechanical strength be permitted to change in such a dramatic manner as to permit fetal expulsion.

It has been suggested that this process is similar to an inflammatory reaction,[4] and Junqueira et al.[3] and Rath et al.[5] have shown that neutrophil invasion of the cervix occurs in labor. Others have proposed that the breakdown of the collagen matrix in the final stages of pregnancy is caused by collagenase and elastase produced not only by leukocytes, as would be found in an inflammatory reaction, but also by the fibroblasts of the cervix, which are known to produce collagenase.[1] Collagenase produced by granulocytes, such as neutrophils, selectively degrades type I collagen,[6] whereas collagenase produced by fibroblasts degrades type I and type III collagen equally. Because type III collagen

Department of Obstetrics and Gynaecology, University of [a] and the Medical Research Council, Reproductive Biol- Centre for Reproductive Biology.[b]

r publication November 23, 1992; revised January 28, pted March 8, 1993.

quests: Rodney W. Kelly, PhD, Department of Obstetrics cology, University of Edinburgh, Centre for Reproductive Chalmers St., Edinburgh, Scotland EH3 9EW.

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still represents a significant percentage of cervical collagen (30%),[1, 7] this would tend to support the idea that collagenolysis in the cervix is a multienzyme process.

The interleukins may play an important role in such a process because they are chemotactic for neutrophils and may represent the signal for the commencement of cervical ripening.

Interleukin-8 (IL-8) was initially identified by several laboratories and given a different name by each on the basis of where it was isolated and the experiments that were carried out. Some of the names assigned to it include monocyte-derived neutrophil chemotactic factor, neutrophil-activating factor, and neutrophil-activating or attracting protein or peptide. In addition, T lymphocyte chemotactic factor was also found to have the same amino acid sequence.[8] Because it appears to be a multipotent compound acting on several cell types, agreement was reached that this compound should be renamed "interleukin-8."[9]

IL-8 is a 72 amino acid peptide belonging to a supergene family of peptides.[8, 10] It attracts neutrophils and causes them to release their *specific* granule contents, which include collagenase, and may also under some circumstances stimulate the release of elastase from the azurophil granules.[11]

Several human cell types have been shown to produce IL-8, including dermal fibroblasts and, more relevant to this work, chorion and decidual cells.[12] Both these cell types were shown to produce IL-8 and, under the influence of phorbol myristate acetate or lipopolysaccharide, to be capable of augmenting their IL-8 output. There is evidence that other cytokines may be involved in the regulation of IL-8 synthesis. In particular, interleukin-1 (IL-1) and tumor necrosis factor (TNF) were shown by Matsushima et al.[13] and Larsen et al.[14] to cause rapid induction of IL-8 messenger ribonucleic acid expression. IL-1 and TNF have also been found to promote prostaglandin and collagenase production in many cell types,[15-19] and prostaglandins are inflammatory mediators known to be involved in cervical ripening.[20] Additionally, IL-8 and prostaglandin E_2 (PGE_2) have been found to act synergistically, PGE_2 reducing the concentration of IL-8 required to cause neutrophil invasion of rabbit skin.[21]

In this paper we report, for the first time, the ability of human cervical explants to secrete large amounts of IL-8 in vitro.

Material and methods

Tissue. Tissue was divided into four categories. Group 1 ($n = 14$) was from women undergoing surgical termination of pregnancy who were given a 1 mg pessary of the prostaglandin E analog Cervagem (16, 16-dimethyl Δ^2-prostaglandin E_1 methyl ester) 2 to 3 hours before the procedure. Group 2 ($n = 9$) was from women undergoing surgical termination of but not pretreated with Cervagem. Cervage to ease the dilatation of the cervix in women. Group 3 ($n = 8$) was from prem nonpregnant patients with no disease of undergoing hysterectomy. These women w ous stages of the menstrual cycle. There was data to show stage-specific differences. Grou was tissue from postmenopausal patients h for curettage for investigation of postm bleeding.

Biopsy specimens were obtained from gr and 4 with a Tru-cut needle. The tissue nonpregnant women (group 3) was obtained Forty-nine samples were obtained, but one was discarded because of discrepancies in weights.

This study had the approval of the local e mittee.

Tissue culture. The needle biopsy speci proximately 10 to 15 mg in weight, 10 to length, and 1 to 2 mm in diameter, were saline solution at 4° C. The tissue was cut sections and placed in 250 μl of culture (RPMI1640, Northumbria Biologicals, Cra U.K.) supplemented with penicillin (50 IU/ml mycin (50 μg/ml, Gibco), gentamicin (20 μg/m St. Louis), L-glutamine (0.3 mg/ml, Northum logicals), and 10% fetal bovine serum (No Biologicals) in a 48-well plate (Costar).

The needle biopsies gave four pieces of culture, two of which were used as controls. Ea 3 cervix provided more pieces, thus allowin treatments to be tested per biopsy. Every used with this group of cervices was applied four separate samples.

The treatments used were phorbol myristat (Sigma) at 10^{-7} mol/L, okadaic acid (S 5×10^{-8} mol/L dissolved in dimethylforma popolysaccharide (Sigma) at 4 μg/ml, and (Boehringer, Mannheim, Germany) at 100 The effect of progesterone was also examined mol/L; this concentration was chosen beca higher than circulating levels and because th concentration of progesterone adjacent to th ductive organs is unknown but assumed to because it is produced locally.

All the tissue was incubated in 95% air and 5% dioxide under humid conditions at 37° C.

Medium was changed at 24-hour intervals, medium containing the same additives as the The harvested medium was split into two porti frozen at −20° C and later assayed for inter and the other treated with methyloximating solu assay of prostaglandins.

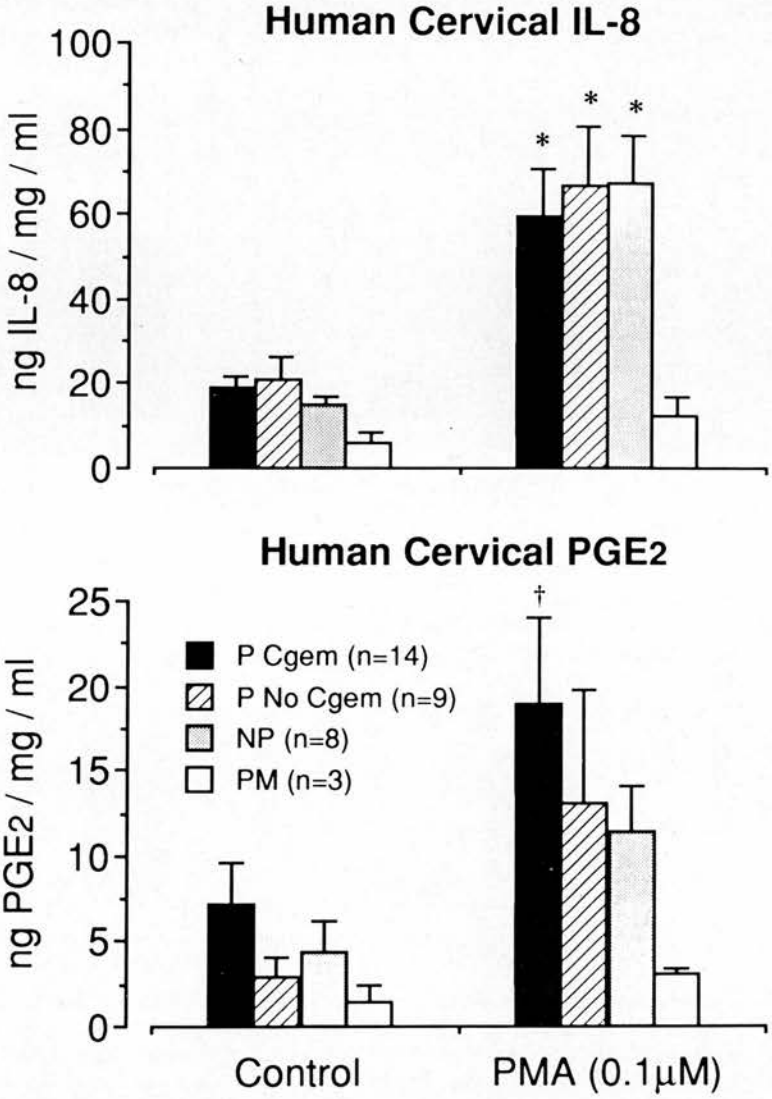

Fig. 1. Effect of phorbol myristate acetate *(PMA)* on IL-8 and PGE_2 production by human cervix (mean ± SE). *Asterisk,* Significantly different from control, $p \leq 0.01$; no significant differences between IL-8 controls; *dagger,* significantly different from control, $p \leq 0.02$; no significant differences between PGE_2 controls; *P Cgem,* group 1; *NP,* group 3; *PM,* group 4.

s. For the IL-8 radioimmunoassay a polyclonal n (ICN Flow) was used with recombinant IL-8 rative Biomedical) labeled with iodine 125 am) by means of chloramine T oxidation. A second antibody separation technique was

es had 100 μl of 100 mmol/L Tris buffer with bovine serum albumin and 0.2% Tween 80. ient for the antiserum and the label was a ontaining phosphate saline buffer.

andards were stored as freeze-dried aliquots nstituted in standard buffer (50% phosphate- saline solution, 50% bovine serum albu- en buffer) just before assay (range 0.097 to 25

The sensitivity of the assay was 0.2 ng of IL-8 per milliliter. Assayed medium contained <0.2 ng/ml. The recovery of known amounts of IL-8 in incubation medium gave a correlation coefficient of 0.998 when compared with the expected values, and the sequential dilution of cervix culture medium (up to eightfold dilution) also gave a good correlation with the expected values (coefficient 0.994). The radioimmunoassay was compared with a commercially available enzyme-linked immunosorbent assay kit for IL-8 (Quantikine), and the results from the measurement of cervix-conditioned medium were closely matched, having a correlation coefficient of 0.968.

The intraassay coefficient for the IL-8 assay was 5.9%, and the interassay coefficient was 12.4%.

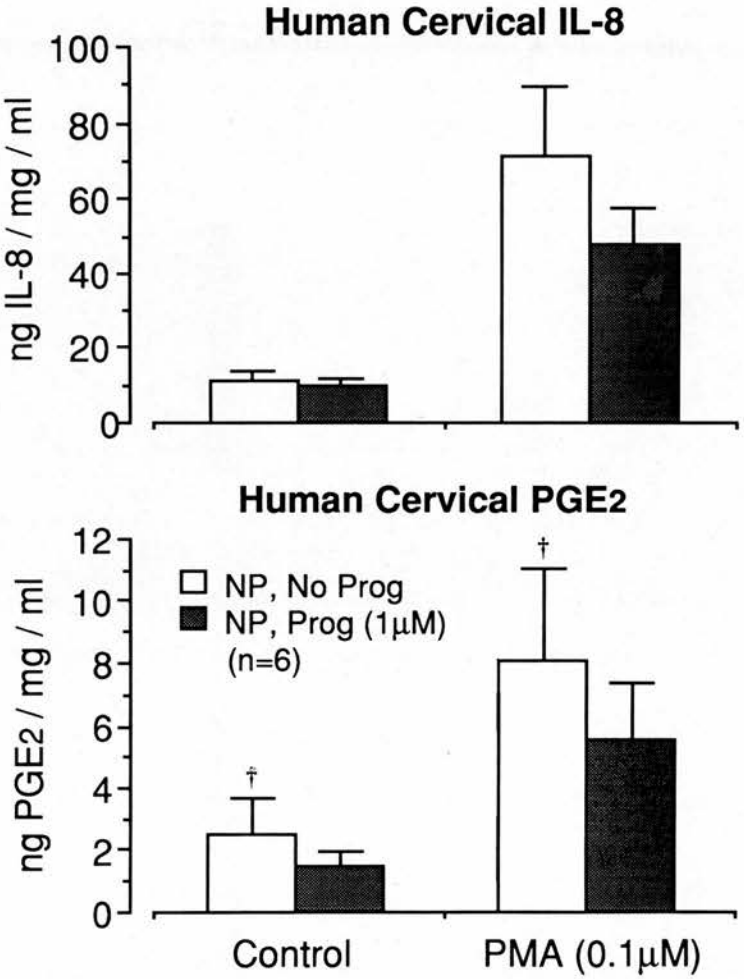

Fig. 2. Effect of progesterone on stimulation by phorbol myristate acetate *(PMA)* (mean ± SE). Progesterone *(Prog)* had no measurable effect on phorbol myristate acetate–evoked stimulation of IL-8. IL-8 output was stimulated significantly above control, $p \leq 0.05$. PGE_2 output was only significantly stimulated by phorbol myristate acetate in cultures where no progesterone was present (*dagger*, $p \leq 0.05$); however, no statistical differences were observed between two control levels or two phorbol myristate acetate–treated levels. *NP,* Group 3.

PGE_2 was assayed as its methyloxime derivative as previously described.[22] The intraassay coefficient for the PGE_2 assay was 6.7%, and the interassay coefficient was 7.6%.

Each sample was assayed in duplicate, and in cases where there was >10% variation between the results the sample was reassayed.

Statistical analysis. Analysis of variance was used in association with the Newman-Keuls test to assign significance.

Results

Interleukin-8 production. Cervix explants from groups 1, 2, and 3 produced similar basal amounts of IL-8. Although group 4 showed reduced production, it was not significantly different (Fig. 1). This expression could be increased by treatment with the protein kinase C activator phorbol myristate acetate in grou and 3 but not in group 4 (groups 1, 2, and 3 significantly different from each other but sig different from group 4, $p \leq 0.05$).

Progesterone was studied with group 3 samp 2), and statistically there was no differenc stimulation evoked by phorbol myristate acet progesterone was present.

Comparison of group 1 and group 3 outpu under the influence of the phosphatase inhib daic acid (Fig. 3) indicated no stimulation curred, and, although the graph may suggest t was in fact a decrease in IL-8 levels in group 3, not reach significance. No statistical differen measurable between these groups.

Fig. 4 shows the results from group 3 tissue with okadaic acid, lipopolysaccharide, TNF,

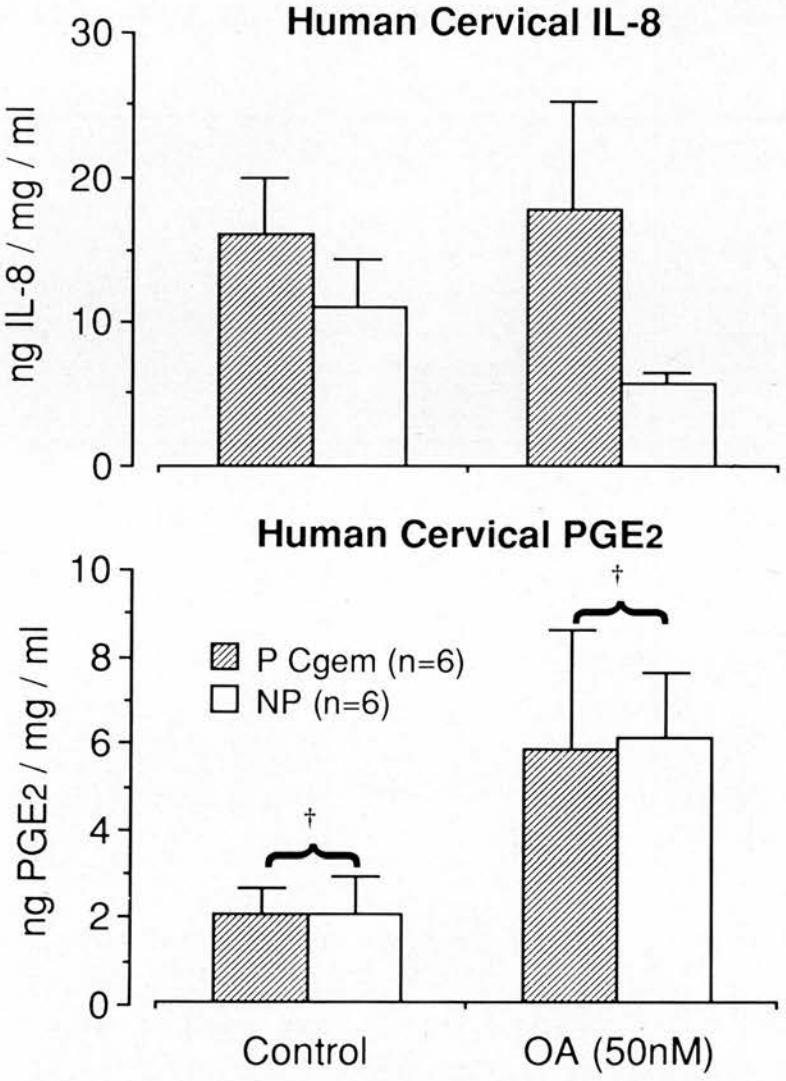

Fig. 3. Effect of okadaic acid on IL-8 and PGE_2 production by human cervix (mean ± SE). No significant differences in IL-8 levels were found. Okadaic acid stimulated PGE_2 output in both groups (groups 1 and 3) (*dagger*, $p \leq 0.05$). No differences in PGE_2 were observed between groups. *P Cgem*, Group 1; *NP*, group 3.

ormamide, the vehicle used for okadaic acid. ntrol, dimethylformamide, and okadaic acid nts were indistinguishable. Lipopolysaccharide NF treatments were significantly higher than (lipopolysaccharide $p \leq 0.05$, TNF $p \leq 0.01$). relation coefficient of 0.93 between the first and days of production indicates that IL-8 produc- nges were maintained for at least 48 hours. The for IL-8 were all based on data obtained from 24-hour incubation period.

aglandin production. Each group of tissue was of producing PGE_2, and again there was no nt difference between the basal levels (Fig. 1). myristate acetate only increased the output of gnificantly above the baseline in group 1.

2 shows the effect of progesterone on phorbol myristate acetate action on group 3 tissue. The control level was unchanged in the presence of progesterone, as was the level on treatment with phorbol myristate acetate. Although this was the case, only the tissue that did not get progesterone had a significant rise in its output of PGE_2.

Okadaic acid treatment significantly stimulated PGE_2 production by groups 1 and 3 ($p \leq 0.05$) (Fig. 3).

No difference between the control and dimethylformamide-treated explants was seen in group 3 tissue (Fig. 4). Okadaic acid caused PGE_2 levels to rise significantly above all the other treatments in this experiment ($p \leq 0.01$), whereas lipopolysaccharide and TNF only reached a significance value of $p \leq 0.05$ when compared with control.

Again there was no significant difference between the

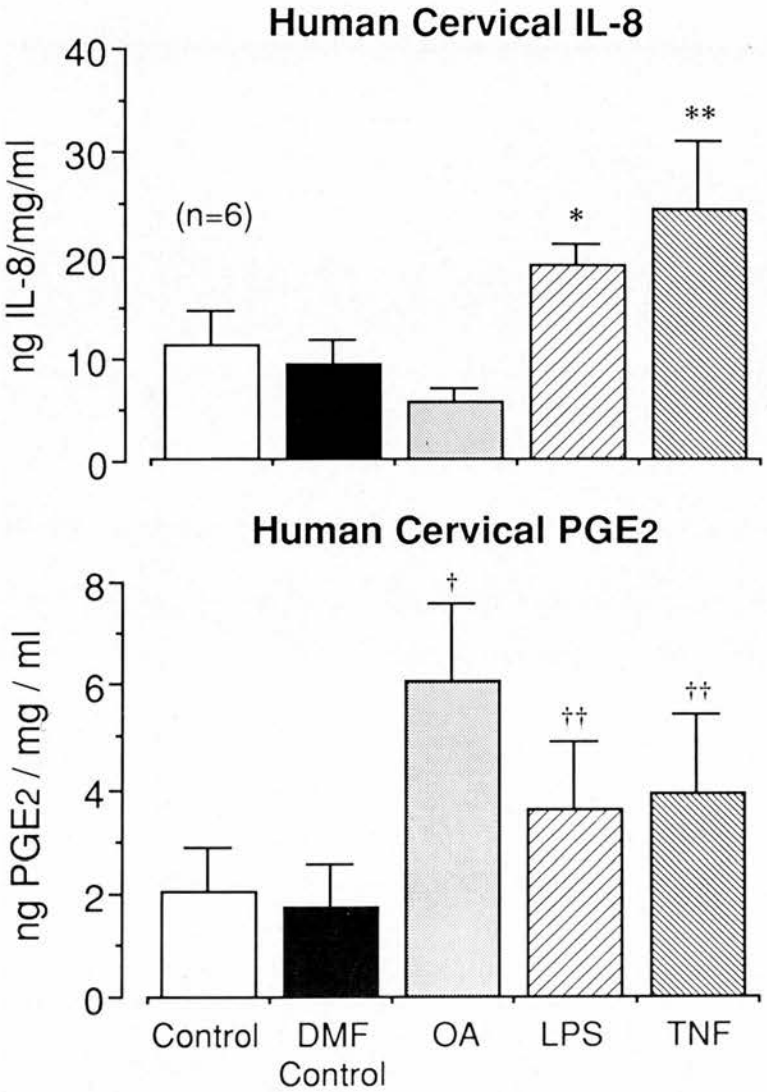

Fig. 4. Effect of okadaic acid *(OA)*, lipopolysaccharide *(LPS)*, and TNF on nonpregnant human cervix (mean ± SE). Okadaic acid vehicle dimethylformamide *(DMF)* and okadaic acid were indistinguishable from the control with regard to IL-8 production. *Asterisk,* Lipopolysaccharide stimulated IL-8 output over control ($p \leq 0.05$); *two asterisks,* TNF stimulated IL-8 output over control ($p \leq 0.01$). PGE_2 levels in control and dimethylformamide treatments were same. Okadaic acid provoked significant increase in PGE_2 output over all other treatments. (*dagger,* $p \leq 0.01$). *Two daggers,* Lipopolysaccharide and TNF caused PGE_2 levels to rise significantly above control ($p \leq 0.05$).

production of PGE_2 on the first and second days of culture; the correlation coefficient was 0.91. The figures for PGE_2 were all based on data obtained from the first 24-hour incubation period.

Comment

The results show, for the first time, that human uterine cervix explants are capable of producing IL-8 and that the cervix can increase its output of IL-8 when stimulated by phorbol myristate acetate. Similarly, PGE_2 output can also be stimulated by phorbol myristate acetate.

The ability of the cervix to produce IL-8 and may be hormonally dependent because tissue postmenopausal women apparently lost its ity to respond to a challenge from phorbol my acetate.

Although it has been shown the IL-8 gene con glucocorticoid-progestin response element[10] an progesterone at 10^{-6} mol/L has an inhibitory ef IL-8 production in choriodecidual cells,[22] the from the experiments reported here show a hi tween-patient variation in basal and stimulated p tion of IL-8, which may explain why the diffe

—d in the presence of progesterone did not reach
nce.

's have shown that lipopolysaccharide and TNF
e IL-8 production in various cell types,[24] and
ılts here indicated that human cervix is also
ed by these compounds. Lipopolysaccharide
'F seemed to have a similar effect on PGE_2 to
IL-8.

naintenance of IL-8 and PGE_2 production dur-
second 24 hours in culture indicated that the
ontinued to be viable. In addition to the data
described, some of the cultures were prolonged
ırther 24 hours, and levels appeared to remain
those stimulated with phorbol myristate acetate.
ıbility of the cervix to produce large amounts of
ıuld account for neutrophil influx seen in two
e studies by Junqueira et al.[3] and Rath et al.[5]
erefore the presence of leukocyte collagenase
d by Osmers et al.[25] The ripening effect of
landins on the cervix, the fact that both prosta-
ı and collagenase production are influenced by
ytokines (e.g., IL-1 and TNF), together with this
ıta concerning the inflammatory mediator IL-8
: cervix, draws us again to the conclusion that the
; of cervical ripening is an inflammatory one.
em is an analog of prostaglandin E, but it does
ect the level of PGE_2 or IL-8 when compared
e other groups. However, it is worth considering
ıe proved role of PGE_2 in promoting cervical
ıg may be through its synergistic interaction with

ough we have shown that phorbol myristate ace-
mulates IL-8 production by the human cervix,
ysiologic control mechanism for its stimulation in
gan remains unknown. However, the interaction
:r inflammatory cytokines such as IL-1 and TNF,
—f which act to stimulate IL-8 in other situations,
likely.

cent report that the origin of a chemotactic factor
abbit cervix was from fibroblasts suggests that the
ı cervical source of IL-8 may in fact also be the
asts.[26]

her work is required in respect of postmeno-
cervical tissue. More samples of such tissue
l be studied, and tissue from women taking hor-
replacement therapy should be examined to
nine if the capacity to induce IL-8 returns. Im-
ıtly, it is necessary to assess the usefulness of early
ıpregnant cervix (premenopausal) as a model for
rm cervical state with regard to IL-8 production.
3 has a dual function, to induce neutrophil migra-
nto tissues and to cause degranulation of the
granules that contain collagenase.[11] Therefore
nay be useful clinically when cervical ripening is
ogressing satisfactorily. Administration of IL-8 in
a gel or pessary formulation may be a safer alternative to prostaglandins for effecting cervical ripening, because any myometrial contractions are likely to be secondary to neutrophil influx.

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Appendix IV

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