STEROID HORMONE AND GROWTH FACTOR ACTION IN NORMAL AND CANCER CELLS

by

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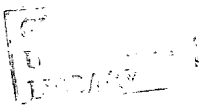

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In the memory of my mother

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ABBREVIATIONS

The standard abbreviations, as recommended in the Biochemical Journal 'Policy of the Journal and Instructions to Authors' (Biochem.J. (1981) 193, 1-27), are used throughout this thesis, with the following additions:

BSA	Bovine serum albumin
BSS	Balanced salt solution
CNS	Central nervous system
C	Cortisol
CBG	Corticosteroid binding globulin
cpm	Counts per minute
D	Dexamethasone
DCC	Dextran coated charcoal
DES	Diethylstilboestrol
dT	Thymidine
E_1	Oestrone
E_2	Oestradiol-17β
EGF	Epidermal growth factor
ER	Oestrogen receptor
ERc	Cytoplasmic oestrogen receptor
ERn	Nuclear oestrogen receptor
FCS	Foetal calf serum
FCS-DCC(4)	Charcoal stripped foetal calf serum, at $4^{o}C$
FCS-DCC(56)	Charcoal stripped foetal calf serum, at $56^{o}C$
FITC	Fluorescein-isothiocyanate
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
^{3}H	Tritiated
KRB	Kreb's Ringer buffer

IP	Induced proteins
MEM	Minimal essential medium
MPA	Medroxyprogesterone acetate
P	Progesterone
PBS	Phosphate buffered saline
PBS-A	Calcium-magnesium-free PBS
PDGF	Platelet derived growth factor
PPO	2,5-diphenyloxazole
PR	Progesterone receptor
RIA	Radioimmunoassay
SD	Standard deviation
SEM	Scanning electron microscopy
SHBG	Sex hormone binding globulin
SCM	Standard culture medium
17-β SDH	Steroid dehydrogenase
SPB	Sorenson's phosphate buffer
T	Tamoxifen
TCA	Trichloroacetic acid
TEM	Transmission electron microscopy

Trivial names are used in the text for the following steroids: aldosterone, cholesterol, cortisol, dexamethasone, diethylstilboestrol, dihydrotestosterone, oestradiol-17β , oestrone, pregnenolone, progesterone.

Full structures and systematic chemical names can be found from "Steroid Hormones" by D.B. Gower (see Ref. Gower, 1979)

Tamoxifen is 1-[4-(2-dimethylaminoethoxy)] phenyl trans-1,2-
-diphenyl-1-butene,

Medroxyprogesteron acetate (MPA) is 6-α-methyl-17-α-hydroxypregn-
-4-ene-3,20-dione acetate.

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SUMMARY

The endometrium is an epithelio-mesenchymal structure. The growth and differentiation of normal endometrial cells is regulated by the interactions of both polypeptide and steroid hormones. An understanding of how hormones affect proliferation and metabolic activities, necessarily involves knowledge of the regulatory processes of these cellular functions. The regulation of the frequency of replication in animal cells is a complex phenomenon for which much of the molecular basis is currently unknown. Cell cultures, which are exempt from tissue and systemic influences are ideal for investigating whether individual growth factors are acting directly on the endometrial cell.

Primary cultures of both normal and malignant epithelial cells were investigated. These cells have been grown both as pure epithelial cultures and as mixed populations of epithelial and stromal cells. The purpose of this study was first, to evaluate some of the critical data now available on various aspects of steroid regulated growth, and second to develop *in vitro* new models of steroid promoted growth that can accommodate current information.

Different cell types, from both rat uterus and human endometrium, were separated by enzymatic and mechanical techniques. Epithelial cells were tentatively identified by comparison of their morphologic features in culture with the well documented features of endometrial cells *in vivo*. Moreover, epithelial nature was also confirmed using immunocytochemical criteria. Growth dynamics of these cells in culture were analysed by the classical techniques of ^{3}H-thymidine incorporation, cell proliferation index and morphological criteria.

Serum performs many functions in cell culture. In addition to providing classical hormones and growth factors, it compensates for the deficiences of defined media, supplying additional nutrients and trace

elements. To determine the minimum requirements for foetal calf serum in cultures of normal and malignant cells derived from endometrium, this study reports a detailed examination of growth requirements of endometrial cells during the early culture. The results presented in Chapter 4 strongly suggest that it is possible to eliminate serum from culture medium and that the main function of serum in cell culture is to furnish hormones and other growth factors. The most important factors are EGF, transferrin, insulin and glucocorticoids. The role of serum in attachment and spreading of these cells was also assessed. Charcoal stripping of serum at $4^{o}C$ enhanced the attachment, whereas, the same treatment of serum at $56^{o}C$ depressed attachment and spreading of normal epithelial cells. Increased growth was observed in endometrial cancer cells in $56^{o}C$-stripped serum. Coating plastic substrates with different preparations increased the speed of attachment of the cells. Culture conditions that were employed resulted in immediate selection of epithelial cells that were able to grow in response to specific growth factors.

The presence of significant but unknown concentrations of biologically available hormones in serum obviously hampers attribution of a response to aparticular growth factor(s). In a detailed study of serum involvement in steroid responsiveness, two diverse observations were made. Firstly, serum seemed to be essential for the inhibitory effects of certain hormones. Secondly stimulation of growth by particular growth factors was most readily achieved in serum-free conditions. Oestradiol stimulated the rate of DNA synthesis in presence of charcoal-stripped serum or serum-free medium. Tamoxifen and progesterone showed wide ranging effects, depending on their concentrations and the serum levels. Medroxyprogesterone Acetate (MPA $10^{-7}M$) required 10% serum to show inhibitory effects. Glucocorticoids

stimulated the process regardless of serum levels. In a tumour cell population, the presence of progesterone receptor seemed to be necessary for depression of growth by progestins. Oestradiol was stimulatory in these tumour cells. Oestradiol plus progesterone increased DNA synthesis regardless of the presence or absence of progesterone receptor.

Studies of cell-cell contact, cell-substrate interactions and cell shape have provided new insight into growth control in differentiated cells. Ultrastructural studies showed oestradiol-treated cultured cells have the appearance of rapidly dividing cells, while progesterone modified cells towards a more secretory type. Cell shape, as judged by microtubules and microfilaments, also appears to be related to hormone supply. Direct evidence shows that there are definite alterations in the quantity or arrangement of cell surface features, cytoskeletal elements, lysosome-like organelles, and rough endoplasmic reticulum in response to hormones. The possible relationship of these changes to increased metabolic activity is discussed.

Analytical and cytochemical probes were used in conjunction with other physiological and biochemical techniques together with electron microscopy to examine cellular heterogeneity. The research described in this thesis indicates that the future holds great promise for an increased understanding of hormones and their actions in cell culture, which, in turn, will provide insight into how these interactions occur in vivo and their relative importance. It also indicates the potential value of primary cell cultures for examining new growth factors and screening new drugs.

CHAPTER 1 - INTRODUCTION

1.1000 STEROID HORMONES AND CELLULAR GROWTH

1.1100 STEROID HORMONES

Normal functioning of a complex biological system requires control signals to coordinate metabolic developments. In mammals two control mechanisms (i) The Central Nervous System (CNS) and (ii) The Endocrine system meet this requirement. In contrast to the CNS, the endocrine system acts more slowly, utilising, instead of electrical, chemical messages (hormones) which are concerned principally with metabolic functions of the body including cellular growth and differentiation.

A hormone is secreted by a specific ductless tissue, the endocrine gland, and is transported in the blood to cause physiological effects on or in cells in remote tissues. Once released into the circulation, the hormone will contact many tissues and organs. However, response is only initiated in specific organs, termed target organs, and is reflected by appropriate metabolic changes.

Chemically, hormones can be divided into three classes:

a) The amino acid derivatives

b) The peptide hormones

c) The steroid hormones

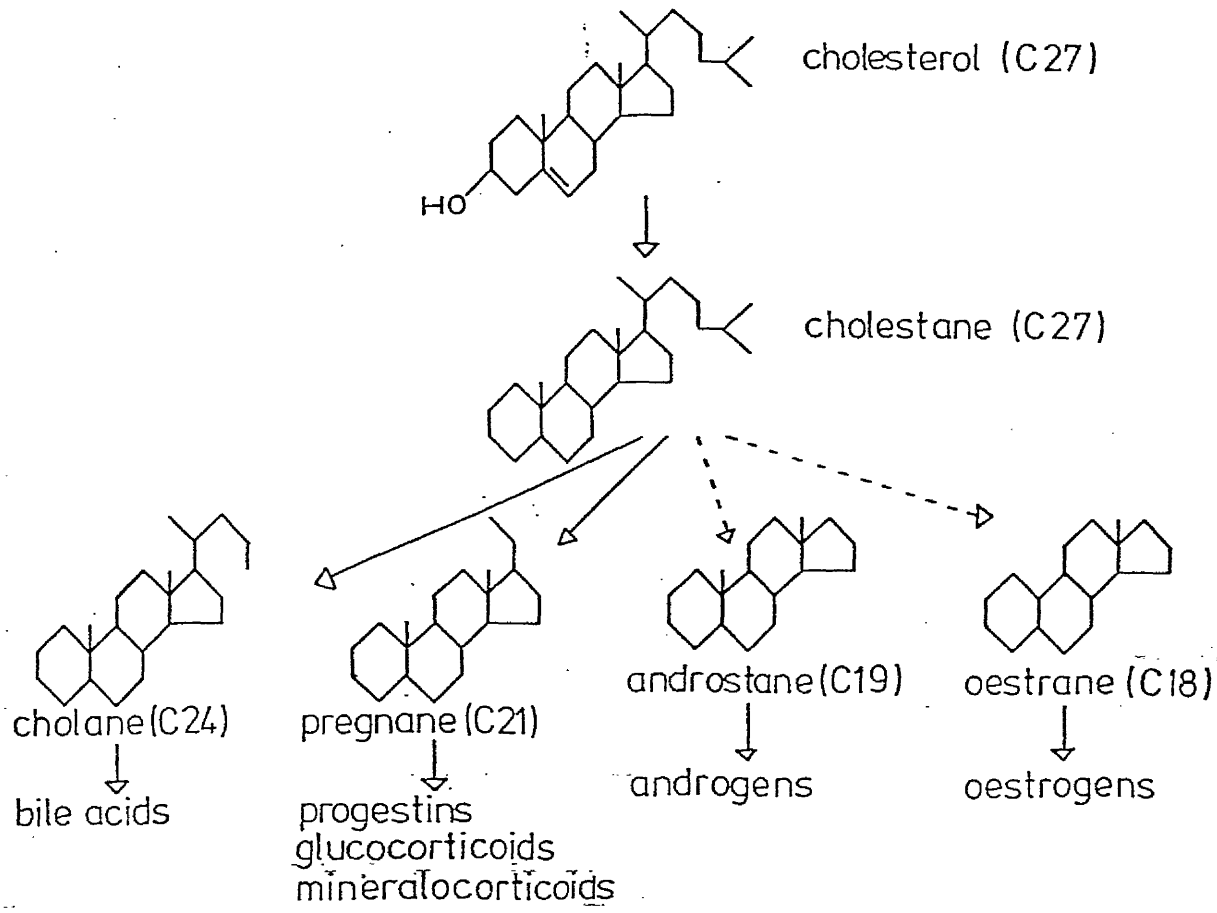
Studies on the molecular mechanism of hormones led to the division of hormones into two groups depending on whether the hormones acted at the cell surface or within the cell. This separation has recently become blurred (Kahn et al., 1981; Posner et al., 1981) because of evidence of internalisation of plasma membrane hormone-receptor complexes. There are six classes of steroid hormones, represented by oestrogens, progestins, androgens, glucocorticoides, mineralocorticoids, and, recently included, vitamin D (Pike, 1982). Steroids are relatively small hydrophobic molecules derived chemically from a common parent

compound, cholesterol. The two main organs synthesising cholesterol are the liver and intestine. The endocrine glands can, therefore, use plasma cholesterol for the synthesis of steroids. However, it has been shown that the adrenal cortex, ovaries and other endocrine glands have the capacity to synthesise cholesterol from acetate (Gower, 1979). In the testis, steroids are synthesised exclusively from acetate. In addition, Ramsey and Nicholas (1972) have shown that the brain also contributes to the body cholesterol pool, but only to a very minor extent. The body cholesterol pool is therefore a balance of the extent of absorption from the diet plus the amount of de novo synthesis relative to excretion. Figure 1.1 shows the various dehydrogenation reactions involved in the production of the final steroid structure. The 27 carbon cholesterol is converted to pregnenolone, a 21 carbon compound, by a series of biosynthetic steps common to all mammalian steroidal hormones (Baird, 1972). Pregnenolone can then be converted to (i) 21 carbon atom progesterone, glucocorticoids and mineralocorticoids (ii) 19 carbon atom androgens and (iii) 18 carbon atom oestrogens. Three six membered rings and one five membered ring are common to all steroids.

The enzymes responsible for catalysing the chemical steps involved in the biosynthesis of sex hormones are present in the adrenal, testis and ovary. The pattern of steroids secreted by the respective endocrine glands is determined by the relative proportion of cell types, the anatomical organisation of the gland, the blood supply and concentration of co-factors and precursors present in the gland and the presence of trophic stimuli (Baird, 1972).

It is important to appreciate that in physiological conditions no single hormone is likely to be secreted exclusively and that the ultimate biological activity is determined by the relative proportions of a number of hormones, together with the morphological state of the target organ.

Figure 1.1: Dehydrogenation reaction involved in the formation of steroid structure


cholesterol (C27)
HO
cholestane (C27)
cholane (C24)
bile acids
pregnane (C21)
progestins
glucocorticoids
mineralocorticoids
androstane (C19)
androgens
oestrane (C18)
oestrogens

1.1200 PHYSIOLOGICAL ACTION OF STEROID HORMONES

Steroids are transported through the blood stream bound to steroid hormone binding globulin (SHBG), albumin (Clark and Peck, 1979) and other plasma proteins. The blood proteins bind steroid with varying affinities and the free hormone level determines the amount of steroid available to the tissue (Westphal, 1970).

Entry of the steroid hormones into the cell is not fully understood. Available evidence (Giorgi, 1980) indicates that steroids enter by simple diffusion, although the cell membrane may have selective permeability for individual steroids (Gorski and Gannon, 1976; Giorgi and Stein, 1981). The presence of a relatively small number (10-20,000/cell) of highly specific receptor molecules is responsible for the retention and, possibly, concentration of hormone within a cell and the presence of such receptors defines a target tissue (Clark and Gorski, 1969; Clark and Peck, 1979; Leake, 1981a).

The general concept involved in the model of the mechanism of steroid hormone action was put forward in 1968 by two independent groups (Gorski et al., 1968; Jensen et al., 1968). The research that led to these theories, and much of the subsequent research on oestrogen action, involved studying its effects on the immature rat uterus (Jensen et al., 1974) and, later, on chick oviduct (O'Malley et al., 1976). After several years of intensive investigation, the general principles still hold true. However, modifications in the overall scheme have been suggested (Linkie and Siiteri, 1978; Sheridan et al., 1979; Martin and Sheridan, 1982).

According to the currently accepted mechanism (Chan and O'Malley, 1976; Leake, 1976), steroids bind to their soluble specific receptors upon entering the cell and this hormone-receptor complex is then "activated" and binds to the chromatin inducing characteristic changes in gene expression.

This results in the modulation of transcription (Leake, 1981a) and subsequent appearance of specific messenger ribonucleic acid (mRNA) molecules (Aziz et al., 1979). This would result in the synthesis of specific proteins and altered cell function. The two step mechanism of action involving activation of the hormone-receptor complex was originally observed for oestrogen (Jensen et al., 1968), but has been shown to be general and can be demonstrated for progesterone (Schwartz et al., 1976), androgens (Mainwa ring and Peterken, 1971), glucocorticoids (Higgins et al., 1973), mineralocorticoids (Edelman, 1968) and vitamin D (Brumbaugh and Haussler, 1974).

1.1300 THE NATURE OF STEROID RECEPTOR

Elucidation of the mechanisms by which growth, differentiation and other metabolic processes are brought about was greatly assisted when it became possible to designate 'steroid target tissues', as those which contained receptors. After detailed investigations, it was realised that the interaction of hormone with target cells is not a simple association effect. Using autoradiographic and ultrastructural techniques, radioactive hormone was found to be located in two separate regions (Toft et al., 1967). The data indicated that bound receptor was principally associated with the soluble and nuclear fractions. This led Jensen to propose the original two step model for the interaction of oestrogen with the uterus (Jensen et al., 1967; see Section 1.1200).

In general, the steroid binding molecules are heat labile and non-dialysable and their protein nature has been demonstrated by their sensitivity to proteolytic enzymes (Toft and Gorski, 1966; King, 1968). They are characterised by high affinity for steroid ($Kd < 10^{-9}$M). Mester et al. (1970) demonstrated the pH profile of oestrogen receptor and the optimum value was found to be 7.0 Ionic

changes on the protein molecules influence receptor-hormone interaction. The importance of sulphydryl groups for the binding of hormone molecules has been demonstrated (Jensen et al., 1967; Muldoon, 1971). Receptor activation is also shown to depend on intact sulphydryl groups (Nielsen and Notides, 1975). The receptor is destroyed by repeated freezing and thawing (King et al., 1978). It has been suggested that steroid receptors are metallo-proteins (Shyamala, 1975) and that activation causes an allosteric change, involving the altered availability of metal ions (Schmidt et al., 1981). King et al. (1978) demonstrated that protein conformation is an integral part of receptor for determining its association with hormone. Lyophilisation has been shown not to alter the protein conformation (Koenders et al., 1980). The concentration of receptors present in normal tissues depends on circulating levels of various hormones (Sarff and Gorski, 1971). This is demonstrated by the cyclic variation of uterine receptors during the ovarian cycle in both rats (Lee, 1982) and humans (Pollow et al., 1975; Soutter et al., 1979).

1.1400 GROWTH KINETICS OF STEROID TARGET TISSUES

The important aspects to be looked at in the control of growth are (i) the intrinsic factors which influence the rate at which biomass increases according to the availability of supply of food or raw material and also to the environmental conditions, (ii) the mechanism which initiates and coordinates cellular division.

Steroids are chemically quite simple molecules, yet their biological activities are exquisitely specific. Their diverse effects include metabolic, morphological and behavioural changes. They can stimulate cell proliferation and cyto-differentiation. A wide variety of biochemical activities coordinately changes in order to cause a

physiological response. In the immature rat uterus, the effects of oestrogen are characterised by hypertrophy followed by hyperplasia. The earliest detectable response is an increase, starting after 15 minutes, in RNA polymerase II activity, which reaches a maximum level at 30 minutes and drops back after 2 hours (Glasser et al., 1972; Borthwick and Smellie, 1975). By 30 minutes there is a detectable stimulation in the synthesis of hnRNA (Knowler and Smellie, 1971, 1973), including many sequences destined to appear as mRNA (Aziz and Knowler, 1978, 1980). This is followed by the appearance of hnRNP particles (Knowler, 1976) and the aggregation of existing ribosomes into polysomes containing newly made mRNA (Merryweather and Knowler, 1980).

Briefly, among the nuclear events that occur during the first hour after oestrogen treatment are:

a) Increased uptake of precursors of RNA into the nucleus

b) Increased rates of synthesis of all three classes of RNA (mRNA, rRNA, tRNA)

c) Increased synthesis of nonhistone chromosomal proteins

d) Increased template activity of chromatin

e) Increased activity of both the nucleolar RNA polymerase (RNA polymerase I) and nucleoplasmic RNA polymerase (RNA polymerase II)

Tomkins and Gelehrter (1972) proposed that steroid hormones may elicit changes in the rates of synthesis of specific proteins by preventing post-transcriptional degradation of specific mRNA. Such results were shown subsequent to glucocorticoid stimulation of liver cells suggesting that early protein synthesis is a prerequisite for many of the later steroid hormone-induced, genetically mediated responses.

In a number of target tissues, steroids stimulate the synthesis of major new secretory proteins. These are valuable markers for monitoring the control and kinetics of the response and have enabled specific probes to be made with which to study the genes that specify such proteins.

Since many protein phosphorylation and dephosphorylation reactions are activated by cyclic nucleotides, various attempts to examine the possible role of cAMP and cGMP in steroid action have been made. Oestradiol caused a transient increase in cAMP within seconds after injection into immature rats (Szego and Davis, 1967). Long term changes in the concentration of cyclic nucleotides after oestradiol treatment of rats have been observed by (Flandroy and Galand, 1976), involving increases in cGMP and decreases in cAMP. The increase in cGMP is inhibited by actinomycin D , suggesting that the increase occurs subsequent to a transcriptional event. The increase in fluid retention is, however, thought to occur independently of transcription, as a result of eosinophil migration to the uterus (Tchernitchin, 1979). Other oestrogen induced but non-receptor mediated responses, include an increase in glycogen and the induction of cervicovaginal antigen (Tchernitchin et al., 1975, 1977). Jensen and DeSombre (1972) reported an early rise in mRNA, phospholipids and glycogen synthesis followed by a rise in total protein, RNA and DNA. The first specific protein to be induced is Induced Protein (IP), which has been recently characterised as 'brain type' creatine kinase (Kaye, 1983).

Late responses to oestrogen include a sustained high RNA polymerase I activity, a second rise in polymerase II (Glasser et al., 1972; Borthwick and Smellie, 1975) and a sustained increase in glucose metabolism (Gorski and Raker, 1973). There is general growth and division of cells, and the receptor must remain in the nucleus for 6-12 hours to elicit these late responses (Clark and Peck, 1979).

There are several more effects of steroids which occur within seconds after steroid-cell interactions involving changes in membrane permeability. Oestrogens, for example, change the rate of water uptake (Ui and Miller, 1963), the rate of nucleotide transport (Billing et al., 1969) into cells, and the movement of lysosomes from the plasma membrane to the nucleus (Szego, 1972). Aldosterone stimulates potassium excretion by a process which is genetically distinct from its protein-induction mediated effects on sodium transport. The rapidity of these responses as well as the lack of sensitivity to actinomycin D, suggests that transcription may not be involved.

Advances that promise further elucidation of steroid mediated growth include specific effects of various steroids at both cellular and molecular levels, kinetic studies on nuclear and cytoplasmic receptor forms, studies on steroid induced gene expression in target cells, in vitro studies, which could explain the relapse of the steroid responsive state into the undifferentiated, autonomous state, and on hormonal control of cell proliferation and differentiation.

1.2000 THE PHYSIOLOGY OF THE UTERUS

1.2100 THE ORGAN

The uterus is a hollow, muscular organ in the pelvis of a female in which, during pregnancy, the growing foetus is protected and nourished until birth. In the human the upper part is broad and branched out on each side into the fallopian tubes. At its lower end, the uterus narrows into the cervix, which leads into the vagina. The whole organ is attached to the pelvic and abdominal walls by the broad ligament of the uterus. Through this ligament, the uterus receives its blood and

nerve supplies. The uterine wall consists of the following layers:

a) The serous membranes, which cover the whole uterine body;

b) The myometrium, which itself consists of three layers - the internal circular muscle, the external longitudinal muscle and, separating the muscle layers, the vascular layer;

c) The endometrium, which consists of the epithelial lining of the lumen, the glandular layer, and the connective tissue (Nalbandov, 1975).

The myometrium is usually the thickest of the three layers. In the non-pregnant uterus it is composed of interlacing bundles of long, smooth muscle fibres arranged in ill-defined layers. Within the muscle is a rich network of arteries and veins supported by dense connective tissue. During pregnancy, under the influence of oestrogen, the myometrium increases greatly in size by both cell division and cell growth (de Brux et al., 1981). At parturition (and occasionally at other times) strong contractions of the myometrium are reinforced by the action of hormones (Sarosi et al., 1983). These contractions expel the foetus from the uterus into the vagina and also constrict the blood supply to the placenta, thus precipitating its detachment from the uterine walls.

1.2200 THE ENDOMETRIUM

The endometrium, the mucous lining of the uterine cavity, is an epithelio-mesenchymal structure, whose biological role is to provide the environment for the implantation and development of the fertilised ovum. It constitutes an outstanding example of the hormonal regulation of growth, morphogenesis and differentiation in animal tissues.

Studies on the structure and function of the endometrium have been extensively reviewed (Norris et al., 1973; Kimball, 1980;

Dallenbach-Hellweg, 1981; de Brux et al., 1981). In this section, the current concepts of endometrial structure and function will be outlined with particular emphasis on the different cell types and on some of the unsolved problems in endometrial biology.

1.2300 STRUCTURE OF ENDOMETRIUM

The endometrium is composed of an epithelium, which forms tubular glands, and a highly reticular stroma containing an extensive vascular network. It can be divided into two layers, differing in their morphology and function. The functionalis (functional layer) undergoes cyclic changes during the reproductive years whereas the basal layer shows few, if any, cyclic changes. The following description refers to the events in the functional layer of the endometrium.

1.2310 EPITHELIUM

The epithelium lining the lumen of the uterus is a simple layer of columnar cells, separated from the stroma by basement membrane. They possess elongated, delicate microvilli on their free surface. During the secondary phase, these microvilli draw back and disappear. Occasionally, in women, some patches of the epithelium may bear cillia (Nalbandov, 1975). The epithelial glands are the most important components of the endometrium. These glands are tubular invaginations of the epithelium and they too are lined with simple columnar epithelium. The distal ends of the glands may be either straight or convoluted, depending on the stage of the cycle at which they are observed. The uterine surface has special areas for the attachment of the placenta, called the cotyledonary areas.

1.2320 STROMAL CELLS

The endometrial stroma consists of pluripotential mesenchymal cells, which at the beginning of the reproductive cycle are uniformly

spindle-shaped, poorly differentiated, and joined to one another by cytoplasmic processes. The elongated nuclei have abundant chromatin. Autoradiographic studies show well defined synthesis of DNA and DNA controlled synthesis of RNA (More et al., 1974).

Padykula (1980), in structures of human endometrial stromal cell populations, classified them into various different types sub-divided into two categories. The resident cells (Stein cells, fibroblasts, lymphocytes and mast cells) are consistently present in the adult endometrium, whereas transient cells (decidual cells, macrophages, blood leucocytes and endometrial granulocytes) appear intermittently reflecting the degree of hormonal stimulation. This classifcation, useful for descriptive purposes, separates into different types cells which may, in fact, have a common origin, and thus, belong to the same lineage. Such is the case of the decidual cells which have been shown by autoradiographic studies in rats (Galasi, 1968) to originate from undifferentiated stromal cells.

1.2330 RETICULAR CELLS

This term is used to define the population of the endometrial stromal cells which have the potential to proliferate and differentiate into more specialised cell types which are peculiar to the endometrium. Thus, reticular cells will include the following:

a) Stem cells: their presence is evidenced by autoradiographic studies (Ferenczy et al., 1979a) but they are not readily identifiable by specific morphological features.

b) Fibroblast-like cells: are characterised by their similarities in shape and ultrastructure to fibroblasts of other organs.

c) Decidual cells: the term decidua refers to the pregnant endometrium. The transformed, large polygonal stromal cells of the decidua arranged in a mosaic pattern are termed decidual cells

(Arias-Stella, 1973). The term predecidual is used for these cells in non-pregnant endometrium. They have a rounded nucleus and abundant cytoplasm containing well developed Golgi apparatus and endoplasmic reticulum which is distended with a dense material. All the histological and ultrastructural studies (Noyes, 1973; More et al., 1974; Dallenbach-Hellweg, 1981) suggest that in the human, decidual cells are derived from undifferentiated stromal cells in a manner similar to that which occurs in rats (Galasi, 1968).

d) Endometrial granulocytes: they have been described by a number of authors (Hamperl, 1955; Feyrter, 1963; Van Boagert, 1975) as small stromal cells with a beam-shaped nucleus together with cytoplasm containing granules. In electron microscopic studies these cytoplasmic structures appear limited by a distinct membrane and resemble secretory granules more closely than lysosomes (Henzl et al., 1972). However, other authors (More et al., 1974) describe them as indistinguishable from lysosomes. It has been suggested that the endometrial granulocyte is a differentiated form of stromal cell alternative to the predecidual cells (Dallenbach-Hellweg, 1981).

1.2340 OTHER CELL TYPES

In addition to cells of endometrial origin, the following cell types are also found in active endometrium:

a) Lymphocytes: They are regularly present in variable numbers in the endometrium and may be derived from the circulating blood or the local lymphoid tissue (Dallenbach-Hellweg, 1981).

b) Mast Cells: They are often found in the stroma surrounding the blood vessels (Robinson et al., 1978). By electron microscopy, mast cells are readily identifiable and differentiated from predecidual cells and macrophages (Sheppard and Bonnar, 1979). Although their physiological role in the endometrium is unknown, recent findings (Foley

et al., 1978) of heparin-like activity in uterine fluid suggests they may be involved in menstrual bleeding.

c) Mononuclear phagocytes: Both monocytes and macrophages have been described in the human endometrium (Feyrter, 1963). Uterine macrophages have similar characteristics to those found in other tissues (Pershall and Weiser, 1970). In other animal species (Padykula and Taylor, 1976) macrophages increased in number during the post-partum regression and their role in tissue remodelling has been proposed (Padykula, 1981).

d) Blood granulocytes: Neutrophils have been reported to invade the endometrium during the late secretory phase (Noyes, 1973). However, other reports (Dallenbach-Hellweg, 1981) claim that invasion occurs only after menstruation sets in. Large numbers of blood granulocytes are only found under pathological conditions.

1.2350 EXTRACELLULAR MATERIAL

1.2351 The Reticular Fibres

The fibrillar extracellular material is composed of reticular fibres which include collagen type III (Nowack et al., 1976), fibronectin (Stenman and Vaheri, 1978) and some non-collagenous proteins (Pras et al., 1974). The distinction between collagen and reticulin is seen by differential staining patterns under light microscopy. Electron microscopic studies have also found that maximal abundance and thickness of collagen fibres occurs in the secretory phase (More et al., 1974) and around predecidual cells (Wynn, 1977).

1.2352 Ground Substance

The amorphous component of the extracellular matrix contains mucopolysaccharides and probably other substances (Harkness, 1964). Information on this subject is almost entirely confined to histochemical studies (Schmidt-Mattieson, 1963) with subjective observations of the

intensity of staining. Considerable variations in staining intensity occur at different times of the cycle and similar patterns of change are seen with nearly all staining methods.

1.2360 BLOOD VESSELS

Endometrial blood vessels differ from those in other organs by their unique structure and their sensitivity to hormonal changes (Ramsey, 1977). During the proliferative phase spiral arteries extend increasingly into the endometrium; capillaries and veins increase in diameter throughout the cycle becoming dilated during the secretory phase.

The growth of spiral arteries results from a progressive formation of media and adrentitia around the pre-capillaries and capillaries thus converting them into arterioles and arteries as the cycle progresses. In the late secretory phase arterioles may be morphologically identified in the sub-epithelial zone of the endometrium. The structure of the veins remains fairly constant throughout the cycle showing a continuous endothelium and a thin media only in the deeper regions of the endometrium.

1.2400 REPRODUCTIVE CYCLE

In the non-pregnant state, the female reproductive system undergoes continuous cyclic changes from puberty to menopause. When ovulation is not followed by the implantation of a fertilised ovum, the proliferated mucosal lining regresses and a new ovulation cycle commences.

1.2410 THE NORMAL OESTROUS CYCLE

This cycle refers to a series of changes taking place in the reproductive tract of the female leading to conditions of heat. In domestic animals, the proliferative uterine mucosa is absorbed rather than shed and the female is receptive to the male only during the period

of ovulation, known as oestrous (or heat). The remaining part of the cycle is called the dioestrous and the whole cycle is known as the oestrous-cycle. Certain species of mammals have clearly defined periods of oestrous of short duration, which recur throughout the year at regular intervals, in a cyclic manner. In rats, where the oestrous cycle has been most carefully studied (Nalbandov, 1975; Johnson and Everitt, 1980) it is described as polyoestrous Table 1.1 relates the changes that take place during the course of a typical oestrous cycle.

A day before the rat is due to come into oestrous, that is on the morning of pro-oestrous, secretion of oestrogen from the developing follicles reaches peak values. During pro-oestrous, uterine glands are rather simple and straight with few branches. Ovulation occurs in the early hours of the following morning.

At the approach of oestrous, the leucocytes disappear and the smear consists mainly of epithelial cells with marked nuclei. The female is then in heat. During the post-oestrous phase, a return of the leucocytes is seen amongst the cornified cells. The female is no longer in heat. The phenomenon of endometrial destruction and regeneration, in rats, occurs through continuous sloughing off of the endometrium and the presence of uterine debris does not seem to be correlated with any hormonal state (Nalbandov, 1975). A similar sequence of events occurs during the oestrous cycle of the sheep (Johnson and Everitt, 1980) and pig (Nalbandov, 1975), although the timings are quite different.

1.2420 THE NORMAL MENSTRUAL CYCLE

The fundamental layer of the human endometrium undergoes cyclic changes which are temporally related to the ovarian cycle and the plasma levels of ovarian steroids (Fig.1.2). These alterations constitute a preparation of the endometrium for the nidation of the ovum. If fertilisation does not occur the endometrium sloughs off and menstruation ensues.

TABLE 1.1: The oestrous cycle of the rat and associated changes in the reproductive organs

Reproduced from Short (1972)

STAGE	EXTERNAL GENITALIA		VAGINAL SMEAR						
	Vulval area	Vaginal wall	Epithelial cells	Cornified epithelial cells	Leucocytes	Mucus	Mating behaviour	Ovary	Uterus
I. Pro-oestrus	Becoming swollen	Dry	+++	+	±	-	±	Follicles maturing	Becoming distended
II. Oestrus (early)	Maximum swelling	Very dry and corrugated	-	+++	-	-	+	Follicles mature	Fully distended
III. Oestrus (late)	Swelling receding	Slightly moist	±	+++	±	-	±	Ovulation	Less distention
IV. Met-oestrus	Not swollen	Moist	+	++	++	-	-	Corpora lutea (C.L.)	Fleshy and pink
V. Dioestrus	Not swollen	Moist	+	-	+++	little, stringy	-	C.L. regressing, follicles growing	Resting condition

Figure 1.2: Menstrual cycle and implantation as controlled by ovarian hormones

(Reproduced and modified from "Histopathology of the endometrium" Dallenbach-Hellweg, 1981)

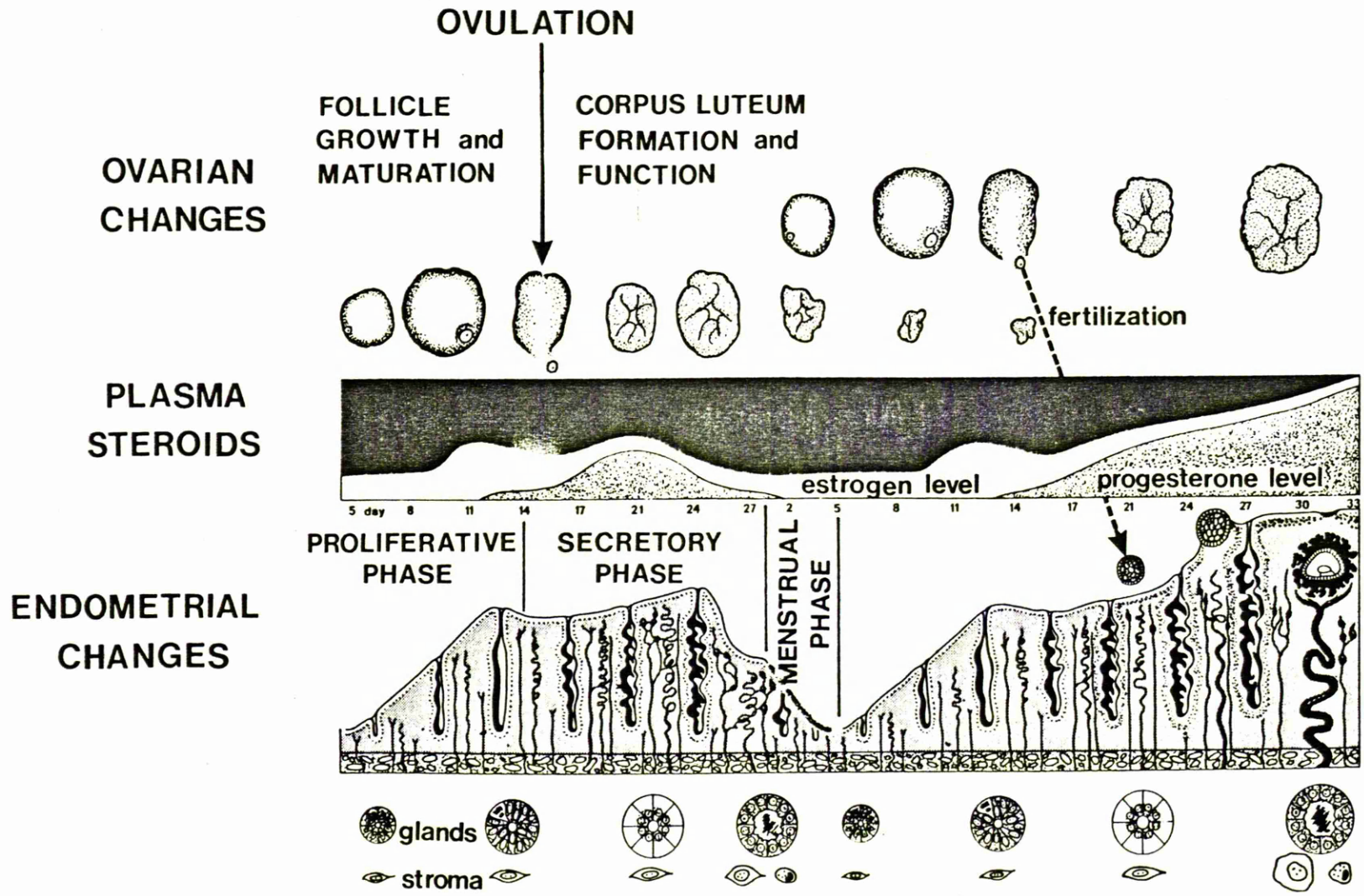


Fig. 1.2 Menstrual cycle and implantation as controlled by ovarian hormones. (Modified from Dallenbach-Hellweg, 1981).

Individual elements of the endometrium show distinct morphological changes throughout the menstrual cycle. The dating of the endometrium refers to the classic 28-day cycle in which ovulation is assumed to occur on day 14. The duration of the post-ovulatory phase shows little variation between individuals (14 days $\pm$ 36h). The pre-ovulatory or proliferative phase ranges from 7 to 21 days and the terms "early" and "late" proliferative phase are preferred to the use of specific stages of the cycle (Wynn 1977). The pre-ovulatory phase of the cycle (proliferative) is concerned with growth while the post-ovulatory (secretory) is concerned with differentiation.

1.2421 The Proliferative Phase

Immediately after menstrual shedding a regenerative phase begins and lasts for a few days during which the denuded endometrium is re-epithelialised. In the following days the dominant feature is cell proliferation with development of all the individual structures, leading to an increase in thickness of the endometrium. The endometrial stroma proliferates to form a deep, richly vascularised stroma resembling primitive mesenchyme. In the epithelium the glands elongate and become tortuous with crowding of the cells. This process is sustained under oestrogen until ovulation.

1.2422 The Secretory Phase

The release of progesterone from the corpus luteum, which is formed after ovulation, promotes production of copious, thick, glycogen-rich secretions by the proliferated endometrial glands.

During the first half of this phase major changes occur in the epithelium. Proliferation ceases and cells engage in secretory activity. During the second half, the histological changes are seen primarily in the stroma. Spiral arteries and extracellular fibres reach their maximal development and predecidual cells and endometrial

granulocytes appear. Around day 24 of a cycle in which pregnancy does not occur glandular involution begins.

1.2423 The Menstrual Phase

In the absence of pregnancy, the proliferated uterine mucosa is shed in a period of bleeding known as menstruation. There is dissolution of the reticular fibres, dissociation of stromal cells and haemorrhaging in the superficial stroma. Further disintegration of the tissue, haemorrhage and sloughing off the functionalis follows. The process of menstruation represents the end point of the cycle of endometrial changes. The first day of menses is usually taken to mark the first day of the 28 day menstrual cycle.

1.2430 ULTRASTRUCTURE

1.2431 Epithelium

The cyclic changes in the ultrastructure of the endometrial glandular cells follow an orderly pattern. In the early proliferative stage, cells are poorly differentiated as manifested by the scant development of the structures related to specialised functions. The changes that follow during the proliferative phase reflect increased mitotic activity and progressive development of organelles related to specialised functions. With the onset of the secretory phase the cells reach their peak in differentiation and functional activity. Proliferation ceases and the biosynthetic and secretory machineries display their maximal development and activity. At the end of the cycle ultrastructural differentiation of the epithelium diminishes and regressive changes are apparent.

1.2432 Stroma

Stromal cells also show characteristic cyclic changes in their ultrastructure. During the first half of the cycle, the cells have scant cytoplasm and poorly developed organelles. They resemble

undifferentiated mesenchymal cells. As the cycle progresses there is a steady increase in the amount of cytoplasm and complexity of the organelles in the stromal cells. They acquire the appearance of mature fibroblasts. Collagen accumulates in the extracellular space during this time. In the second half of the post-ovulatory phase major changes take place in the stromal cells and predecidual cells and granulocytes appear. The most outstanding feature of predecidual cells is the formation of gap junctions. With the human endometrium these junctions are found only in predecidual and decidual cells (Lawn et al., 1971).

1.2440 REGENERATION

Studies by Ferenczy (1976, 1980) and Nogalis-Ortiz et al. (1978) indicate that, in the case of women, regeneration following the menstrual slough starts between day 2 and 3 of the cycle. The process is initiated by migration of the epithelial cells from the residual gland stumps and the persistent surface epithelium adjacent to the denuded areas. Autoradiographic studies (Ferenczy et al., 1979a) showed that after migration the regenerating epithelium shows increased DNA synthesis. Migration and proliferation continue until endometrial repair is completed around day 5 of the cycle. Stromal cells also show increased DNA synthesis in regenerating areas during this period.

Although there is general agreement in the sequence of events during endometrial regeneration (Ferenczy, 1980), controversy still exists amongst morphologists about the extent of the tissue shedding at menstruation and the participation of persistent secretory tissue in the regeneration process. However, despite the accumulated data no uniform conclusions and, consequently, no understanding of the mechanisms of endometrial tissue loss and its remodelling have been reached. There are three schools of thought. The traditional view favours the concept of total loss of the functionalis layer followed by re-epithelialisation

of the denuded basalis (Ferenczy, 1979a,b, 1980; Dallenbach-Hellweg, 1981). One alternative view advocates that desquamatisation of the endometrial mucosa involves only the upper 1/3 - 1/2 of the functionalis and this is supported by recent studies (Flowers and Wilborn, 1978; Nogalis-Ortiz et al., 1978). Others suggest that following variable loss of the functionalis the new surface epithelium derives from the residual endometrial stromal fibroblasts (Irwin, 1982).

In general, irreversible cell injury leads to impaired cellular integrity and continuity and results in regeneration. It is also conceivable that regeneration to replace lost tissue is related to temporary loss of inhibitors of tissue growth. There is yet no clear indication as to the validity of either of these opposing views. However, possible variations in regeneration processes between both different uteri and different areas within individual uteri should be borne in mind when evaluating the available information.

1.2450 HORMONAL REGULATION OF THE REPRODUCTIVE CYCLE

Hormonal control of the mammalian cycle is complex. In the rat external stimuli, mediated via the Central Nervous System (CNS) crucially affect cyclicity. The CNS-hypothalamic system, the pituitary, the ovary and the endometrium are various elements of a functionally coherent system for controlling the cyclic release of gonadotrophin, essential for ovulation and conception (Yen, 1978).

Two phenomena are most important for control of the cycle in primates. The first is the variable hypophysial sensitivity to luteinising hormone releasing hormone (LHRH) during the cycle (de Brux et al., 1981). Leutinizing Hormone (LH) response is maximal during the pre-ovulatory period, whereas, later, gonadotrophin rises show a progressive decrease after injection of a fixed doze of LHRH. The LH response then decreases during the luteal phase (Yen and Lein, 1976).

These variations in hypophysial responsiveness are clearly dependent on circulatory oestrogen levels (Yen and Tsai, 1972; Yen, 1978). The second phenomenon is the autonomy of the mediobasal hypothalamus, which is not dependent on a signal from the preortic area for induction of the pre-ovulatory LH peak (de Brux et al., 1981).

Yen et al. (1972) have suggested that there are two pools of pituitary gonadotrophins. One would be readily releasable and discharged in response to a brief pulse of LHRH. A second reserve pool would then be activated and released after a more prolonged LHRH stimulation. This pool would be dependent on the level and duration of oestradiol secretion (Yen et al., 1972). Based on these studies, a hypothesis integrating the total regulation of the reproductive cycle can be postulated. The sensitivity of the hypophysis would be enhanced by the rhythm of oestradiol secretion.

In the case of rat, a peak in oestradiol secretion in the pro-oestrous phase triggers the ovulatory surge of LH during the same day and ovulation occurs in the early hours of the following day. The pre-ovulatory surge of progesterone secretion is almost coincident with the LH peak (Short, 1972). The corpus luteum secretes large amounts of progesterone in response to LH stimulation and since the release of LH is also facilitated by progesterone (de Brux et al., 1981), the whole sequence of events is beautifully integrated.

The blood levels of Follicle Stimulating Hormone (FSH) and prolactin are also elevated in late pro-oestrous, and the act of mating presumably stimulates gonadotrophin release still further, so as to produce a functional corpus luteum.

Clinical observations in women following ovariectomy, corpus luteum removal or discontinuation of hormone therapy suggest that similar regulatory mechanisms also operate in the human (Frazer and Diczfalusy,

1980; Dallenbach-Hellweg, 1981). The menstrual cycle is again a repetitive expression of the operation of the hypothalamic-pituitary-ovarian system, associated with structural and functional changes in the target tissue, the uterus. An increase followed by a marked reduction of the FSH/LH ratio is typically associated with cyclic release of gonadotrophins during the reproductive phase (Yen, 1978). The circulating levels of gonadotrophin, oestrogen, androgen and progesterone during the normal ovulatory cycle in women exhibit a cyclic pattern. Follicular phase begins with a rise of serum levels of FSH and a concomitant initiation of follicular growth before the end of the previous cycle. An increase in LH follows a rise in FSH. Secretion of oestrogen is at fairly constant levels during the first portion of the follicular phase, whereas the latter half is characterised by an increase in oestrogen levels, which reach a maximum, just a day before the LH peak. A rise of plasma oestrogen levels is accompanied by a decrease in FSH levels and a small rise in LH secretion. This divergence in secretion of FSH and LH is probably related to (1) a preferential inhibitory action of oestrogen on FSH release (Yen and Tsai, 1971) and (2) an increase in inhibin secretion by the maturing follicle, with selective inhibition of FSH secretion (Yen, 1978).

Several days before onset of the midcycle LH surge, plasma levels of androgen rise (Ross et al., 1970). Progesterone levels show no significant change prior to the midcycle surge, although one study has reported a small rise on the day before (Gautray, 1981). During the ovulatory phase, there is a rapid rise in the plasma LH levels, which leads to the final maturation of the graafian follicle and then follicular rupture. Although there is a simultaneous increase in FSH levels (Yen, 1978), its role is not understood. Shortly before the onset of the midcycle LH surge and prior to ovulation, plasma oestradiol

levels drop (Gautray, 1981) and plasma progesterone begins to rise. Following ovulation, the follicular cells are luteinised, and the post-ovulatory follicles become increasingly vascularised. The most important feature of the luteal phase is the marked increase in progesterone secretion by the corpus luteum. There is also, in parallel, a second increase in oestradiol levels, reaching a peak smaller than that in the follicular phase. As progestins and oestrogens increase, plasma LH and FSH decline throughout most of the luteal phase, but FSH begins to rise at the end of the luteal phase to initiate follicular growth for the next cycle.

The change precipitating menstruation is the fall in the blood levels of ovarian hormones (Hisaw and Hisaw, 1961). In both cycling and ovariectomized animals, prolonged administration of oestrogen and progesterone prevents the onset of menstruation (Kraemer, 1980), and induces extensive decidualisation of the stroma with involution of the glands and spiral arteries (Dallenbach-Hellweg, 1981). Cessation of progesterone with or without oestrogen treatment induces menstruation (Hisaw and Hisaw, 1961). Follicle growth regulation requires both pituitary and intra-ovarian messengers. The importance of plasma levels of pituitary hormones is well known and insufficient FSH secretion has been demonstrated in many menstrual cycle disorders.

Another cellular mechanism that is important for luteolysis is cell desensitisation or down regulation. This is concerned with regulation of peptide hormone receptor numbers and target cell responses and usually results from increased concentrations of the homologous hormone (Catt et al., 1979). This process is particularly marked in the testis and ovary after exposure to elevated gonadotrophin levels.

1.2460 BIOCHEMICAL CHANGES IN THE ENDOMETRIUM

The dynamic nature of endometrial tissue has been shown by biochemical studies (O'Grady et al., 1978; Richardson and MacLaughlin, 1978; Wilson, 1980). Early work on carbohydrate metabolism and lysosomal enzyme activities has, in recent years, been superseded by studies of steroid hormone metabolism and of hormone receptors. Biologically, the endometrial cells are programmed to respond to a normal sequence of hormones designed to cause the endometrium to proliferate, differentiate and produce glycoproteins, and, in the absence of pregnancy, to undergo an orderly regression and remodelling in preparation for a fresh cycle. An alteration has been reported in these events when the endometrium is subjected to unopposed oestrogen (Flowers et al., 1983). According to them the absence of "oestrogen-inhibiting" progestins allows the excessive endometrial growth during the normal follicular phase and is sustained thereafter, if the absence of progesterone continues. In the absence of progesterone there is no direction to the glands or stromal cells to use their energy to manufacture glycoprotein. Thus endometrium is free to use all of its energy for growth rather than for the production of secretory products. Progesterone-induced invagination and differentiation are either absent or suppressed.

Progesterone is the principal agent that allows the normal menstrual cycle to be maintained (Flowers et al., 1983). This hormone modulates the normal hormonal milieu within the cell, so that each cytologic function occurs in proper sequence. At the time of menstruation, the enzymes released by extracellular lysosomes derived from leukocytes and fragmented cells, result in the digestion of the reticular fibres (Flowers and Wilborn, 1978).

In the human, on cycle days 1-2 the functionalis becomes disorganised, containing predecidual stromal cells admixed with epithelial glandular cells. Moreover synthesis of nuclear DNA is near zero level in the secretory functionalis layer of the endometrium as determined by a lack of nuclear radiothymidine incorporation (Ferenczy et al., 1979a,b) These findings are consistent with ultrastructural observations and also agree with those of Epifanova (1977), using histological estimation of mitotic indices. As the effect of oestrogen steadily intensifies, the endometrium gradually changes. The epithelial cells are low columnar only during the early stage of the cycle. Their cytoplasm contains little RNA (Dallenbach-Hellweg, 1981). Their nuclei appear small and oval. The chromatin is dense. Later in the pre-ovulatory phase, the epithelial cells become compressed into tall columnar cells. Their nucleoli soon become apparent and the average DNA content of the nucleus increases (more cells in S and G2 phase) . Just before ovulation, the cytoplasm of epithelial cells increases and RNA accumulates. The nuclear-cytoplasmic ratio shifts gradually in favour of the cytoplasm, which is more clearly seen in the post-ovulatory phase. The RNA content of cytoplasm gradually falls with the passage of time but the nucleoli remain large. When progesterone levels decrease in the latter stage of the cycle, synthesis and activity of prostaglandin F_2 (PGF_2) and, to a lesser degree, of PGE_2, increase (Ferenczy, 1980). Finally the endometrium greatly contracts because of the fall in both progesterone and oestrogen levels. The glands collapse, assuming a saw-toothed appearance and the predecidual stroma becomes very dense. Golgi apparatus of the stromal and glandular cells involutes and the remaining histologically detectable RNA disappears.

In the absence of conception, acid phosphatase synthesis and activity increases in glands and predecidual cells during the last stage of the cycle preceding menstruation (Ferenczy, 1980). Menstruation is characterised by haemorrhages in the superficial stroma. The cause of menstruation is still incompletely understood. The most fundamental experiments carried out until now were in monkeys (Witt, 1963). From the results, it is assumed that following the premenstrual fall of both steroids, the endometrium loses water and greatly shrinks, causing the spiral arterioles to collapse and kink. Smooth muscle cells contract and capillaries become more fragile. In addition, the fall in progesterone activates fibrinolytic enzymes and induces the release of relaxin, which in turn brings about dissolution of the stromal fibres. The stromal cells dissociate and the functionalis breaks down to be discharged (Dallenbach-Hellweg, 1981). Thus, ovarian steroid levels influence both lysosomal enzymes and prostaglandins leading to menstrual degeneration of the endometrium.

Menstruation represents two main processes. First is the loss of tissue and second, the restoration and renewal of parts of the functionalis. The mechanism of induction of endometrial proliferation during the menstrual period appears to be independent of hormonal stimuli (Ferenczy, 1980).

1.2461 Enzymes of steroid metabolism

Several steroid-metabolising enzymes vary in activity throughout the cycle (Tseng and Gurpide, 1974; Pollow et al., 1975; Gurpide, 1978; Fleming and Gurpide, 1981). Following the work of Tseng and Gurpide (1972), oestradiol-17β dehydrogenase activity has been well demonstrated in human endometrium and its changes have been correlated with hormonal status. This enzyme converts oestradiol to oestrone and is located mainly in glandular epithelium (Pollow et al., 1975;

Seublinski et al., 1976). In other words, this enzyme plays an active role in oestrogen induced cell division by reducing the availability of oestradiol.

Other enzymes, which contribute towards steroid metabolism are 5α and 5β reductases. These two enzymes ensure that any excess of progesterone is rapidly converted to its less active metabolites. During the pre-ovulatory phase, both of these enzymes are active, whereas in the post-ovulatory phase the metabolism of progesterone to reduced products is prevented by the increased activity of 20-dihydroprogesterone dehydrogenase, which occurs primarily in the oxidative direction (Tseng et al., 1972). Overall circulating progestins can reduce the effectiveness of oestradiol by (1) reducing the synthesis of oestrogen receptor protein, and (2) reducing the available oestradiol by stimulating intracellular oestradiol-17β dehydrogenase activity (King et al., 1978).

1.2462 Steroid Receptors

Hormone-dependent growth of a particular tissue is assumed to correlate with the presence of specific receptors for that particular hormone on or within the cells of that tissue. Receptors for both oestrogens and progestins have been demonstrated in the human endometrium (Pollow et al., 1975; Richardson and MacLaughlin, 1978; Soutter et al., 1979; King et al., 1981) as well as in rat endometrium (King and Mainwaring, 1974; Clark and Peck, 1979; Kirkland et al., 1981). Both types of receptors have been found in both epithelial and stromal cells (Pavlik et al., 1979; Fleming and Gurpide, 1981; King et al., 1982). The cytoplasmic oestrogen receptor from stroma had a higher affinity for oestradiol than did the epithelial receptor (King et al., 1981a,b), whereas progesterone receptor had similar binding affinities in epithelial and stromal tissues (de Brux et al., 1981; Dallenbach-Hellweg, 1981; King et al., 1981, 1982).

The number of cytoplasmic oestrogen receptors is dependent upon the serum level of oestrogens (Sarff and Gorski, 1971). This should lead to a regular variation of the number of receptors during the oestrous cycle of the rat (Buchi and Keller, 1980). However, maximum concentration has been variously reported at pro-oestrous (Feherty et al., 1970), oestrous (Lee and Jacobson, 1971) or late dioestrous (Nalbandov, 1975).

These conflicting data are not surprising, considering the fact that there are two oestrogen receptor systems (Tchernitchin, 1979), each having its own pattern of cyclic fluctuation and both distributed unevenly in different segments of the uterus. The histochemical studies show that the only conspicuous cyclic change of intracellular oestrogen receptor concentration occurs in the cytoplasm of luminal epithelium (Lee, 1982). This concentration reaches the peak level probably in late pro-oestrous, but drops after ovulation. According to Lee (1982) it continues to decline and hits the lowest point in met-oestrous.

It has been proposed that the synthesis of progesterone receptors is part of the genomic response to oestrogen stimulation (Milgrom and Baulieu, 1970; Rao et al., 1973). Lee (1982) provides cytochemical evidence that both oestrogen and progesterone receptors fluctuate in tandem during the oestrous cycle, but do so only in the epithelial cells.

Rat uterus has also been reported to contain androgen receptors (Buchi and Weber, 1983). According to them, androgen receptor concentration showed a regular change during the oestrous cycle. They hypothesised that androgens play a physiological role in the biology of the uterus and that oestogen is a prime stimulator of androgen receptor synthesis during the cycle. They also concluded that the hormonal control of the cytoplasmic androgen receptor of the rat uterus is very similar to that of the oestrogen and progesterone receptors.

Endometrial receptors for ovarian steroids in women show changes in concentration and subcellular distribution during the menstrual cycle similar to those described in rat uterus. An increase has been reported in the concentration of oestradiol receptor (Pollow et al., 1975; Soutter et al., 1979b) during the proliferative phase. This is related to the plasma oestradiol surge, leading to synthesis of more receptor in the cytoplasm and to nuclear translocation of hormone-receptor complexes. The available data are consistent with the concept that oestradiol stimulates de novo synthesis of both oestrogen and progesterone receptors (Richardson and MacLaughlin, 1978). The post-ovulatory decrease in the oestradiol receptor is related to progesterone effects on the conversion of oestradiol to oestrone (Mester et al., 1974) and the inhibition of oestradiol receptor synthesis (Hsueh et al., 1975). Consequently, there is also a decrease in oestrogen-dependent synthesis of progesterone receptor, as the luteal phase progresses. In addition, it has been observed that progesterone directly "inactivates" its own receptor (Tseng et al., 1977). However only cytoplasmic receptor sites decrease immediately after ovulation, whereas the nuclear levels of both receptors initially remain constant (Rao et al., 1974; Pollow et al., 1975; MacLaughlin and Richardson, 1976; Soutter et al., 1979b; Levy et al., 1980; de Brux et al., 1981). The nuclear progesterone receptor concentration soon increases reflecting raised translocation and/or prolonged nuclear retention time (Levy et al., 1980). The changes in receptor levels follow a similar pattern in epithelial and stromal cells (King et al., 1982). The content of total and nuclear oestrogen receptor of epithelial cells is similar to that of stromal cells. However, the cytoplasmic progesterone receptor content in the epithelium is appreciably higher than in the stroma (King et al., 1982).

During the menstrual cycle the concentration of nuclear progesterone and oestrogen receptors does not exceed that of cytoplasmic receptors because the plasma levels of both hormones are far below the values needed to saturate the receptor sites. Levels of progesterone receptor are always several fold higher than those of oestrogen receptor during the same phase of the menstrual cycle (de Brux et al., 1981; Dallenbach-Hellweg, 1981; King et al., 1982).

1.2470 TISSUE DISTRIBUTION

Histochemical studies have shown (see Section 1.2462) that biochemical changes during the reproductive cycle are not uniform throughout the endometrium but rather located in specific cell types (Nenci et al., 1980; Lee, 1982). The major detectable changes are localised in the epithelial cells, whereas in stromal cells, histochemical changes only become evident in the second half of the post-ovulatory phase (Lee, 1982).

1.3000 EFFECTS OF OVARIAN STEROIDS ON THE ENDOMETRIUM

All the cells of an organism have the same genetic constitution, but show marked differences in their responses, if any, to individual hormones. In a number of target tissues, steroid hormones stimulate the synthesis of major new secretory proteins (King and Mainwaring, 1974; Sutcliffe et al., 1982; Lejeune et al., 1983). These are valuable markers for monitoring the control and kinetics of the response and have enabled specific probes to be made with which to study the genes that specify such proteins. The process of cell renewal through tissue degeneration in the endometrium during each ovarian cycle is an event which is under strict control by ovarian steroids (Schmidt-Mathiesen, 1963; Leroy et al., 1981; also see Section 1.2000). If successful fertilisation occurs, then at implantation, the endometrium of rodents

undergoes a series of morphological and biochemical changes leading to the formation of decidual tissue. This process is characterised by proliferation into epithelioid decidual cells of stromal fibroblasts, while most of the epithelial tissue disappears (de Brux et al., 1981). Biochemically, the decidual cell reaction entails an increase in alkaline phosphatase activity, collagen breakdown and also increased DNA, RNA and protein synthesis (Heald, 1976). At least three new proteins appear in the uterine lumen during decidualisation and concentrations of a number of other intra- and extra-cellular proteins are simultaneously increased (de Brux et al., 1981; Sutcliffe et al., 1982; Lejeune et al., 1983).

1.3100 IN VIVO

The response of the immature rat uterus to steroids is a complex phenomenon involving numerous specific biochemical events which occur in a temporally ordered sequence. This overall response can be divided into (1) a set of early tissue responses, thought to represent the preparations for growth, and (2) a set of later responses involving DNA synthesis, cell division, and true tissue growth itself (Gorski and Gannon, 1976; Clark and Peck, 1979).

The temporal relationship between the cyclical changes in the endometrium and the plasma levels of ovarian steroids provides strong circumstantial evidence on the role of oestrogens in inducing proliferative changes and progestins in inducing events involved with differentiation. This causal relationship has been substantiated by studies on women receiving hormonal therapy (Dallenbach-Hellweg, 1981; Lubbert et al., 1982).

A characteristic cell proliferation during early pregnancy can be elicited by administration of oestradiol to ovariectomised mice (Martin and Finn, 1968) and rats (Leroy and Galand, 1969; Tachi et al., 1972). Similarly secretory and predecidual changes can be induced with progestin therapy (Vladimirsky et al., 1977; Lejeune et al., 1983).

Recent studies (Lubbert et al., 1982) have shown that sequential steroidal therapy induces, in women with gonadal dysgenesis, cyclic changes of endometrial oestrogen and progesterone receptor levels and oestradiol dehydrogenase activity similar to those seen in normally cycling women.

Oestrogens are known to produce an increase in uterine blood flow and volume (Majid and Senior, 1982) as well as an increase in uterine weight (Cowan and Leake, 1979). It has been demonstrated that true tissue growth does not merely result from hormonal stimulation of early responses in the target tissue (Anderson et al., 1972; Gorski and Raker, 1974) but, rather, involves interactions occurring at different times after hormone administration (Anderson et al., 1972) and possibly at different nuclear sites (Clark and Peck, 1979). The induction of the synthesis of a specific uterine protein called 'Induced Protein' (IP) is the earliest known biosynthetic tissue response after oestrogen administration (Means et al., 1972). Synthesis of IP is detectable within 40 minutes (Barnea and Gorski, 1970) and is preceded by synthesis of mRNA, which is detectable by 10 min (De Angelo and Gorski, 1970). This synthesis can be induced by physiological concentrations of oestrogen in vitro (Katzenellenbogen and Gorski, 1972) and can be inhibited by some anti-oestrogens (Katzenellenbogen and Katzenellenbogen, 1973).

The stimulation of RNA polymerase activity by oestrogen in the uterus is again one of the earliest biochemical responses observed after hormone administration (Borthwick and Smellie, 1975). A similar response is observed with non-steroidal oestrogen antagonists in the rat uterus (Kurl and Borthwick, 1980). Inhibition of uterine RNA polymerase by a single dose of progesterone was reported by Kurl and Borthwick (1981).

Increased DNA synthesis is usually accompanied by an apparent increase in DNA polymerase activity. Two principal molecular species of DNA polymerase α and β have been found in rat uterine cells (Harris and Gorski, 1978). They showed that the oestrogen-stimulated immature rat uterus displays increased cellular DNA polymerase activity, which correlates with increased rates of DNA synthesis and cell proliferation. They also indicated that oestrogen treatment apparently increased DNA polymerase α activity with less effect on DNA polymerase β. The uterus shows three states in terms of DNA synthesis and DNA polymerase activity (1) the unstimulated resting level, (2) the oestrogen stimulated state of increased activity, and (3) the refractory state in which levels of DNA synthesis returned to the lower resting levels. The cellular mechanism responsible for this refractory state remains unknown. In the initial report of this refractory state occurring in the rat uterus following oestrogen treatment (Stormshak et al., 1976), it was suggested that oestradiol induced the accumulation of some product that limits the ability of the cells to respond to additional oestrogens. They also suggested that metabolic changes occurring sometime prior to mitosis but which are essential to sustained cell division, are being inhibited.

Stack and Gorski (1983) reported the inability of oestradiol to stimulate uterine DNA synthesis in very young rats. On the other hand they have shown that Diethyl Stilbestrol (DES) stimulates DNA synthesis at the earliest age tested. Therefore, the neonatal rat uterine cell does not lack the cellular response. Epithelial cell death has also been reported in the uterus after oestrogenic stimulation of DNA synthesis (Martin et al., 1973).

As mentioned earlier in Sections 1.1300 and 1.2462, the sequential presentation of different hormones at the target tissue is

often accompanied by changes in receptor populations, and in sensitivity of the responding tissue to hormones. There is now abundant evidence that some hormones modulate the levels of their own receptors or of receptors for other hormones and thereby influence tissue sensitivity to multiple hormones (Clark and Peck, 1979; Katzenellenbogen, 1980). It has been accepted that the level of intracellular oestrogen-receptor in the uterus responds to oestrogen (Martel and Psychoyos, 1978). However, evidence has been offered by recent studies that progesterone, alone and in combination, also influences this level (Mester et al., 1974; Martel and Psychoyos, 1978). According to Mester et al. (1974), the increase in Oestrogen Receptor (ER) was due partly to cell proliferation but also to a rise in the cellular concentration of receptor. This has also been confirmed by Hsueh et al. (1976), who suggested that after translocation, replenishment of the cytoplasmic oestrogen receptor can be blocked by pretreatment with progesterone. This has been confirmed by King et al. (1982). It has been shown that steroid binding to cytoplasmic receptor, translocation, and binding of the complex to nuclear acceptor sites are all absolutely necessary to produce genetically-mediated physiological responses (King and Mainwaring, 1974; Clark and Peck, 1976, 1979 Leake, 1976, 1981a). This has been confirmed by Soutter and Leake (1978), showing that the stimulation by oestradiol of thymidine kinase activity corresponds with the amount of hormone-receptor complex bound in the nucleus. In premenopausal women progestin administration during the proliferative phase increased isocitrate dehydrogenase, alkaline phosphatase (Jelinek et al., 1978) and oestradiol dehydrogenase (Gurpide, 1978) activities and reduced total oestrogen receptor (Tseng et al., 1977) to levels similar to those found in the secretory phase. In women receiving oestrogen therapy, progestins induced changes in enzyme activities similar to those mentioned above for premenopausal women and

also reduced nuclear oestrogen receptor levels (King et al., 1980, 1981a,b). All these findings which support the classical model of oestrogen action (Gorski et al., 1968; Jensen et al., 1968) whereas Tchernitchin (1979) proposed that two, possibly three, independent mechanisms of oestrogen action exist in the uterus, each one mediating a separate group of parameters of oestrogen stimulation.

Sutcliffe (1976) published data in search of a new progesterone dependent human foetal protein. He showed its presence in human amniotic fluid (Sutcliffe et al., 1978) and gave further explanation in support of this alpha uterine protein (Sutcliffe et al., 1980; Horne et al., 1982). Joshi et al. (1980a,b) also reported the production of a protein in the human endometrium, which was dependent on progesterone. Recently both these groups discovered similarities and described these two proteins as one (Sutcliffe et al., 1982).

Sarosi et al. (1983) showed a decrease in the amplitude of electrically stimulated contractions of uterine horn segments from oestrogen pretreated rats. According to them pretreatment with progesterone also results in a significant decrease in the amount of relaxin needed to produce inhibition of the contraction amplitude. These results are in agreement with those of Beck and associates who demonstrated that human relaxin and progesterone synergised to decrease the amplitude of spontaneous human uterine strip contraction in vitro.

Anti-oestrogens increase rat uterine weight (Cowan and Leake, 1979; Majid and Senior, 1982), the increase, over a 24 h period, being similar to that produced by oestrogen. However, the events following nuclear binding of anti-oestrogen/receptor complex differ from those produced by oestrogen despite the fact that both types of compounds are able to produce an initial uterotrophic response (Clark and Peck 1976, 1979; Majid and Senior, 1982).

All anti-oestrogenic activity is not receptor mediated (Clark and Peck, 1979) Majid and Senior (1982) reported anti-oestrogens binding to type I oestrogen receptor, whereas Sutherland et al., (1980) showed, in addition, a specific anti-oestrogen protein receptor. Anti-oestrogen treatment brings about a marked alteration in the distribution of oestrogen receptors (De Boer et al., 1981) and only about 10% remain in the cytoplasm (Katzenellenbogen et al., 1977) after high doses.

Tamoxifen can act as an effective oestrogen-like agonist in stimulating RNA synthesis (Waters and Knowler, 1981). However, it stimulates transcription on a totally different time scale to oestrogen (Waters et al., 1983). These authors also showed the biphasic stimulation in transcriptional response after exogenous oestrogen administration. Tamoxifen activates all the enzymes of DNA synthesis (Leake et al., 1975), but fails to induce significant cell division (Cowan and Leake, 1979). This relates to polyploidy in uterine cells (Martin, 1978). Anti-oestrogen pretreatment also blocked the oestrogen mediated increase in uterine blood flow (Majid and Senior, 1982), indicating that the uterine blood flow response may also be mediated through the oestrogen-receptor mechanism.

In rats the tritiated tamoxifen is converted to a more polar metabolite(s) that is selectively accumulated in the uterine nuclear fraction in combination with receptor (Hayes et al., 1981). Borgna and Rochefort (1979) have also shown the selective accumulation of a polar metabolite from tamoxifen. Another oestrogen-like compound, Lignan, showed a significant decrease in the rate of RNA synthesis in rat uterus (Waters and Knowler, 1982).

The comparative effects of oestrogens and progestins may be similarly summarised. Oestrogen caused an increase in nuclear and nucleolar size in epithelial cells of rat endometrium (Clark, 1971),

whereas Tachi et al., (1974) showed progesterone to be responsible for enlargement of nucleoli and also for massive accumulation of the granular components in stromal cells of ovariectomized rat endometrium. This suggests that progesterone affects the process of maturation of ribosomal RNA in the nucleolus. A transformation of the chromatin from a condensed to a dispersed state was shown after several days of treatment with oestrogen (Sirtori and Bosiso-Bestetti, 1967). Some sort of chromatin dispersion was reported to occur within 15 and 30 minutes of oestradiol treatment in the endometrium of ovariectomised rats (Vazquez-nin et al., 1978). Patrice et al. (1980) showed that the dispersion of chromatin in interphase nuclei of rat endometrium is induced by oestradiol rapidly (within an hour).

They also tried to specify the relationship between the effect of oestrogen on chromatin dispersion and on transcription in the same uterine cells. They presented ultrastructural and biochemical evidence that transcription is stimulated when chromatin has been decondensed by oestrogen. Template activity has been shown to rise correspondingly in chick oviduct after oestrogen stimulation (O'Malley et al., 1976). Le Goascogne and Baulieu (1977) studied changes in nuclear bodies in epithelial cells of rat endometrium and reported an increase in number with diethylstilbestrol and a depression with progesterone, but they could not show any effect of oestradiol treatment.

In summary, steroids have varied and extensive effects on the endometrium. They increase the blood supply causing hyperaemia, they can enhance capillary permeability and uptake of water, electrolytes and amino acids by the cells of endometrium. The rates of glycolysis and certain other aspects of carbohydrate metabolism are increased, as is O_2 consumption. Steroids increase the uptake of the nucleotide precursors of RNA, the activity of RNA polymerases, the template activity

of the chromatin, the synthesis of phospholipids and proteins and the numbers of ribosomes in the cell.

1.3200 DIFFERENTIAL RESPONSES OF UTERINE CELL TYPES TO STEROIDS

The uterus represents a complex and dynamic community of numerous cell types, each susceptible to the regulatory action of oestrogens and progestins. Oestradiol acts differently, in terms of DNA synthesis and mitosis, on endometrium and myometrium (Tachi et al., 1972). Robertson et al. (1971) have shown differences in oestrogen receptor in these tissues. Progesterone showed little effect on the myometrium, whereas oestradiol caused a large increase in the content of myometrial oestrogen receptor, compared with soluble protein and total DNA (Mester et al., 1974). According to these authors, the oestradiol effect on myometrium was significantly counteracted by a simultaneous progesterone administration. Hsueh et al. (1976) studied the whole uterus of immature rats and reported that progestins blocked the oestrogen-induced increase in concentration of receptors, whereas Martel and Psychoyos (1978) concluded that progestins, at the endometrial level, acted synergistically with oestrogens and at the myometrial level acted as an oestrogen antagonist. One of the earliest responses, IP synthesis, indicates that this action of oestrogen is the same in both these tissues (Katzenellenbogen and Leake, 1974) implying that divergence may occur at some point after IP synthesis. Therefore, progestin-oestrogen interaction occurs differently in each uterine tissue and probably for each major cell type.

Endometrium consists mainly of stromal and epithelial cells (McCormack and Glasser, 1980; Dallenbach-Hellweg, 1981). Epithelium can further be divided into the luminal and glandular fractions. The epithelial elements have important functions during the pre-implantation

stages of pregnancy, and stromal cell proliferation is a crucial feature of the decidual reaction. Several investigations of endometrial growth, based upon mitotic count, have indicated that exogenous oestrogens produce surface and glandular epithelial cell proliferation in the endometrium of various experimental animals (King and Mainwaring, 1974; Clark and Peck, 1979) and humans (Ferenczy, 1979a,b; Dallenbach-Hellweg, 1981; de Brux et al., 1981). The location of tritiated oestradiol was monitored using autoradiography, and was shown that 86% of the hormone became localised in the epithelial tissue of immature rat uteri(Stumpf, 1968). In more recent studies, McCormack and Glasser (1980) fractionated cell types from immature rat uteri and measured the amounts and concentration of oestrogen receptor present in each cell type. Epithelial and stromal cells from untreated animals contained a higher concentration of nuclear oestrogen receptor than myometrial cells and epithelial cells also contained the highest concentration of cytoplasmic receptor. Although myometrial cells have the lowest concentrations they contain 84-89% of the total uterine receptors, by virtue of the preponderance of myometrium in the whole uterus. Epithelium and stroma account for 5% and 10% of receptors respectively. Following oestrogen treatment cytoplasmic receptors increase in all cell types (McCormack and Glasser, 1980). Keefar (1982) compared autoradiographic-immonocytochemical data with biochemical results and concluded that the duration of retention of tritiated oestradiol in both stromal and epithelial cell types varies and that the whole of this process is independent of the concentration of oestrogen. Lee (1982) provided cytochemical evidence that both oestrogen and progesterone receptors fluctuate in rat endometrium during the oestrous cycle but only do so in the epithelial cells. She further emphasised that the concentration of receptors in stroma is comparatively low, low enough to serve as a reference for observation of the receptor-rich endometrial epithelium.

Similarly in the case of women, the reduction of oestrogen receptor content after progestin administration occurred in both epithelial and stromal cells and was correlated with a decrease in DNA synthesis in both cell types (Ferenczy et al., 1979a,b; King et al., 1981c, 1982). On the other hand isocitrate and oestradiol dehydrogenase activities are only increased in the epithelial cells. The lack of effect of progestins on stromal oestradiol dehydrogenase is especially important. There is considerable evidence that at least part of the anti-oestrogenic effect of progestins on human endometrium is mediated via the induction of this enzyme (Gurpide, 1978; King et al., 1980, 1981a) which by preferentially metabolising oestradiol to oestrone (a weaker oestrogen) would reduce effective intracellular oestradiol levels. Progestins affect both DNA synthesis and nuclear oestrogen receptor levels in stromal cells without affecting their oestradiol dehydrogenase activity.

As mentioned in the previous section (see Section 1.3100), progesterone dependent alpha uterine proteins (Sutcliffe et al., 1978, 1980) can only be observed in luminal epithelial cells of the human endometrium (Horne et al., 1982). Kirchner (1979) also has shown the presence of uteroglobin in luminal and glandular epithelial cells of the endometrium but not in stroma or myometrium. Ricketts et al. (1983) confirmed this in vitro.

One further observation from the autoradiographic analysis is that hypertrophy occurs in the stroma and myometrium while hyperplasia occurs in the luminal epithelium of oestrogen-treated mouse uteri (Martin et al., 1973). Concerning endometrial cancer in women, various opinions have been emerging. Evidence that endometrial carcinoma may be a mixture of receptor containing and receptor lacking cells, has created a more complicated situation. Variation of oestrogen receptor content in

various sections of the same tumour (Castagnetta et al., 1983) has supported the idea of metabolic differences among similar cells of one tumour. Thus our current knowledge of the differential responses of uterine cell types to various steroids is somewhat sketchy, and further investigations may help to explain some of the ambiguous results obtained from studies of the whole uterus as a single entity.

1.3300 IN VITRO STUDIES

Appreciable progress has been made in elucidating the way in which sex steroids affect the reproductive tract in experimental animals (King and Mainwaring, 1974; Katzenellenbogen and Gorski, 1975), but the nature of the experimental model imposes certain difficulties in interpreting data so obtained. Virtually all of the data have been obtained by administering hormones to animals in vivo which raises problems as to the nature of the hormone that is active at the cellular level. Thus, an endometrial culture system provides a unique opportunity to examine hormonal affects in a controlled environment. There is the hope that culture techniques may have a place in investigating differences between normal and malignant tissues, and in providing some prognostic guide to the clinical course of disease in individual patients. Various attempts have been made with short and long term incubations of tissue slices, organs, or isolated cells, as distinct from homogenates or cell free systems.

1.3310 TISSUE INCUBATIONS AND ORGAN CULTURE

Efforts to study in vitro structurally intact endometrial tissue are not new. As early as the 1920s, attempts were made to maintain endometrial tissue in culture (Traut, 1928). Randall, Stein and Stuermer were the first group to systematically investigate the cytodynamic properties of human endometrial tissue derived from biopsies (Randall et al., 1950). They maintained tissue fragments immersed in

fluid media for up to 35 days. Hughes et al. (1969) placed endometrium on filter membranes supported by stainless steel grids in organ culture dishes.

The direct effect of progesterone, inducing secretory changes in proliferative phase endometrium, has been verified using this system (Csermely et al., 1969). Various studies have been attempted on oestrogen interaction with rat uterus (Ruh et al., 1973). The stability of steroid receptors in this system (Peck et al., 1973), biochemical viability of tissue (Thomas, 1978) and steroid metabolism in human endometrium (Hausknecht et al., 1982) have all been examined. Recently, Kaufman et al. (1980) have maintained human endometrial tissue as differentiated organ cultures for periods exceeding 6 months. Such cultures were subjected to cyclic hormonal changes similar to those seen in the menstrual cycle in vivo. During these in vitro cycles, the changes in histology and DNA synthesis were reported to follow patterns similar to those seen in vivo. However no quantitative data on labelling indices were presented.

Endometrial responses to oestradiol in vitro include an increase in RNA synthesis (Wilson and King, 1969) and in the activation of glucose-6-phosphatase and lactic dehydrogenase (Wilson, 1969). Nordqvist (1970) found that addition of a very high concentration of progesterone to human endometrial organ cultures reduced RNA and DNA synthesis. Glycogen accumulation was induced in proliferative endometrium by progesterone but not by oestradiol (Hughes et al., 1969). Progesterone-induced glycogen synthesis in vitro was not affected by oestradiol but was inhibited by actinomycin D, implying its dependence on RNA and protein synthesis (Shapiro et al., 1980). Tseng and Gurpide (1978) showed that oestradiol dehydrogenase activity in proliferative human endometrium was increased by incubation of the tissue

in medium containing progesterone. Addition of oestradiol to the medium had no influence on the activity of the enzyme. Two different groups (Hirsch et al., 1977; Shapiro and Forbes, 1978) have reported that the increased synthesis of an endometrial soluble protein(s) with an approximate molecular weight of 50,000, which occurs during the secretory phase in vivo, can also be induced in vitro. However, the identity and function of this protein are not known. Another observation made by Hausknecht et al. (1982) using human endometrial tissues, indicates a conversion of the main circulating C19 steroids to dehydrotestosterone.

A great deal of effort has been expended on the development of an in vitro system for relating physiological response to the levels of oestrogen receptor in rat uterine cells. However, with the exception of the induction of an oestrogen dependent uterine protein (IP) by Gorski and co-workers (Katzenellenbogen and Gorski, 1972; Ruh et al., 1973), all attempts at the development of an in vitro system of organ culture which is responsive to physiological concentrations of oestradiol have failed (Peck et al., 1973; Rao and Talwar, 1979). Peck et al. (1973) do not agree with the reports of Gianopoulos and Gorski (1971) and Sarff and Gorski (1971), that the uterine cytoplasmic receptor is stable under in vitro incubation conditions. They conclude that oestrogen receptor is very unstable when uteri are incubated at elevated temperatures in the absence of oestradiol. Thomas (1978) reviewed the in vitro studies on the immature rat uterus as well as human endometrium and concluded that, given the correct conditions, sensitive macromolecules are able to survive in culture and the loss of cytoplasmic receptors must relate to some deficiency in conditions used for culture, rather than to an inherent limitation in the technique.

1.3320 CELL CULTURE

The differential patterns of steroid and polypeptide activities in organ and cell cultures may reflect different requirements of these two groups of hormones for cell interactions. Thus, the two kinds of culture systems yield complementary information about the function of individual and coordinated cellular components of endocrine tissues.

The question of whether a culture is authentic usually involves whether it has a tissue specific function. In the case of cell cultures, this requirement would be superimposed on the need to undergo a useful number of population doublings. In embryonic development both processes are essential, and "terminal differentiation" precludes further cell division. In such cases further cell division depends on the presence of apopulation of 'stem cells'. The stem cells may also give rise to new secretory cells. After embryogenesis, stem cells may be rapidly lost, as is probably the case with neural tissues. The question of the existence and continued function of stem cells is a basic unsolved problem of development, which is central to the understanding of carcinogenesis.

Primarily, cell culture systems bypass the homeostatic mechanisms that persist in studies performed in whole animals. In addition, the homogenous population of target cells growing in long term culture permits the study of hormone action on precise genetic events and the metabolic form of any growth factor is, potentially, easily monitored.

1.3321 Mixed uterine cell cultures

Monolayer cell cultures are frequently established from suspensions of single cells prepared by the enzymic dissociation of organ fragments. The resultant primary culture is a mixed cell population which contains many of the cell types present in the tissue of origin.

Robertson et al. (1961) reported that cells cultured from human endometrium, were predominantly fibroblastic cells with a minority of epithelioid cells. Later Chen et al. (1973) found that epithelioid elements gradually disappeared while fibroblastic cells continued to divide in culture. Both of these groups reported an increase in precursor incorporation after oestradiol stimulation. Pietras and Szego (1975) studied early membrane effects of oestrogen in rat uterine mixed cell populations, with consequent alterations in the cytoplasmic profile of ions, which may be critical to later metabolic and mitogenic effects of the hormone. In another study, oestrogens stimulated cell proliferation in 12 cultures out of 19 from different human endometria (Pavlic and Katzenellenbogen, 1978) In some of them, growth was also stimulated by androgens and glucocorticoids. Sananes et al. (1978) examined the process of modification of stromal cells into decidualised cells in rat endometrium and demonstrated that the average life span of the decidualised cells in vitro is comparable to the development of decidualisation in vivo. It has also recently been shown by using this system, that the stimulation of progesterone receptor levels in primary cultures of human endometrial cells is an extremely sensitive indicator of responsiveness to oestrogen (Eckert and Katzenellenbogen, 1981). Halme et al. (1980) reported that rat uterine mixed cells have the potential to synthesise collagenase prior to subculture and this enzyme production is suggested to be under hormonal control.

1.3322 Cultures of individual cell types

The uterus has a complex array of cell types each of which may have very different hormone sensitivites (Martin and Finn, 1968; Satyaswaroop et al., 1979; McCormack and Glasser, 1980; King et al., 1982). Analysis made on the whole organ in culture or cultures of mixed cell types, cannot therefore be automatically translated as indicating

responses in one particular cell type. Variable proportions of different cell types occur with changes in the physiological state of the uterus (Noyes et al., 1950; Dallenbach-Hellweg, 1981). This creates difficulty in interpreting the results obtained from mixed cell populations.

Study of the initiation of the decidual response (O'Grady et al., 1974; Heald et al., 1975; Sananes et al., 1978), of receptor modifications (Fleming et al., 1980) of morphological alterations (Berliner and Gerschenson, 1976; Liszczak et al., 1977; Vazquez-Nin et al., 1979), of molecular mechanism of blastocyst attachment to epithelial cells (Ricketts et al., 1983), of dynamics of oestrogen receptors (McCormack and Glasser, 1980; Fleming and Gurpide, 1981) and of other growth responses (Satyaswaroop et al., 1979; Kirk and Irwin, 1980; Gerscthenson et al., 1981; Fleming and Gurpide, 1982; Dorman et al., 1982) led to the idea that there would be considerable advantage in having separate preparations of epithelial and stromal cells.

Identification of factors and hormones that stimulate proliferation of a given cell type is an essential initial step in elucidating the regulatory mechanisms of growth and differentiation of that particular cell type. In contrast to the previous findings that epithelial cells are less serum dependent than fibroblasts (Dulbecco and Elkington, 1973), Kirk and Irwin (1980) found that human endometrial stromal cells showed a lower serum requirement than endometrial epithelial cells for attachment to the substratum and early growth. They also demonstrated that there are positive synergistic effects between oestrogen, insulin and/or dexamethasone and to, a lesser extent between progesterone and insulin.

Stromal cultures showed great variability in their proliferative responses to both oestradiol and progesterone. Kirk et al. (1978)

observed a signficant shift in the growth response of stromal cells to physiological levels of progesterone following very small changes in oestradiol levels. Progestin antagonised both the oestrogenic stimulation of growth and of radiolabelled thymidine incorporation but, enhanced the incorpor.ation of radioactive uridine and amino acids into RNA and proteins respectively. On the other hand, Liu and Tseng (1979) reported that oestradiol and Medroxy Progesterone Acetate (MPA) together shortened the generation time of both epithelial and stromal cultures derived from a proliferative endometrium. Trent et al. (1980) reported that the incorporation of radioactive thymidine was stimulated in stromal cultures by oestradiol and inhibited in epithelial cultures by progesterone. Fleming et al. (1980) found that the levels of nuclear oestrogen receptors were increased by oestradiol and reduced by MPA in stromal cells. They failed to conclude whether steroids affected the quantities of receptor in the cells or only affected the time at which the peak of oestrogen receptor concentration appears.

Progesterone had a clear inhibitory effect upon the division rate of epithelial cells from rabbit endometrium (Gerschenson et al., 1974). They also showed the antagonism between DES and progesterone (not seen in the presence of Epidermal Growth Factor) on the cell division rate. Berliner and Gerschenson (1976) showed an increase in size of nucleoli in response to DES and increased amounts of rough endoplasmic reticulum (RER) in the presence of progesterone. It has also been suggested that only glandular epithelial cells are responsive to oestrogen while luminal cells are not (Gerschenson et al., 1979).

Gerschenson et al. (1981) reported that primary cultures of rabbit endometrial cells produce a factor or factors which inhibit proliferative responses to 17-β oestradiol. According to them, such inhibitory activity can only be seen when cells are treated with progesterone or plated at higher density than usual.

Liu and Tseng (1979) studied oestradiol metabolism in separated human endometrial epithelial and stromal cells. They found a high rate of oxidation of $E_2 \longrightarrow E_1$ in both fractions of secretory endometrium. However, the conjugate enzyme, sulfotransferase, which further metabolises both E_2 and E_1 to form water soluble metabolites, was only active in glandular epithelial cell cultures. Ricketts et al. (1983) showed, by immunofluorescence studies in cultures of isolated cells, that uteroglobin is present only in epithelial but not stromal cells. They further demonstrated that neither progesterone nor oestrogen altered uteroglobin secretion rates.

Echeverria et al. (1980) described two types of epithelial cells from rat uterus and characterised them as of short and long term survival in culture. Both types showed elevated RNA synthesis in response to oestradiol.

Ziegler and Gurpide (1982) characterised a type of cell from human endometrium as prolactin producing. These attach slowly to the culture dish. The decline in the rate of prolactin production observed after several days in culture may be due to the loss of the capacity of the cells to synthesise the hormone or to a disproportionate proliferation of cells that do not produce prolactin. Fleming and Gurpide (1982) agreed on loss of response in cultured cells with passage of time and gave a reason that the selection of fast growing cells changes the characteristics of the subcultures. Ricketts et al. (1983) also reported the decline in uteroglobin secretion with prolonged culture and, in their view it may reflect deterioration of cell function or progressive hormonal deprivation. Vallet-Strouve et al. (1982) also showed a similar decline in 17-β-Steroid Dehydrogenase (SDH) activity with time in culture of ovine myometrial cells. They observed that this loss in a given subculture is reversible and may reflect a change in

cofactor or enzyme equilibrium. The gradual decrease observed in successive subcultures parallels the slowing of cell growth and overall protein synthesis, probably reflecting tissue ageing.

Fleming and Gurpide (1981) found very rapid and apparently reversible changes in oestrogen binding which may indicate the existence of other mechanisms, apart from protein synthesis and degradation, for regulating the concentration of oestrogen binding sites. They suggested that the measurement of levels of specific oestrogen binding sites in cell cultures should take into account the possibility of significant temporal variations detectable by daily or hourly assays and dependent on endogenous and exogenous factors affecting the metabolic state of the cells. However, the inconsistent pattern of hormonal effects on cell proliferation reported in different studies does not permit general conclusions to be drawn at present. Better characterisation of cultures and improved culture conditions should help to overcome some of the present difficulties in studying ovarian steroid effects *in vitro*.

1.4000 CELL-CELL INTERACTIONS

During development of the uterus, tissue interactions play an essential role in several specific events that lead to the morphogenesis and maturation of the endometrium.

1.4100 STROMAL-EPITHELIAL INTERACTIONS

During embryonic and neonatal morphogenesis the epithelial differentiation induced by gonadal steroids in sexual organs is mediated by the stroma (Kratochwil *et al.*, 1979; Heuberger *et al.*, 1982). Moreover, the possibility of various growth factors acting indirectly on epithelial cells via stroma, either dependent on epithelial contact with the stromal matrix or by stimulating stromal fibroblasts, which then stimulate the epithelium, has been discussed (Gospodarowicz *et al.*, 1977,

1983; Peehl and Ham, 1980). Thus, the final changes in the epithelium might not result from a direct action of sex steroids on these cells but, from their effect upon the stroma. The bulk of the evidence leads to a conclusion that the mesenchyme (a) provides a physical substrate for the attachment and orientation of epithelial basal cells and (b) produces diffusible factors which promote epithelial growth and differentiation (Chan and Haschke, 1983). These results, while implicating the stroma in epithelial responses do not directly demonstrate the mechanisms of stromal function in hormone action. There is also evidence for the influence of epithelium on mesenchymal functions (Irwin, 1982).

Although epithelial/mesenchymal interactions are firmly established as important for normal embryonic development (Kratochwil et al., 1979), their role in the control of adult tissue has received little attention. The endometrium provides a useful model system for studying such interaction by mixing separated epithelial and stromal cells. Epithelial cells can be either plated on or within homologous stroma, as mixed cultures, or physically separated from each other but maintained as co-cultures sharing the same culture medium (Kirk and Irwin, 1980). They also suggested that co-culturing coverslip cultures of epithelium and stroma results in inhibition of stromal growth by the epithelium.

However, the fact that primary cultures of normal and/or transformed cells are difficult to establish and maintain has afforded little opportunity for detailed analysis of growth behaviour and the interactions either between these cell types with or without various transforming agents. In contrast, cells of fibroblastic origin can be established and maintained in vitro with relative ease. Harris (1983) presented evidence that each type of epithelium has its own 'fingerprints' of cytoskeletal proteins. Heckman (1983) confirmed this finding and concluded that growth failure in cultured epthelium is

related to organ and species specificity rather than to the problem of fibroblast overgrowth.

1.4200 PRODUCTION AND DEGRADATION OF EXTRACELLULAR MATRIX

Rat uterine cells play an active role in the synthesis of precursors of the extracellular matrix and this synthetic activity is modulated by oestrogens (Dyer et al., 1980) and possibly progestins (Padykula, 1981). Fibroblasts in other organs participate in the degradation of the extracellular matrix by producing collagenase and proteolytic enzymes (Werb et al., 1977). During the postpartum regression of the rat uterus there is a large increase in uterine collagenase activity (Jeffrey et al., 1971).

Primary cultures of various cell types have been reported to secrete several proteins that have been shown to be components of extracellular matrices (Alitalo et al., 1980). Halme et al. (1980) reported rat uterine mixed cell populations as capable of production of collagen in primary culture (also in organ culture conditions). Although circumstantial evidence suggests a role for endometrial stromal cells in the maintenance and remodelling of the extracellular matrix (Dyer et al., 1980) there is no direct evidence as yet to support it.

1.4300 DIFFERENTIATION AND DE-DIFFERENTIATION

Differentiation is defined as a process of unidirectional and irreversible transformation under normal physiological conditions. This process is accomplished through the ordered control of gene expression. It seems obvious that structural changes in the regulatory DNA could easily lead to a misprogramming of gene function during cellular differentiation. The external environmental stimuli required to transform a fully differentiated normal cell into another must be very and tightly controlled since such an event rarely occurs.

Since endometrial epithelium is a continuously renewing tissue, it may be suggested that it comprises groups of cells capable of long-term self-maintenance i.e. exhibiting the properties of stem cells.

The proliferation of these stem cells is hormone independent (Prianishnikov, 1978) and the nearest descendants of stem cells become hormone-sensitive in the presence of oestrogen (Satyaswaroop and Martel, 1981). According to the model proposed by Prianishnikov (1978), the stem cells of endometrial epithelium proliferate throughout the menstrual cycle and give daughter cells which pass through the following three stages of development: oestrogen sensitive cells, oestrogen-progesterone sensitive cells, and progesterone sensitive cells. He further claimed that the ratio of cells in the three classes is regulated by the levels of various hormones secreted by the ovaries. Disturbances in the secretion of these hormones could lead to changes in the population size or ratio of separate cell types and pathological alterations in endometrial tissues. Such a transformed pathway may be termed de-differentiation, which is believed to reflect in the loss of phenotypic traits and the evolution of malignant cells. Investigations of differentiation and carcinogenesis using cultured human cells are yielding exciting results. Kaufman's laboratory recently described the clonal growth of stromal cells from human endometrium and modulation of their differentiated state by hormones and nutritional factors (Dorman et al., 1983).

On the basis of constant regeneration and cyclic changes of cell proliferation, Satyaswaroop and Martel (1981) proposed a model for the study of differentiation. According to them the process of re-epithelialisation of the postmenstrual endometrium starts in the basalis layer where primitive stem cells undergo cell division to give rise to daughter cells, half of which (precursor cells) may be sensitive

to oestradiol. The other half remain as stem cells, thereby maintaining a constant stem cell pool. Under the influence of oestradiol and/or the micromilieu, these precursor cells proliferate and differentiate into cells containing oestrogen receptors. Oestradiol then acts through the receptor mediated mechanism to set off another wave of proliferation and differentiation of these cells to cells containing progesterone receptors. These cells then respond to progesterone via the progesterone receptor system to differentiate into mature secretory cells.

Gerschenson studied rabbit uterine epithelium and also found four normal mechanisms to be operating: cell proliferation, migration, loss (cell death/or sloughing), and terminal differentiation(Gerschenson and Fennell Jr. 1982). They also concluded that ovarian hormones differentially regulate some of these mechanisms. Disturbances in one or several of these intrinsic mechanisms involved in the growth of the uterine epithelium could result in hyperplasia which become independent of ovarian hormones.

Table 1.2 is self explanatory and shows that different types of endometrial hyperplasia could be attributed to derangement of two steps in the path by which the uterine epithelium grows and differentiates. According to Gerschenson and Fennell, Jr. (1982) similar phenomena could also be operational in endometrial carcinoma. Whereas, Richardson and MacLaughlin (1978) indicated that an oestrogen milieu unopposed by progesterone predisposes the endometrium to neoplastic transformation.

Satyaswaroop and Martel (1981) claimed endometrial carcinoma to be an aberration of endometrial cell differentiation. They presented a scheme, parallel to normal cell differentiation, the derangement of which leads to endometrial cancers of varying degrees of differentiation. Due to the proliferative characteristics as well as the different degree of differentiation of the malignant tissue, they suggested that stem cells

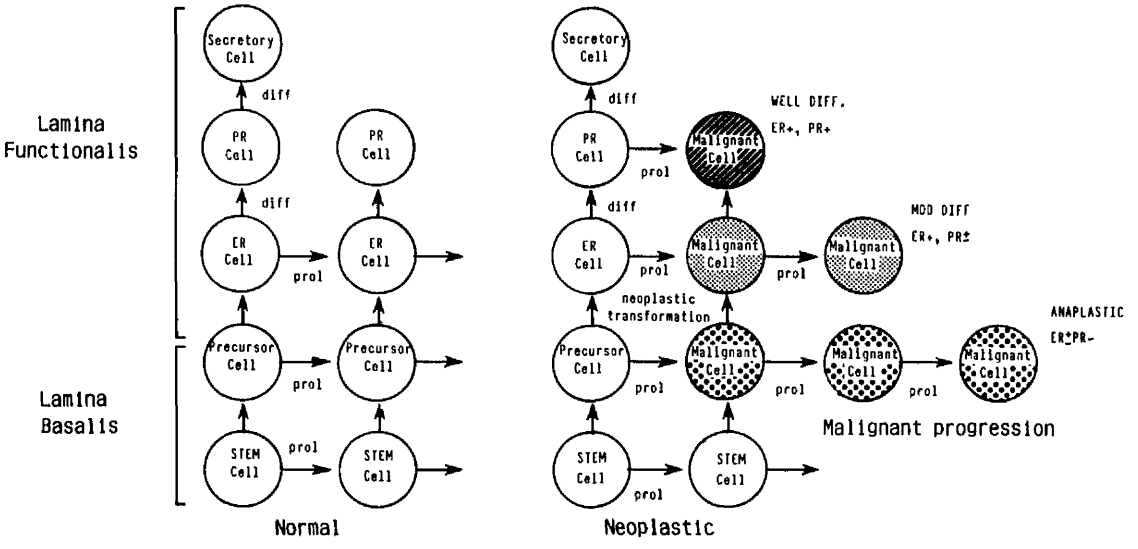
Table 1.2: Deranged mechanisms for uterine epithelium growth

Reproduced from Gerschenson & Fennell (1982)

Figure 1.3: Scheme of endometrial carcinoma as a defect in stem cell differentiation

Reproduced from Satayaswaroop & Mortel (1981)

Types of Hyperplasia	Cell Proliferation	Cell Migration	Cell Loss	Terminal Differentiation
Simple	Increased	Normal	Normal	Normal
Simple	Normal	Decreased	Normal	Normal
Cystic	Normal	Normal	Decreased	Normal
Adenomatous	Increased	Decreased	Normal	Normal
Polypoid	Increased	Normal	Decreased	Normal
Atypical	Increased	Normal	Decreased	Impaired
Atypical	Increased	Decreased	Decreased	Impaired



are potential targets for neoplastic transformation. Therefore, it has been inferred that stem cells which are responsible for growth and differentiation in regenerating tissues may be, at least in some cases, the cellular site of malignant transformation (Mintz, 1978). The conception of endometrial carcinoma as a defect in stem cell differentiation, suggested by Satyaswaroop and Martel (1981), is represented in Figure 1.3. The normal endometrial cell differentiation is shown at the left hand side of the scheme and its derangement leading to tumours of varying degrees of differentiation is depicted on the right.

According to this model, the neoplastic transformation may originate at any level of the hierarchy of progressively specialising cells. Malignant transformation results in an enhanced proliferative capacity with a simultaneous diminution in potential for differentation.

The last stage of differentiation in endometrial epithelium is terminated by cell death (Prianischnikov, 1978; Satyaswaroop and Martel, 1981). There is evidence that apoptosis is a normal physiological process in the regulation of cell populations, because such cell death does not induce acute inflammation even when many cells in a tissue undergo the process simultaneously (Duvall and Wyllie, 1983). It is conceivable that apoptosis may be part of a wider surveillance mechanism for recognising and deleting cells with damaged or altered DNA including potentially malignant cells, without invoking the immune system.

1.4400 ORIGIN OF CANCER

Considerable research goes into the question: "What is a cancer?" More specific questions still to be ansered are why, where, and when human cancers develop, and what is the subsequent behaviour of aggregates of such cells. The concept of tumour progression was coined by Foulds (1949). According to him, karyotype diversity, immunogenic specificity, drug resistance, hormone dependence, metastatic potential

and degree of cellular differentiation are some of the properties that can change as a tumour develops. There is increasing recognition of the concept that most naturally occurring cancers result from a complex interaction between endogenous (host) and exogenous (environmental) factors.

At first glance, the suggestion that a tumour cell need not be capable of unlimited proliferation may appear incompatible with the usual concept of tumour cells. However, the central characteristic of a tumour cell is not its ability for indefinite division potential but rather its ability for uncontrolled proliferation within and through normal tissues of the host to form the clinical tumour. The growth of a tumour is not just a function of mitotic rates but cell loss is probably a factor of critical importance in determining the tumour mass.

The "commitment theory" proposed by Holiday et al. (1977) suggests that, beginning in embryonic life through to the end of the adult stage, there are cells in various tissues of the body that have the potential for an infinite number of divisions. Such cells are termed "uncommitted", and as they divide, one or both daughter cells may retain the potential for an infinite life span, or they may lose the ability to divide indefinitely and become "committed". Moreover, this theory considers that reversion of a committed cell to an uncommitted cell is prohibited.

In contrast, Petricciani (1980) proposed that human tumours arise from the relatively few uncommitted cells present in the body at birth since these remain through to old age and death. These uncommitted cells form a unique class existing at very low frequency in every human and which may, on rare occasions, undergo changes either spontaneously or, more frequently, in response to a mutagenic or carcinogenic agent to become neoplastic. This model predicts that an in

vitro tumour cell population may consist of upto four classes of cells: normal cells that are either committed or uncommitted and that are derived by reversion of tumour cells; and tumorigenic cells that are either committed or uncommitted.

Most recently, Waterfield et al. (1983) presented a model of how a normal cell could be changed into a cancer cell and ultimately form a tumour. Platelet derived growth factory (PDGF) is produced in wounds by clotting blood and has a special power to make cells around a wound grow and heal (Westermark, 1983). Waterfield and his colleagues discovered a structural similarity between PDGF and a cancer virus. They termed this unusual protein, the key substance, which makes certain cancer cells multiply uncontrollably.

Another view of cancer is that it results from an impairment of the cell differentiation process. Mostly, tumours are composed of proliferation compartments and differentiated compartments. The result is a caricature of the renewal process of normal tissues. This leads to the suggestion that inducing differentiation might be alternative to cytotoxic therapy (Pierce & Cox, 1978). The discovery of agents that cause differentiation is as important as the discovery of agents that kill tumour cells. Paul (1978) reinforced the idea of treating cancer by inducing and promoting differentiation. He explained cancer, as essentially a disturbance of the 'cell computer' and this process may not necessarily be an irreversible alteration of DNA. The question, when and how carcinogenic stimuli interact with stem cells or differentiated cells is still unanswered. The basic process involved is a change in growth control. Our understanding of normal growth control is still so limited that much basic work is required before cancer reversal is realistic.

1.4500 ENDOMETRIAL CARCINOMA

A variety of theories have been advanced to explain the development of neoplastic cells in animals and humans (Prianisanikov, 1978; Petricciani, 1980; Lucas, 1981; Satyasworoop, 1981; Gerschenson and Fennell, Jr. 1982; Gusberg, 1982). However, convincing evidence in support of any theory of human tumorigenesis has yet to be developed and confirmed in independent laboratories.

Everything known about the hormone dependence of normal endometrium (Dallenbach-Hellweg, 1981) and its implications for carcinogenesis might be itself a monograph about the hormonal aspects of endometrial cancer (Kaufman et al., 1980; Kimura and Okada, 1981; Lucas, 1981; Gusberg, 1982; Ziel, 1982). Koss et al. (1980) described progesterone and oestrogen as important not only in the growth and differentiation of normal endometrium but also in the development of endometrial hyperplasia and cancer. He further explained oestrogen as an essential precursor of endometrial hyperplasia. Ferenczy (1979b) concluded that endometrial hyperplasia is produced, at least in its early stages, by an oestrogenic environment in the absence of progesterone.

Numerous investigators have examined oestrogen and progesterone receptor concentration in human endometrial carcinoma (Tseng and Gurpide, 1972; Pollow et al., 1975; MacLaughlin and Richardson, 1978; Soutter and Leake, 1978) and according to the majority, most of the more differentiated grades, contained oestradiol and progesterone receptors but at variable concentrations. Several authors (Ehrlich et al, 1978; Creasman et al., 1980) have deemed progesterone receptor concentration as the best index of hormone responsiveness of endometrial carcinoma. The degree of tumour differentiation and circulatory hormone levels may account for the variablity in receptor levels. In addition, carcinoma of the endometrium may co-exist with proliferative, secretory or hyperplastic endometria.

Endometrial hyperplasia includes a wide range of epithelial abnormalities characterised by varying degrees of morphologic alterations and cellular changes (Dallenbach-Hellweg, 1981). Based on data accumulated on sex steroid receptor regulation in normal endometrium, high levels of ER and PR should be expected in endometrial hyperplasia. Indeed Janne et al. (1980) confirmed the presence of high levels of cytoplasic oestrogen and progesterone receptors in hyperplastic endometrium. Likewise, Gurpide et al. (1978) found concentrations of ER and oestradiol-17β hydroxy steroid dehydrogenase activity in endometrial hyperplasia, similar to those in proliferative phase endometrium.

Tumour heterogeneity limits the prognostic value of conventional receptor assays in biopsies. Evidence that breast cancer in some women reflects heterogenous population of oestrogen receptor positive and negative cells have been obtained by cytochemical methods (Pertschuk et al., 1980). Some tumour cells exhibit a preponderance of nuclear fluorescent staining, some show a combination of nuclear and cytoplasmic fluorescence, while in others cytoplasmic fluorescence dominates. ER levels in receptor positive cells may vary between cells.

Derangement in one or more of the mechanisms for normal uterine epithelium proliferation and differentiation could result in endometrial hyperplasia and/or carcinoma (Satyaswaroop and Martel, 1981; Sommers, 1982; also see in Section 1.4300). According to Gerschenson and Fennell, Jr. (1982) derangements in any of the variables described earlier, could result in hyperplasia. However, malignant growth could occur only as a result of a further event involving viral, chemical or physical injury. At the same time it is also possible that de-differentiation could result in neoplastic transformation. For instance, administration of oestrogens to patients with defects in the mechanisms of cell loss, and lacking negative feedback through oestrogen

inhibitor factors, could result in the selection of cell populations with increased growth ability and invasive properties. These cell populations could eventually become oestrogen-independent as a result of either further selection or a mutagenic event.

There is no conclusive evidence that steroid hormones are carinogens (Gerschenson and Fennell Jr, 1982). Nevertheless, a large body of evidence exists, suggestive of their playing an important role in prostate (Kaighn, 1980) breast (Jensen, 1981) and endometrial (Kimura and Okdad, 1981) neoplasia.

It seems important to define the cell of origin in the different types of endometrial carcinoma, because it could have important therapeutic implications. Information is being acquired on cell specific factors controlling both growth and differentiation. It is also clear that exogenous factors do not necessarily act individually but interact (Kaighn, 1980). These interactions must be understood and controlled if meaningful *in vitro* carcinogenesis studies are to be carried out. An exciting approach is the hormonal cycling experiments reported by Kaufman's laboratory *in vitro* (Kaufman *et al.*, 1980). An understanding of the influence of such changes on susceptibility to carcinogens could provide approaches to prevention as well as cure.

1.5000 PROBLEMS IN ENDOMETRIAL BIOLOGY

The endometrium has been the object of extensive morphological and biochemical studies for the past 50 years. The recent advances in the molecular aspects of steroid hormone action have undoubtedly improved our understanding of the endometrial function. However, there is still a poor knowledge of how the morphological and biochemical changes are reflected in functional changes.

1.5100 MECHANISMS OF HORMONE ACTION

The steroid binding studies in the endometrium leave no doubt that this tissue responds directly to the major ovarian hormones, oestradiol and progesterone, via receptor-mediated mechanisms. The cyclic variations in receptor levels and the correlations with plasma steroid levels constitute strong circumstantial evidence of the key roles receptors must play in cellular proliferation and differentiation during the menstrual cycle. It is crucial, however, that direct associations be made between receptors and the specific functions they regulate in the cell (MacLaughlin and Richardson, 1979). The problem of identifying oestradiol and progesterone induced products is further complicated by several recent observations. First, there may be a third uterine steroid receptor, which is specific to androgens (Chambon *et al.*, 1977). This has been confirmed by Buchi and Weber (1983), although the role(s) for this receptor is unknown. Further, several polypeptide hormones including insulin (Demers *et al.*, 1977), prolactin (Moodbidri *et al.*, 1974) and prostaglandins (Abel and Kelley, 1983) have been implicated in the normal control of endometrial functions. Suitable *in vitro* systems should help to overcome some of these problems.

1.5200 USE OF ENDOMETRIAL CELL CULTURE IN CANCER RESEARCH

The changes induced *in vivo* by administration of exogenous hormones (Section 1.3100) have established the role of ovarian steroids in regulating many endometrial functions. However, the complexity of the *in vivo* situation makes it difficult to demonstrate whether the observed changes result from a direct steroidal effect on specific cell types. On the other hand some hormonally regulated functions may not be readily apparent under *in vivo* conditions.

It is evident that various endometrial functions including secretion as well as the synthesis of glycogen and some enzymes can be

affected by ovarian steroids in vitro. Some other functions have been more elusive to in vitro analysis. Perhaps the most remarkable case is that of oestrogen stimulated cell proliferation. It was first demonstrated in human endometrial cells in vitro almost a decade ago. However, there is still little information about oestrogenic regulation of growth in endometrial cells. A major drawback has been the use of mixed cultures which do not permit an interpretation of the results with respect to cell type. On the other hand, studies using separated cell types have yielded inconsistent and often conflicting results. Potentially, this system affords the comparison of the functional properties of epithelial and stromal components at the different stages of carcinogenesis. The lack of success in demonstrating some hormonal effects in vitro could be the result of some deficiency in the culture conditions or an inherent limitation of the in vitro techniques (Thomas, 1978). It has also been proposed (Sirbasku and Bensen, 1980) that oestrogens stimulate proliferation of target cells indirectly by inducing the production of growth factors in other tissues. Clearly hormone responsive endometrial cultures provide a useful system to clarify these points.

The end result of these studies have implications not only for understanding the origin of neoplastic cells, but also for therapy design, since it has shown that malignant cells differentiate and become terminal (Pierce & Cox, 1978).

1.6000 THE AIMS OF THE STUDY

It is not clear how many of the controls of normal cell behaviour have to be altered to create the commonest forms of cancer. Since most epithelial cells probably have a more complex programme of cell renewal than fibroblats, several steps may be involved in the

progression of a normal epithelial cell to a neoplastic one. Different steroids may act as initiator and/or promoter for endometrial cancers. Although we understand the mechanism by which steroids induce alterations in the synthesis of proteins in target cells through the steroid receptor system, there is still a gap in our understanding of the growth promoting activity. To that end, a model system is required to study direct effects of steroids on growth.

To date attempts to establish a cell culture system from endometrial epithelium, in which a consistent response to ovarian steroids can be demonstrated, have met limited success. Such a system, when reproducible, would be a useful tool to study general mechanisms of steroid hormone action and, in particular, problems of endometrial cell biology.

In this context the aims of the investigations involved in this project were to establish a successful method by which endometrial cells could be introduced routinely into culture. A reproducible method to isolate and characterise cell types was sought. The primary aim of this research was to gain an understanding of the growth requirements of individual cell types from normal and malignant endometrium. Two of the main objectives were:-

a) to investigate the effects of ovarian steroids on endometrial epithelial cells in primary culture

b) to determine what factors would modify the responsiveness of cultured endometrial cells to ovarian steroids

Another, more optimistic, purpose was to examine hormone-induced changes in endometrial epithelial cells with the special goal of correlating an *in vitro* phenotypic marker with *in vivo* tumorigenicity.

CHAPTER 2 - MATERIALS AND METHODS

2.000 MATERIALS AND METHODS

2.100 REAGENTS

2.110 FINE CHEMICALS were obtained as follows:

Norit A activated charcoal	Sigma, London
Bovine Serum Albumin (BSA)	Sigma, London
Deoxyribonucleic Acid (DNA) (calf thymus type V sodium)	Sigma, London
Dextran T70	Pharmacia, Sweden
Sucrose (AnalaR)	Fisons, England
Trizma base	Sigma, London
Methanol	Fisons, England
Ethanol	Fisons, England
Acetone	Fisons, England
HEPES	Cambridge Research Biochem.Ltd. England

All other reagents used were BDH "AnalaR" grade.

2.120 RADIOCHEMICAL used was (^{3}H-methyl) thymidine (47 Ci/mmol), supplied by Amersham International.

2.130 PHOTOGRAPHIC ITEMS

D-10 Developer	Kodak Ltd., England
AR 10 Film grain (Autoradiographic stripping film)	Kodak Ltd., England
K-2 nuclear emulsion	Ilford Ltd., England
Amfix	May & Baker, Dagenham, Kent
Kodak Electron Microscopy film 4489	Kodak Ltd., England
Panatomic-X	Kodak Ltd., England
HP4 & HP5	Ilford Ltd., England

2.140 ANTIBODIES

The monoclonal antibody (LE61) was kindly supplied by Dr.E.B.Lane, ICRF, London.

Fluorescein conjugated goat and rabbit anti mouse IgG was obtained from Sigma, London.

2.150 ENZYMES

Trypsin (1:250)	Difco Laboratories, Detroit
Trypsin (Bovine Pancreas)	Sigma, London
Collagenase type III	Worthington, England
Crude pancreatin	Gibco Biocult, Paisley
DNAse	Worthington, England
RNAse	Worthington, England

2.160 INHIBITORS

Soy-bean trypsin inhibitor	Sigma, London

2.170 SCINTILLATION MATERIALS

Toluene (AnalaR Grade)	Koch-light Labs, England
2,5, diphenyloxazole	Koch-light Labs, England

2.200 STANDARD SOLUTIONS

2.210 PHOSPHATE BUFFERED SALINE (PBS)

made up w/v in distilled water to a final pH of 7.2	0.8%	NaCl
	0.02%	KCl
	0.115%	Na_2HPO_4
	0.02%	KH_2PO_4
	0.0133%	$CaCl_2\ 2H_2O$
	0.01%	$MgCl_2\ 6H_2O$

For Ca and Mg free PBS (PBS-A) the respective salts were omitted.

2.220 SORENSEN'S PHOSPHATE BUFFER (SPB)

0.1 M $Na_2 HPO_4/NaH_2PO_4$ buffer pH 7.2

2.230 KREBS RINGER BUFFER (KRB)

	0.6%	NaCl
	0.035%	KCl
made up in distilled water	0.16%	KH_2PO_4
(w/v) and stirred overnight	0.1%	Glucose
at 4^oC at pH 7.4	0.1%	Methyl cellulose
	0.21%	$NaHCO_3$
	0.0005%	Phenol Red

2.240 EARLE'S BALANCE SALT SOLUTION (BSS)

	6.8%	NaCl
	0.4%	KCl
	0.2%	$MgSO_4\ 7H_2O$
w/v in distilled water	0.14%	$NaH_2PO_4\ 2\ H_2O$
	0.39%	$CaCl_2\ 6\ H_2O$
	0.0015%	Phenol Red
	0.1%	Chloroform

2.250 COUNTING FLUID

	0.7%	NaCl
w/v in distilled water	1.05%	Citric acid
	0.1%	Mercurie Chloride

2.260 BUFFERED SALINE

	0.58%	NaCl
w/v in distilled water	0.37%	EDTA
pH 7.0	0.12%	Tris

2.270 GELATIN-CHROME-ALUM

Gelatin	5g
$CrK(SO_4)_2$ 12 H_2O	0.5g
Formaldehyde (40% v/v) solution	5ml
Photo-flo	1ml

Total volume adjusted to 1 litre

2.300 TISSUE CULTURE MATERIALS

2.310 CULTURE VESSELS were obtained as follows:

Tissue culture flasks ($25cm^2$, $75cm^2$)	Falcon, Division of Becton Dickinson Co., U.K.
Plastic Nunc Petri Dishes (35mm, 65mm)	Gibco Biocult Ltd., Paisley
Costar multiwell trays (24 x 16mm diameter)	Costar, Cambridge, Mass. U.S.A.
Glass coverslips (10mm, 13mm diameter)	Chance Propper Ltd., England

2.320 FILTERS

Nalgene filter units were obtained from Nalge Co., Sybron Corporation, Rochester, N.Y.

Swinnex Filtration Units	Millipore, London

2.330 DISPERSION SOLUTIONS

2.331 Trypsin

2%	Trypsin
0.0115M	$CaCl_2$

in KRB buffer (Section 2.230)

2.332 Trypsin and Collagenase

0.35%	Trypsin
0.5%	Collagenase

in KRB buffer

2.333 Trypsin and Pancreatin

0.5%	Trypsin
2.5%	Pancreatin

in PBS-A (Section 2.210)

2.334 Trypsin and Versene

	0.25%	Trypsin
	0.3%	Tri sodium citrate
Solution A - w/v in distilled	0.61%	Sodium chloride
water and sterilised by	0.0015%	Phenol Red
filtration		
	0.8%	NaCl
	0.02%	KCl
Solution B - w/v in distilled	0.11%	Na_2HPO_4
water and autoclaved at 15 lbs	0.02%	$KH_2 PO_4$
pressure for 20 minutes	0.02%	Versene (EDTA)
	0.0015%	Phenol Red

One part of Solution A mixed with 4 parts of Solution B immediately before use.

2.335 Collagenase

200U/ml collagenase type III in Eagle's medium (MEM)

All these solutions were filter sterilised using G.S. membrane filters (0.22μ, Millipore)

2.340 MEDIA

2.341 Standard culture medium (SCM)

This consisted of Eagle's MEM with Earle's salts without glutamine. Although containing bicarbonate - CO_2, this medium was additionally buffered with 25mM HEPES (Gibco Biocult, Paisley).

2.342 Additional media

MCDB-104 and medium 199 were obtained from Gibco Biocult Ltd., Paisley.

2.343 Antibiotics

Penicillin	Sigma, London
Streptomycin	Sigma, London
Fungizone (Amphoterinic B)	Gibco Biocult, Paisley

2.350 SERUM

The culture medium was generally supplemented with foetal calf serum (FCS) obtained from Gibco Biocult Ltd., Paisley or Flow Laboratories, Irvine, Scotland.

2.351 DCC adsorbed

To extract steroids Dextran Coated Charcoal was used at a final concentration of 0.25% charcoal and 0.0025% Dextran.

The serum was shaken either 12 hours at $4^{o}C$ or 2x45 minutes at $37^{o}C$ or $56^{o}C$. After adsorption DCC particles were separated by centrifugation at 10,000 rpm (15,000g) for 10 min. Removal of the remaining charcoal particles and sterilisation were accomplished by sequential filtration through Millipore 0.45μm and 0.22μm pore size membranes.

2.352 Dialization of serum

Foetal Calf Serum (FCS) was dialysed for 48h against 50 volumes of constantly stirred deionised water at $4^{o}C$ with a change of water every 8h. Visking dialysis tubing (Scientific Instruments Centre, London) was boiled and soaked in several changes of glass-distilled water to remove impurities before use.

2.353 Heat-inactivation of serum

Serum was inactivated at $56^{o}C$ twice for 45 min.

2.360 ADDITIONAL SUPPLEMENTARY SOLUTIONS

2.361 Glutamine

0.2M glutamine was prepared as a stock solution and stored at -20°C. Just before use it was diluted 1:100 in MEM.

2.362 Penicillin/Streptomycin (P/S)

10,000U/ml	Penicillin
1%	Streptomycin

Made up w/v in distilled water and sterilised by millipore filtration using G.S. membranes (0.22μ). Immediately prior to use, it was diluted 1:100 with MEM.

2.363 HEPES

2.5M HEPES (N-2-Hydroxyethylpiperazine - N-2-ethanesulphonic Acid) was made in distilled water (pH 8.1) and diluted in medium (1:100) before use.

2.364 Hormones and Growth Factors

were obtained as follows:

Oestradiol-17 β	Sigma, London
Progesterone	Sigma, London
Cortisol	Sigma, London
Dexamethasone	Sigma, London
Tamoxifen	ICI, England
Medroxyprogesterone Acetate (MPA)	Upjohn Ltd., U.S.A.
Insulin	Sigma, London
Transferrin	Sigma, London
Collagen type VI	Sigma, London

Rat tail collagen was kindly provided by

Dr.John Lackie, Dept. of Cell Biology, Glasgow University

Fibronectin	B.R.L., England
E.G.F.	B.R.L., England
Gelatin	Sigma, London

Steroid hormones and related molecules (oestradiol-17 β, progesterone, cortisol, Tamoxifen, MPA and Dexamethasone) were dissolved and stored in absolute alcohol at 4°C (for a maximum of 3 months). Bovine insulin (1mg/ml) was made up in 0.1 N HCl; EGF (1μg/ml) and transferrin (1mg/ml) were prepared in PBS. A stock solution of fibronectin (1mg/ml) was made in urea; Gelatin and collagen were prepared in distilled water.

All these solutions were filter sterilised (0.22μ).

2.370 FIXATIVES

2.371 Formal Saline

w/v in distilled water	0.5%	NaCl
	1.5%	$Na_2 SO_4$
	0.4%	Formaldehyde

2.372 TCA

5% Trichloroacetic acid (w/v) in distilled water was prepared and stored at 4°C.

2.373 Glutaraldehyde

25% was obtained from TAAB Laboratories, England and diluted in Sorenson's phosphate buffer.

2.374 Osmium Tetraoxide

was obtained from TAAB Laboratories, England, and diluted to 1% in Sorenson's phosphate buffer.

2.380 STAINS

2.381 Giemsa

30gms giemsa were mixed with 1980ml glycerol, heated at 56°C for 2 hours and added to 1980ml methanol. After mixing, this solution stood at room temperature for 7 days and was then filtered through green's 904/2 filter paper.

2.382 Uranyl Acetate

1% uranyl acetate was dissolved in distilled water.

2.400 MISCELLANEOUS

Sterile disposable scalpels were obtained from Gillette Surgicals, England.

Filters for sterilisation (0.45μ, 0.22μ) and prefilters were obtained from Millipore, London.

Depex was obtained from Hopkin and Williams, Essex; Propylene Oxide and Araldite mixture from EM Scope Laboratories, Ashford, Kent and metalled grids from TAAB Laboratories, England.

NBD Phallacidin (7-Nitrobenz-2-oxa-1,3-Diazole Phallacidin) was obtained from Molecular Probes Inc., Texas.

Disposable plasticware was obtained from Sterilin, Teddington, England.

Glass microfibre filter discs and GF/C filters (2.5cm dia) were obtained from Whatman Ltd., England.

Glassware was washed and rinsed in glass distilled water and finally in deionised water. To sterilise, it was autoclaved at 15mm pressure for 20 min.

2.410 LIVE-STOCK

2.411 Rats

Immature (16-23 days old) female rats were Albino Wistar strain(Glasgow University Colony).

2.412 Human Tissue

Endometrial, normal and cancer tissues were obtained as currettings either at hysterectomy or during dilation and curettage (D & C) from the Department of Gynaecology at the Western Infirmary, Glasgow. Samples were collected under sterile conditions in BSS containing antibiotics and maintained on ice during transport to the laboratory.

2.500 METHODS IN CELL CULTURE

2.510 CELL SEPARATION

2.511 Isolation of rat uterine mixed cells

This was carried out by a modification of the method described by Williams and Gorski (1973). Uteri were excised from 16-23 day old rats, trimmed free from adhering fat and mesentry and, finally, minced with razor blades. The mince was incubated for 20 min at 37°C in KRB containing 2% trypsin and 0.0115M $CaCl_2$. Incubations were carried out in 25ml universal tubes at a tissue to volume ratio of 2 uteri/ml. Tubes were shaken constantly while submerged in a water bath. The tissue pieces were then centrifuged and washed twice in KRB at room temperature. The washed tissue pieces were resuspended in KRB containing 0.5% collagenase and 0.35% trypsin. After incubation at 37°C for 40 min, the tissue pieces were drawn into and slowly expelled from capillary pipettes. Repetition of this process several times in a 40 min period resulted in the nearly complete dissociation of the tissue pieces into individual cells. The resulting suspension was centrifuged and washed twice in KRB. The last wash contained trypsin inhibitor (100 μg/ml). Finally, the cell suspension was passed through a double layer of gauze to separate single cells from tissue pieces and cell clumps.

All centrifugations in the cell preparations were for 5 min at 200g.

2.512 Fractionation of uterine cell types

Epithelial cells were isolated from immature rat uteri by a slight modification of the method described by McCormack and Glasser (1980). The uteri were blotted, slit longitudinally, and placed into an ice-cold solution of trypsin (0.5%) and pancreatin (1.5%) in phosphate buffered saline without Ca^{++} and Mg^{++} (see Section 2.333). Uteri

(2 uteri/ml)) were incubated at 4°C for 1h. The universal tube containing the enzyme solution and uteri was then placed at room temperature for 1h. The whole mixture was then vortexed for 10 sec. The supernatant containing the epithelial cells was transferred through a double layer of gauze, to another conical universal tube containing 1/10 vol of stripped 4°C foetal calf serum. The uterine mince was washed twice with 5ml PBS-A, and the washes were added to the epithelial cell suspension. The suspension was then centrifuged for 5 min at 1000 rpm (200g).

2.513 Isolation of Human Endometrial Epithelial cells

Isolation was carried out as described by Kirk and Irwin (1980), with an additional separation procedure. Endometrial curettings were rinsed several times with BSS (containing antibiotics), to remove excess red blood cells. The tissue pieces were minced into cubes (approximately $1mm^3$) with razors. The minced sample was then digested with 50 volumes of collagenase solution (Section 2.355) overnight (15-20h) at room temperature (22°C) in $75cm^2$ Falcon flasks. Stromal clumps were reduced to single cells by vigorous shaking of the total digest which was then transferred to 25ml universal bottles. After settling under gravity for 5 min the top two thirds of the suspension (stromal-rich fraction) were removed with a pasteur pipette and the remaining third (the epithelial-rich fraction) was resuspended with fresh medium (MEM), the process being repeated another two times. The epithelial fraction was finally centrifuged (1000 rpm, 200g for 5 min) and the pellet was washed with MEM. Residual stromal cells were substantially removed from the epithelial structures by plating the partially purified epithelium in $75cm^2$ Falcon flasks in SCM containing 10% FCS at 37°C in a 95% air - 5% CO_2 atmosphere. Under these conditions the residual stromal cells were attached to the plastic within

45 min, leaving the epithelial structures floating in the medium. The floating cells were transferred to and grown in petri dishes and multiwell plates.

2.514 Epithelial Cell Preparation from Human Endometrial Cancer Tissue

This was performed by the same method (see Section 2.513) as for normal human endometrial cells.

2.520 CULTURE CONDITIONS

All primary cultures were incubated at 37°C in a humidified 95% air - 5% CO_2 atmosphere. Petri dishes, flasks or multiwell plates with or without glass coverslips were used. Eagle's MEM with Earle's salts was used for routine cultures. Penicillin (100U/ml), Streptomycin (100µg/ml), Fungizone (3µg/ml), Glutamine (2mM), and HEPES (25mM) were also included in the standard medium. In addition, foetal calf serum (FCS) at the concentration of 10% was included. The cells were allowed to settle down, attach to the substrate and start growth in the standard culture medium for the first 12h, after which medium was changed with the appropriate experimental media. 1ml (each well of a multiwell tray), 2ml (35mm petridish), 5ml (65mm petri dish), 5ml (25cm^2 flask) and 20ml (75cm^2 flask) volumes of fresh media were used.

2.521 Check for contamination

Samples of each batch of the medium and all other sterile solutions were tested for one week in brain/heart infusion broth at 37°C.

Monolayer primary cultures were checked in routine tests (at the Wellcome Cell Culture Unit) for contamination by mycoplasma using a staining (Paul, 1970) method.

2.522 Determination of cell viability

Viability was tested by the method of exclusion dye staining with 0.05% trypan blue (Adams, 1980).

2.523 Subculturing

The medium was removed from the cells to be subcultured and they were washed with BSS. Trypsin/versene solution (Section 2.334) was then added to cover the layer of cells and removed after 15-20 sec exposure.

The same process was repeated once more. Then a small amount of trypsin/versene solution was added to the cells and their behaviour at 37°C was noted. When the cells were released from the surface of the flask, fresh medium was added. This resuspended the cells and stopped the action of trypsin. The suspension could then be counted and inoculated into different flasks as desired.

2.524 Growth Experiments

The cells isolated from either rat or human tissues were resuspended in SCM, the concentration of viable cells was determined (Section 2.511) and adjusted accordingly with SCM supplemented with 10% FCS. The whole suspension was divided equally into experimental dishes or wells. Unless otherwise indicated, 12h after plating the culture medium was replaced with fresh, experimental medium, which was renewed thereafter every 48h. When steroids were added the final concentration of ethanol in the media of both steroid-treated and control cultures was identical and was never in excess of 0.1%.

2.525 Serum-free cultures

Cells were grown, as described in Section 2.524, with whole serum (FCS) at 10% for the first 12 h. Cultures were then washed twice with serum-free medium (SCM) and maintained in this medium with added hormones and growth factors for another 48h.

2.526 Substrate coating

Fibronectin was prepared in urea (as described in Section 2.364). The concentration used ranged between 1-15 μg/ml. 0.5ml (to each well of a multiwell plate) and 1ml (35mm petri dish) of fibronectin solution (diluted in SCM) was added and incubated at 37°C for 30 min.

The fibronectin solution was evaporated to dryness and the dish was rinsed with SCM at least 3 times.

A thin layer of gelatin (1%) and rat tail collagen was placed inside the dish to be coated. After 20-30 min at room temperature, the solutions were evaporated and the substrates were washed thoroughly with SCM.

2.530 CELL HARVESTING

For routine harvesting, the method was based on using trypsin/versene solution (as described in Section 2.523). The cell suspension so gathered was divided appropriately to determine protein content and/or DNA content, radioactivity and/or cell number.

2.540 CELL FIXATION

2.541 Cell Fixing Using Methanol

Medium was removed from cells to be fixed and BSS was then added to cover the cells to remove any remaining medium. This was poured off, and a (1:1 v/v) mixture of BSS and methanol was added to the culture. After this was discarded methanol alone was added to cover the cells, and exposure maintained for 10 min at room temperature. Cells were finally air dried.

2.542 Cell Fixing Using Formal Saline

Medium was evaporated and the monolayer was washed first with BSS and then with formal saline (see Section 2.371). Cells were then fixed in fresh formal saline for 15 min at room temperature.

2.550 CELL STAINING

Giemsa stain (see Section 2.381) was poured onto fixed monolayers to cover the cells and allowed to stand for 1 min. This was then diluted with 10 volumes of tap water, and discarded after another 9 min. The cells were then washed thoroughly with water until no more stain could be detected in the washings.

2.560 ANTIBODY LABELLING

Cells were fixed for 20 sec in acetone. The coverslips were then covered with a 1/10 dilution of LE61 (Lane, 1982) and left at room temperature for 30 min. The coverslips were washed twice with PBS for 15 min. The coverslips were finally covered with a film of fluorescein-labelled anti-mouse IgG, developed in rabbit for another 30 min. After washing, the preparation was examined with a Leitz photomicroscope using U.V. illumination.

2.570 HISTOCHEMICAL ANALYSIS OF RECEPTOR

Histochemical assays of oestrogen and progesterone receptors in rat uterine mixed cells (in the form of pellets) were kindly performed by Professor L.P. Pertschuck, Department of Pathology, State University of New York. He employed fluorescein conjugated steroid molecules (Pertschuck et al., 1980). Oestrogen receptor in monolayered cells (on coverslips) was kindly cytochemically assayed by Dr.Shanthi Raam, Lemuel Shattuck Hospital, Boston, using a monoclonal antibody initially raised against human oestrogen receptor (Raam et al., 1981).

2.580 FLUORESCENT STAINING OF ACTIN

Cells grown on glass coverslips were thoroughly washed and fixed with formal saline (Section 2.542). After washing with PBS (x2) each coverlsip was incubated with 3 units of NBD phallacidin in 100 μl of PBS at room temperature for 30 min. The coverslips were washed and viewed through a fluorescent microscope.

2.600 MICROSCOPY

2.610 LIGHT MICROSCOPY

Fixed and stained cells were viewed through an Olympus EHT microscope.

2.611 Phase Contrast

Living cells were viewed by phase contrast microscopy using an Olympus IMT inverted research microscope equipped with an Olympus OM2 camera.

2.612 Fluorescent Microscopy

The cells labelled with fluorescein (FITC) conjugated compounds were examined in a Leitz photomicroscope using U.V. illumination. Appropriate filters to reduce background fluorescence were also used.

2.620 ELECTRON MICROSCOPY

2.621 Transmission Electron Microscopy (TEM)

Monolayered cells were processed for transmission electron microscopy by using the method described by Kuhn (1981).

Cells for this purpose were cultured in 35mm petri dishes. The cell monolayers were fixed in situ for 1h at room temperature with 2.5% glutaraldehyde (Section 2.373). After post-fixation in osmium tetraoxide (Section 2.374), the cells were dehydrated in graded ethanol. Immediately before the change to propylene oxide, 3.5mm squares were cut in the culture layers with a scalpel. After replacing the ethanol with propylene oxide, the culture vessels were gently shaken. Within seconds the cut-out culture squares detached from the dish as the propylene oxide dissolved the plastic material. They were immediately decanted and embedded in Araldite Mixture. These araldite blocks were polymerised at 60^{o}C for 24-48h. Ultrathin sections (Sco A^{o}) were cut down with glass knives using an LKB ultramicrotome and stained with uranyl acetate after being picked up on copper grids. These sections were then viewed in a Philips 301 electron microscope. The film used was Kodak electron microscopy film 4489.

2.622 Scanning Electron Microscopy (SEM)

Monolayered cells on glass coverslips were fixed with 2.5% glutaraldehyde (Section 2.373) for 1h at 4°C, 37°C or room temperature. After washing the coverslips with SPB, the cells were post fixed with osmium tetraoxide (Section 2.374) for 1h at room temperature. After washing the coverslips with SPB, cells were either freeze dried (drier made in the Department Zoology, Glasgow University) or dehydrated through graded alcohols (30, 50, 70, 90, 100%) and critical point dried (Polaron Equipment Ltd., England) from liquid CO_2. In both cases the dried samples were gold-sputter coated (Polaron Equipment Ltd., England) and examined in a Philips SEM500 scanning electron microscope at a specimen tilt angle of 33°. Film used for scanning electron microscopy was Ilford HP4.

2.700 QUANTITATIVE PROCEDURES

2.710 CELL COUNTING

2.711 Visual

Cultures were harvested as described in Section 2.530, resuspended in SCM by gentle pipetting to obtain a monodispersed suspension and the concentration of cells determined using a haemocytometer under phase contrast microscopy. A minimum of 200 cells were counted for each determination.

Number of colonies, number of cells per colony and number of radio-labelled cells in fixed monolayers were also counted visually through an ordinary microscope (Olympus VHT).

2.712 Electronic

Cultures were harvested by a routine method described earlier (see Section 2.350). The cell suspensions (100 µl) were diluted with 10 ml counting fluid (see Section 2.250) and three successive counts made on

each sample using a Coulter Model Electronic Cell Counter (Coulter Electronic Ltd., England).

2.713 Cell Spreading

The medium was aspirated and the cultures on coverlips were washed twice with BSS to remove unattached cells. Each coverslip was then viewed by phase contrast microscopy and at least five randomly selected areas per coverlip were evaluated. Fully spread cells with clearly visible nuclei were counted in these areas.

2.720 CELL FRACTIONATION

2.721 Sonication

Cell suspensions in TCA (10%) or water were sonicated using a microprobe - Soniprobe type 7532A (Dawe Instruments, England) at setting 4, with 100 watts for 2x5 sec bursts with a gap between bursts to allow the sample to cool down.

2.730 ^{3}H-THYMIDINE INCORPORATION

2.731 Autoradiography

For autoradiographic analysis cells were cultured in multiwell plates containing 13mm round glass coverslips. Medium was replaced with a fresh one containing ^{3}H-methyl thymidine (2μCi/ml) for the last 3h. The medium was removed and after thorough washing the cells were fixed with formal saline for 30 min at room temperature. After washing with distilled water, cells were extracted twice with distilled water and, finally, with ethanol and dried. Coverslips were mounted (cells uppermost) with DPX Mountant (BDH), onto microsope slides. These slides, with coverslips attached, were dipped in gelatin/chrome alum (see Section 2.270) and dried at room temperature. They were then either covered with AR10 fine grain autoradiographic stripping film or dipped in diluted K2 emulsion. The slides were then dried in a steam of cold air and placed in a light-tight box containing silica gel and maintained at

4°C. After 4 days exposure, the film was developed in D19 developer for 5 min, washed with water, fixed with 20% Amfix for 4 min, washed again in running tap water and the cells stained with 5% Giemsa for 30-40 sec. Finally samples were destained in tap water and air dried. A second coverslip was mounted on top with DPX. Labelling indices were determined by counting labelled and unlabelled nuclei in randomly selected microscope fields. At least 1000 nuclei were counted in each culture.

2.732 Measurement of Radioactivity

Unless otherwise indicated for pulse-labelling experiments, cells cultured in 65 mm dishes or in $25cm^2$ flasks were labelled by replacing the culture medium with fresh but similar medium containing 1 μCi/ml ^{3}H-methyl thymidine, and the cultures returned to the incubator. After 2h the medium was removed and the cultures washed twice with cold PBS. The cells were harvested (see Section 2.530) and cell pellets collected by centrifugation (200g/5 min). Cell pellets were suspended in ice cold 5% TCA and fractionated as described in Section 2,721. Aliquots were then used for the determination of acid soluble and acid insoluble counts, together with protein and DNA contents.

Acid-soluble radioactivity was extracted with 5% TCA at 4°C (30 min). Under these conditions the acid extract was pooled and the radioactivity measured in neutralised aliquots by liquid scintillation (Irwin, 1982). Aliquots (500 μl) were added to 10ml Toluene PPO (5g/l) scintillant and counted at 30% efficiency in a Searle Mark III Liquid Scintillation Counter.

Another aliquot was incubated with 10% TCA for 30 min at 4°C. Acid insoluble counts were retained after repeated washings on 0.45 m millipore filters. After drying, the filters (GF/C) were solubilised in Toluene PPO (5g/l) and counted at 30% efficiency in a Searle Mark III Liquid Scintillation Analyser (Lippman et al., 1976).

2.740 PROTEIN ESTIMATION

An aliquot of the suspension to be assayed was adjusted where necessary to a final volume of 0.5ml with distilled water. Protein was assayed by the method of Lowry et al., (1951) using BSA as a standard.

2.750 DNA DETERMINATION

A slightly modified micro-assay (Brunk et al., 1979) was used to determine DNA quantities. A crude cell lysate (sonicated) was diluted (upto 1ml) in buffered saline (see Section 2.260) and sonicated twice for 5 sec at the lowest level. The homogenate was incubated with RNAse (10μg/ml) at 37^{o}C for 30 min to inhibit any influence of RNA present in the homogenate. Then a fluorescent stain bisbenzimidazole (Hoechst H33258), at a concentration of 100mg/ml, was added and the total volume was adjusted to 3ml. The samples were excited at a wave length of 365nm and the fluorescence emitted was read at 450nm using a Hitachi (MPF-2A) fluorescence spectrophotometer. The intensity of fluorescence from each sample was compared with the fluorescence produced by standard quantities of calf thymus DNA.

2.760 MEASUREMENT OF STEROIDS IN SERUM

The concentrations of steroid hormones in FCS, DCC-FCS, heat-inactivated and dialysed serum were measured by radioimmunoassay and were kindly performed at Glasgow Royal Infirmary by Dr.G.Beastall using the methods described by Cook et al. (1977).

2.800 STATISTICAL METHODS

Statistical parameters of mean ($\bar{x}$) and standard deviation (SD) were computed using a scientific calculator (Texas Instruments). Where indicated the differences between control and experimental groups were tested for statistical significance using students 't' test. The difference of $P < 0.05$ was considered significant.

CHAPTER 3 - ISOLATION AND CHARACTERIZATION OF UTERINE CELLS IN PRIMARY CULTURE

3.000 INTRODUCTION

One of the earliest reports of human endometrium in culture appeared in 1928, when Traut observed in endometrial cultures an outgrowth of fibroblastic cells, which he interpreted as stromal elements (Traut, 1928). The following thirty years produced much data (Hirsch and Jones, 1933; Randall et al., 1950; Papanicolaou and Maddi, 1958) on endometrial cells with two distinct morphologies, interpreted as having an epithelial or stromal origin. Moore (1956) was the first to describe three different cell types in endometrial outgrowths. Apart from the epithelioid cells arranged in sheets and the elongated fibroblastic cells, he observed the outgrowths of polygonal cells which appeared clearly different from the epithelial cells and, when fixed and stained, resembled the cells in the stroma. However, he pointed out limitations in trying to establish the nature of the cultured cells based only on their morphology in the light microscope.

Figge (1960) was the first to demonstrate specific products histochemically to try to establish the origin of cultured endometrial cells. Figge's conclusions lacked the support of convincing experimental evidence, but his work shows the merit of using biochemical markers to characterise endometrial cell cultures.

It is clear from the literature cited that morphological heterogeneity in endometrial cultures was noticed by the early investigators and it became generally accepted that the polygonal cells growing in continuous sheets were likely to be derived from epithelial structures, while the spindle or stellate shaped 'fibroblastic' cells were derived from stroma. However, for a long time no evidence appeared which could define the nature of these two cell types and establish their origin. Moreover, recent studies using human endometrial cultures (Pavlik and Katzenellenbogen, 1978) and rat endometrial cells (Pietras

and Szego, 1973; Sananes et al., 1978; Halme et al., 1980) have ignored the problem of cell identity.

Recently, Liszcazk et al. (1977) demonstrated ultrastructural similarities between cells with epithelioid morphology in endometrial primary cultures and the epithelial cells in the intact tissue. The morphological characteristics common to both included junctional complexes and surface microvilli. Such characteristics in cultured cells can perhaps now be used to justify the epithelial origin (Wynn, 1977).

Early work by Heald et al. (1975) demonstrated a method for the preparation of epithelial and stromal cells from rat uterus. Later, Bitton-Casimiri et al. (1977) isolated epithelial cells from rat uterus by trypsinisation and cultured them successfully.

Kirk et al. (1978) reported for the first time the separation of human endometrial tissue into the epithelial and stromal components. They differentiated the two components on the basis of morphological criteria. Later, Satyaswaroop et al. (1979) reported similar ultrastructural findings in primary cultures derived from isolated endometrial glands. The findings of Kirk et al. (1978) regarding the morphological characteristics of both cell types were confirmed in pure epithelial and stromal cultures by Trent et al. (1980), who showed, in addition, ultrastructural similarities between cultured stromal cells and the reticular cells in the stroma of intact endometrium. A number of other investigators have tried this approach and related morphological features of cultured epithelial cells from rat endometrium (Vazquez-Nin et al., 1979; Echeverria et al., 1980) and from rabbit endometrium (Gerschenson et al., 1974, 1979; Ricketts et al., 1983).

Despite these facts, the precise origin of cultured endometrial epithelial cells is still difficult to establish. This is principally

because no specific biochemical or cytochemical markers have been described, which permit absolute recognition of either individual cell types or their interactions. The work to be described in this chapter attempts to characterize cultures derived from rat and human, normal and cancer epithelium, using morphological and immunocytochemical criteria.

3.100 PRODUCTION OF CELLS FROM INTACT TISSUES

The cell suspensions needed to initiate primary cultures were obtained by dispersing cells from the parent tissue using various techniques.

3.110 RAT UTERUS

3.111 Mixed cells

The procedure described in Section 2.511 gave reasonable yields of free cells from immature rat uterus with a minimum of experimental manipulations. More than 90% of the dissociated cells existed as individual cells with large prominent nuclei (Fig. 3.1a). The remainder of the cells were found in clumps of 2-10 cells. The dispersion of the cells from large clumps by repeated pipetting in the presence of enzymes, was found extremely helpful with minimum loss of cell viability (checked both by dye exclusion (Section 2.522) and by growing ability). The use of trypsin inhibitor during the final washes of the cell suspensions was found unnecessary. Serum at 10% was enough to antagonise the residual enzyme activity.

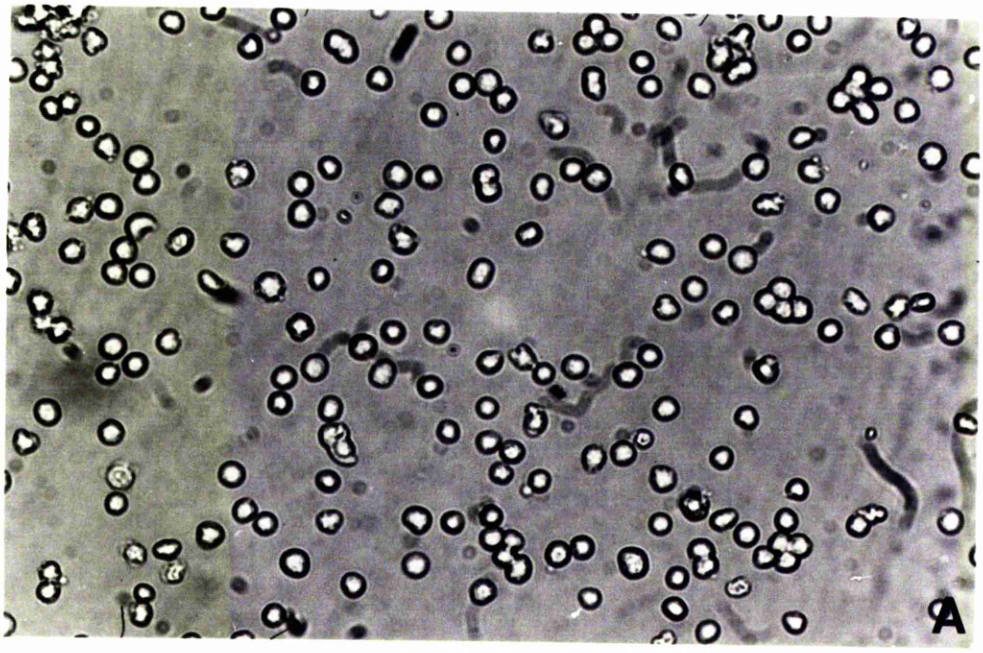
3.112 Epithelial cells

Epithelial cells separated by the method described in Section 2.512 were examined for purity and damage using phase contrast microscopy. Such preparations contained insignificant contamination by other cell types. As is seen in Figure 3.1b, the suspension contained mainly intact glands. The epithelial cells, immediately after isolation, were capable of prompt attachment and growth. Isolated

Figure 3.1: Isolated uterine cell types

a) Immature rat uterine mixed cell population. This is a preparation from whole uteri at the end of the enzymatic dispersion (see Section 2.511). Note the cells are, in the main, single cells rather than clumps. (Phase contrast x 220).

b) Glandular epithelium separated from the endometrium of the immature rat (see Section 2.512). Note that intact glands and a few single cells (Phase contrast x 100).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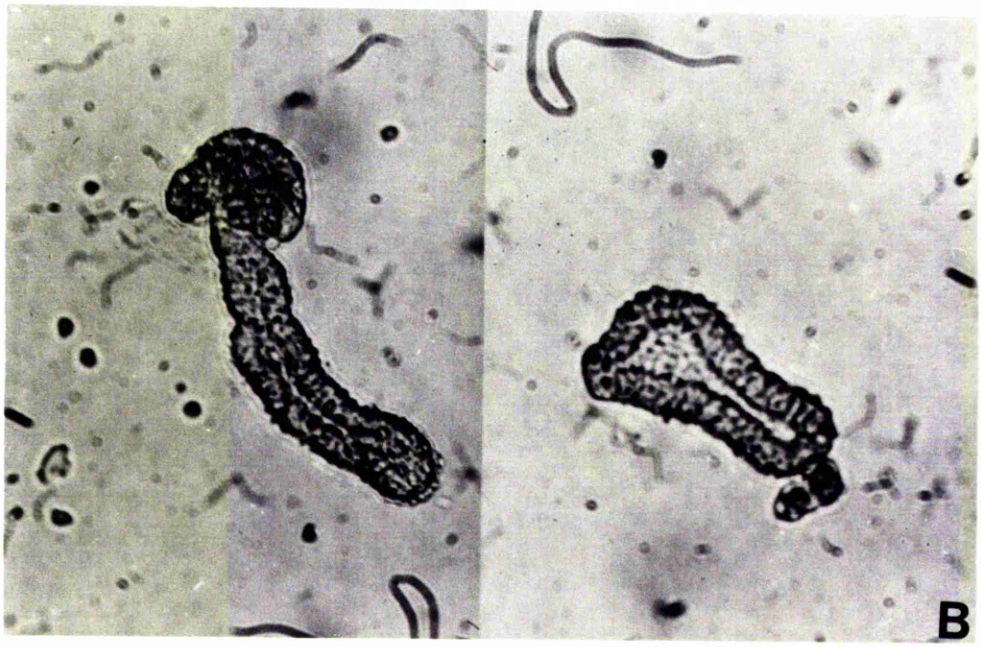
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single epithelial cells and a very few stromal cells also settled down and spread on the substrate. Growth of stromal cells was, at least partially, limited by controlling serum factors (details in Chapter 4).

3.120 HUMAN ENDOMETRIUM

3.121 Epithelial cells

The separation procedure, outlined in Section 2.513, involved enzymatic dissociation of the tissue and subsequent fractionation of the components on the basis of size. The size of minced endometrial tissue pieces was found to be critical, in that, fragments smaller than $1mm^3$ were further broken down into single cells which were difficult to separate from single stromal cells. This method was found optimal for collagenase digestion and produced cleanly separated recognisable glandular structures. Digestion of proliferative endometrium was easier and gave greater cell viability than that of endometrium from the secretory phase.

3.122 Human endometrial cancer cells

The same method as described for the isolation of normal cells (Section 2.513) was used successfully for cancer cells. No significant difference was observed in yield of viable cells after incubating tissue fragments in collagenase for 2 or 3 days.

3.200 CHARACTERISTICS OF PRIMARY CULTURES

3.210 CELLULAR BEHAVIOUR IN EARLY CULTURES

3.211 Cell attachment

Most of the cells in mixed cell populations were stromal and fibroblastic in nature. The process of attachment started immediately after plating in optimum culture conditions (Section 2.520; Fig. 3.2e). Within 12-24 hours after seeding, almost all viable cells were observed to have settled down and spread (Fig. 3.2 a-d). The epithelial cell fraction from rat uterus was observed to complete the attachment process

Figure 3.2: Attachment and early growth of rat uterine mixed cell populations (Fibroblasts)

a) Fibroblasts after 3h in culutre

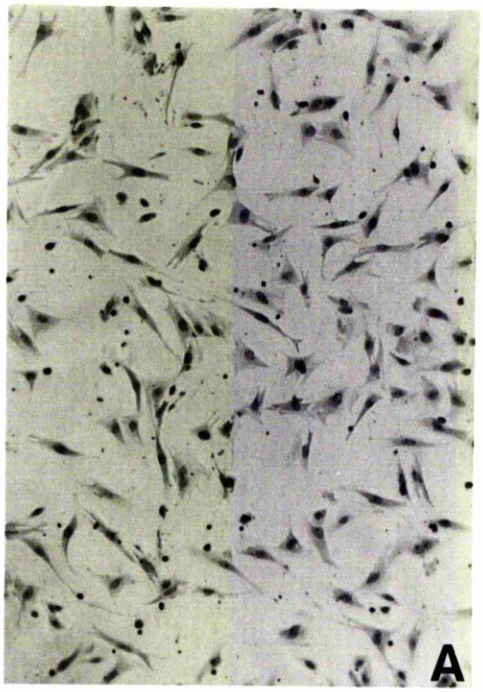
b) Fibroblasts after 6h in culture showing increased spreading

c) The same culture after 12h of incubation

d) The same culture photographed at 24h

(a-d) Cells were fixed with methanol (see Section 2.541) and stained with Giemsa (see Section 2.550) (a-d) x 80

e) Fibroblastic cells in the process of attachment, 45 min after suspension in culture medium (Phase contrast x 180).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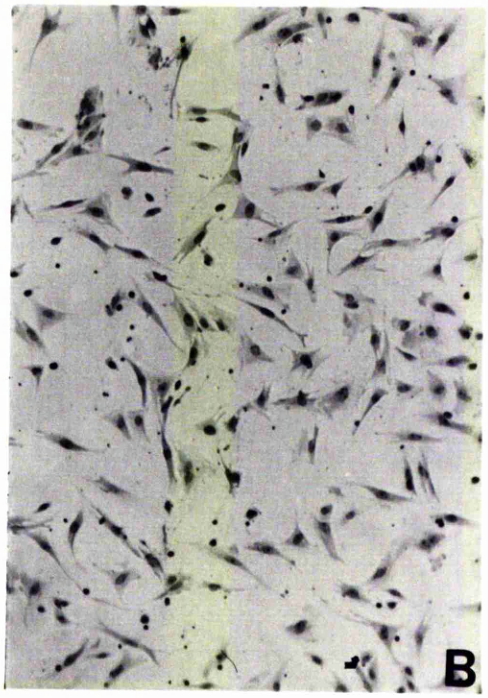
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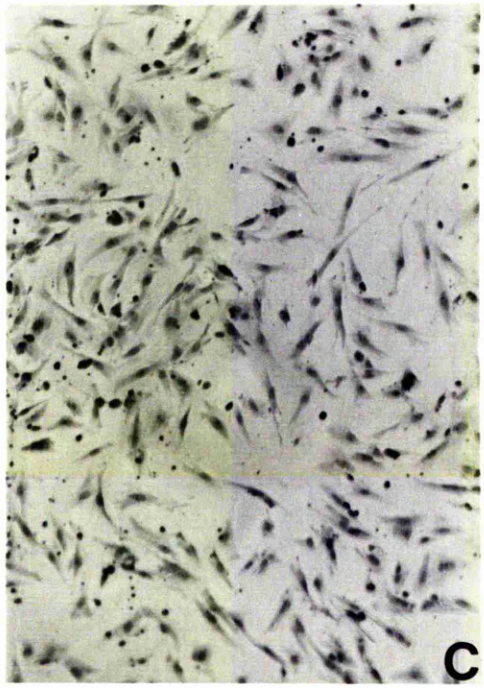
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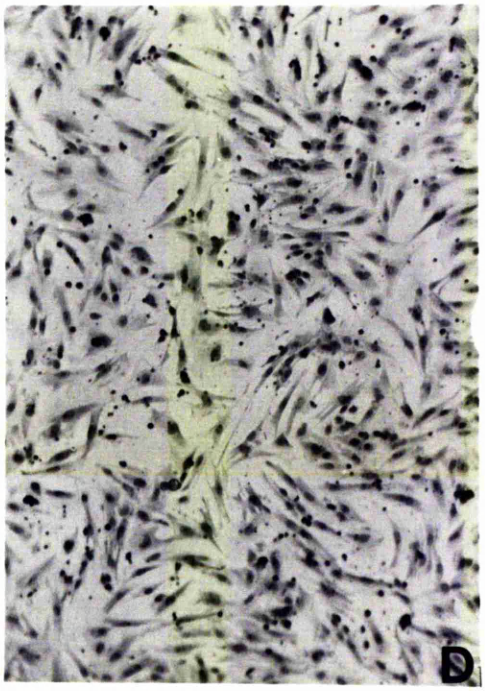
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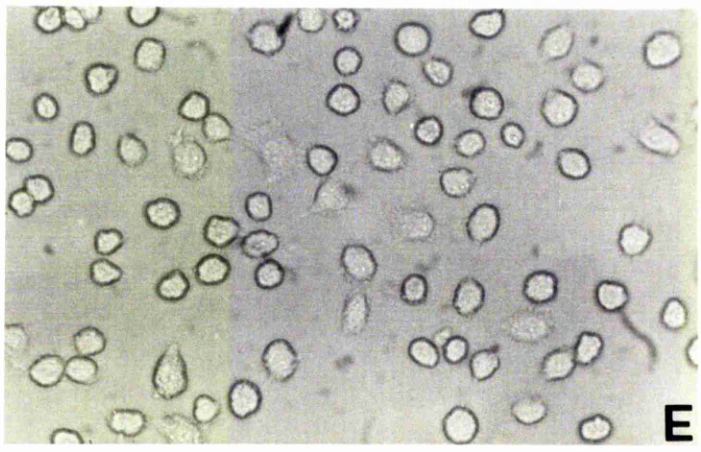
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within 6-8 hours (Fig. 3.3 a-d), though a few epithelial fragments continued to settle down until the end of the first day. Human endometrial normal and cancer epithelial cells were found to take longer to attach to the substratum. The human cells did not complete the process until 24h after initial plating. In these cells, the first medium was not changed until either 24h had elapsed or the apparent end of the attachment period. Occasionally the cells attached to the substratum but did not grow.

3.212 Cell growth

Mixed rat uterine cells were found to be actually dividing during the latter part of the second day of culture. DNA synthesis was also observed by autoradiography, as early as the second day of incubation (data not shown). At the end of the third day, the substrate was partially covered with a population of well spread, fibroblastic-like cells (Fig. 3.4a), frequently showing mitotic figures. After three days in culture, the cells were larger and much more numerous. Very little intercellular space was left (Fig. 3.4b). Many cells were binucleated. There was no contact inhibition and several layers of cells were overlapping (Fig. 3.4c). These fibroblasts formed a sheath of cells after 4-7 days (depending upon initial plating density). The sheath of cells contained monolayered parts and colonies of epithelial cells (Fig. 3.4d). These epithelial colonies were preserved throughout the first passage. On rare occasions, these were also seen in the second passage.

The epithelial cell population from immature rat endometrium was capable of prompt growth after attachment. These cells displayed a consistent and characteristic morphology and outgrowth resulted in typical epithelial colonies. Figure 3.4 (a,b,c) represents the gradual development of one epithelial colony with the lapse of time. Epithelial

Figure 3.3: Colony formation from immature rat endometrial epithelial cells

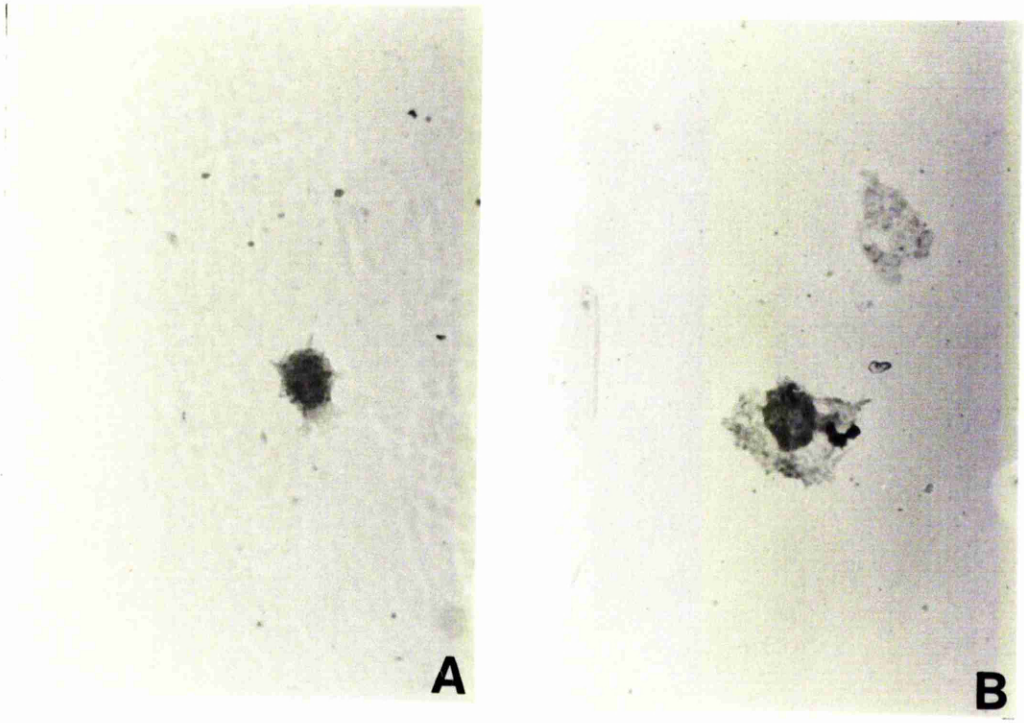
a) Attached piece of glandular epithelium after 2h in culture x 32

b) Glandular epithelium after 4h of incubation x 32

c) A growing epithelial colony after 6h of culture x 160

d) A well grown colony of epithelial cells at the end of the first day x 80

Cells were fixed with methanol (see Section 2.541) and stained with Giemsa (see Section 2.550) after appropriate timings.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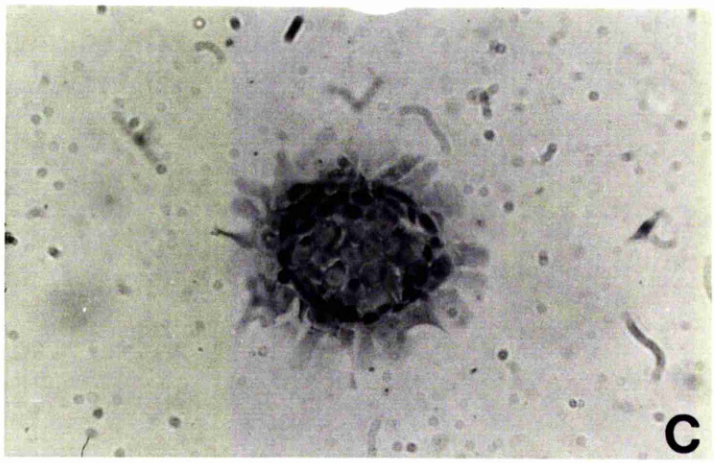
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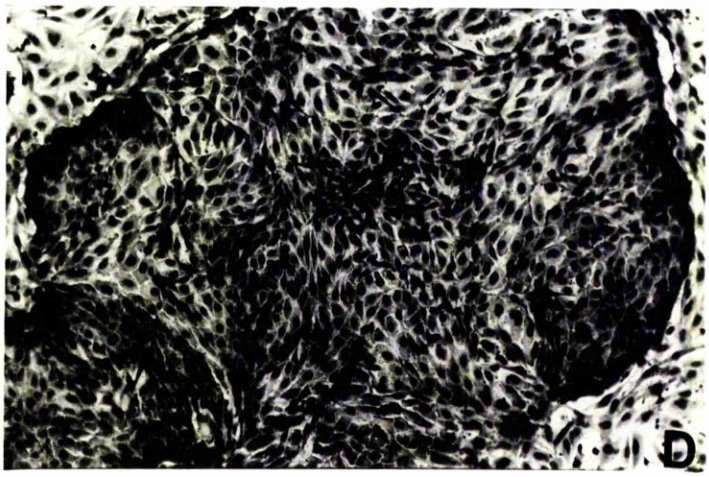
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Figure 3.4: Growth pattern of rat uterine mixed cells

a) Fibroblast-like cells during the third day in culture x 80

b) Fibroblasts growing after 3 days

Note partial monolayer x 80

c) Fibroblasts overlapping each other x 250

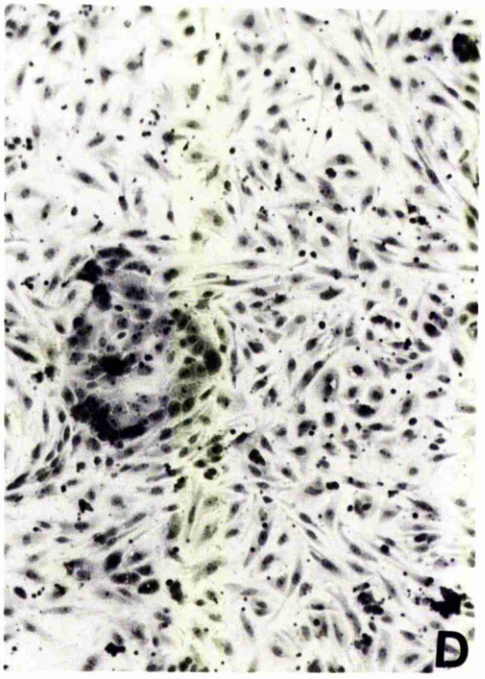
d) Epithelial cell colony growing amongst fibroblasts x 80

Cells were fixed with methanol (see Section 2.541) and stained with Giemsa (see Section 2.550)


A


B


C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cell outgrowth started as soon as the fragments settled down. Very small colonies from clumps can be observed after 2-3 h of seeding. Isolated viable cells flattened more rapidly than those in groups or colonies.

At the start of growth, the epithelial cells exhibited at least 4-5 doublings during the first day of culture. This rate of division was reduced to 2-3 doublings during the second day, which is still more rapid than in vivo division. Roughly, only one division was observed during the third day. At least three types of cells showing epithelial characteristics were observed during the second day in culture (Fig. 3.5 a,b). Each type showed different growth behaviour and morphology. Multinucleated cells and sometimes extra-large cells were also observed (Fig. 3.6a). Multinucleated cells were present even in early cultures (6h) and they frequently occupied the central part of individual colonies. In most cases, the cells with more than one nucleus were found to be polygonal, whereas the cells at the edges of the colony were spindle shaped, elongated, and sworling towards the centre of the colony (Fig. 3.3). Extraordinarily large cells were only noticed after the first day (Fig. 3.6a). Large vacuolated cells were seen after a week in culture (Fig. 3.6c). These cells were cultured for a maximum of two weeks, after which degeneration was observed (Fig. 3.6b). However the experiments described in this study were performed within the first week after plating.

In the case of human normal and cancer cells, after initial plating, the epithelial glands attached to the substrate and cells began to migrate from them after 12-24h. The plating efficiency was variable and seemed to depend on the integrity of the glands immediately after digestion. At least 3 types of explants were again seen in the culture from normal tissue (Fig. 3.7a,b). Explants originating from normal

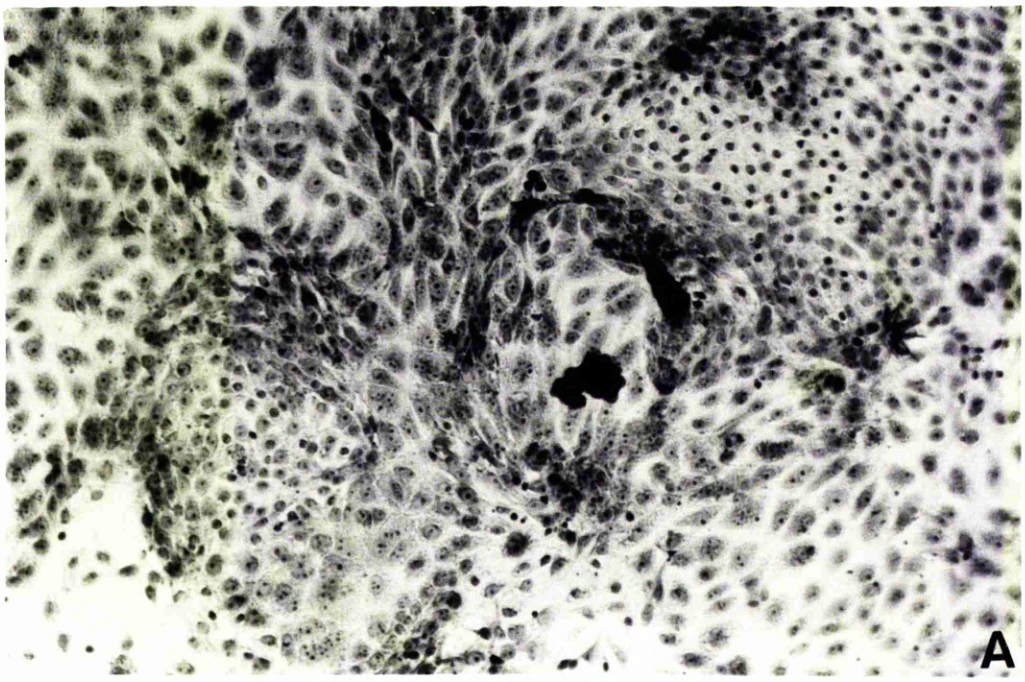
Figure 3.5: Distinct epithelial cell types from rat endometrium

Endometrial epithelial cells were isolated as described in Section 2.512 and established in culture for 3 days. Colonies as appear are of different types

a) Comparatively homogeneous cell population in each colony and colonies of different cell types, as can be seen, are growing close to each other x 160

b) Two different epithelial cell populations (at least morphologically) in the same colony x 160

Cells were fixed with methanol (see Section 2.541) and stained with Giemsa (see Section 2.550)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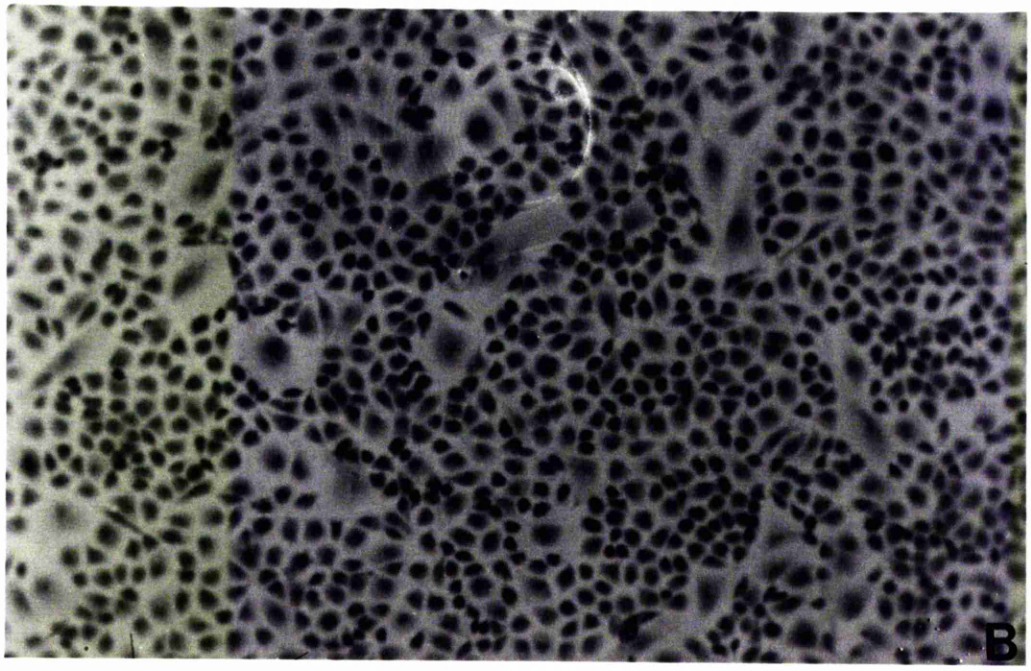
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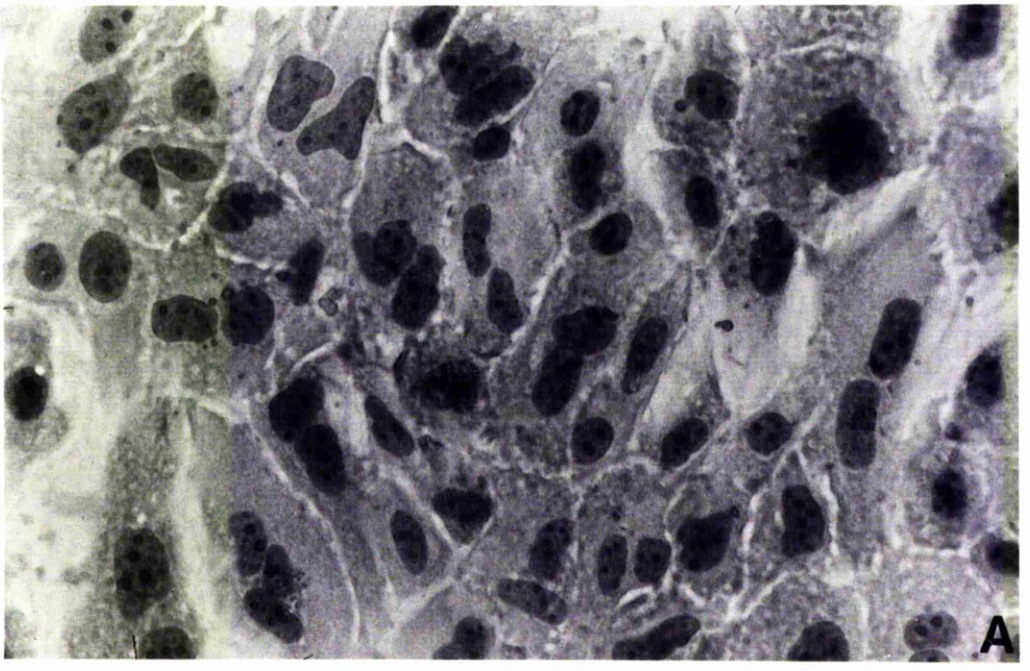
Figure 3.6: Growth behaviour of rat endometrial epithelial cells

Epithelial cell cultures were established from rat endometrium as mentioned before (see Section 2.512, 2.520). Cells with the following characteristics could usually be observed within a colony

a) Multinucleated cells scattered among monolayered cells x 230

b) Endometrial epithelial cells degenerating after two weeks in culture x 250

c) Epithelial cells with large vacules x 250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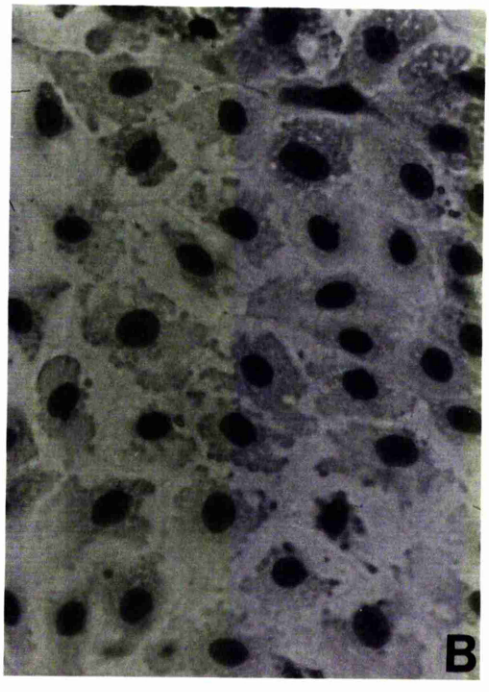
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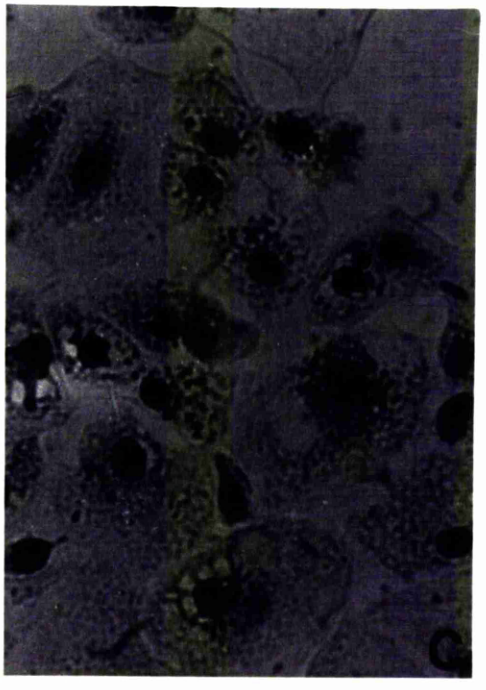
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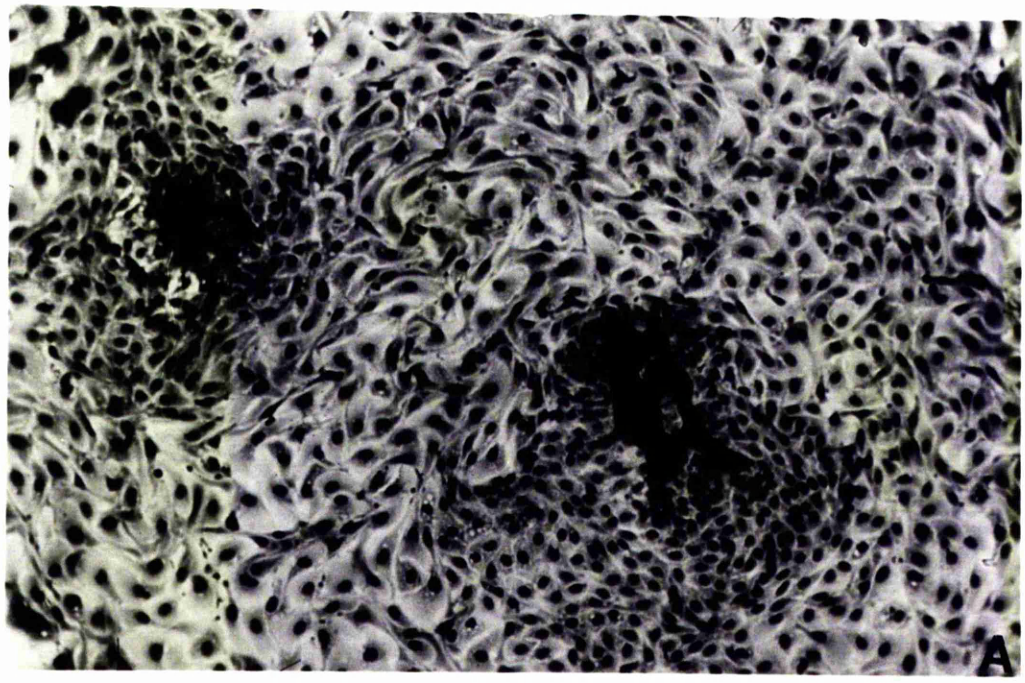
Figure 3.7: Human endometrial epithelial cells in primary culture

a) Epithelial cells from normal human endometrium showing the various morphologies within a single culture x 120

b) Colonies growing out from glandular tissue. Note the sworling behaviour of the cells x 120

Cells were fixed with methanol (see Section 2.541) and stained with Giemsa (see Section 2.550)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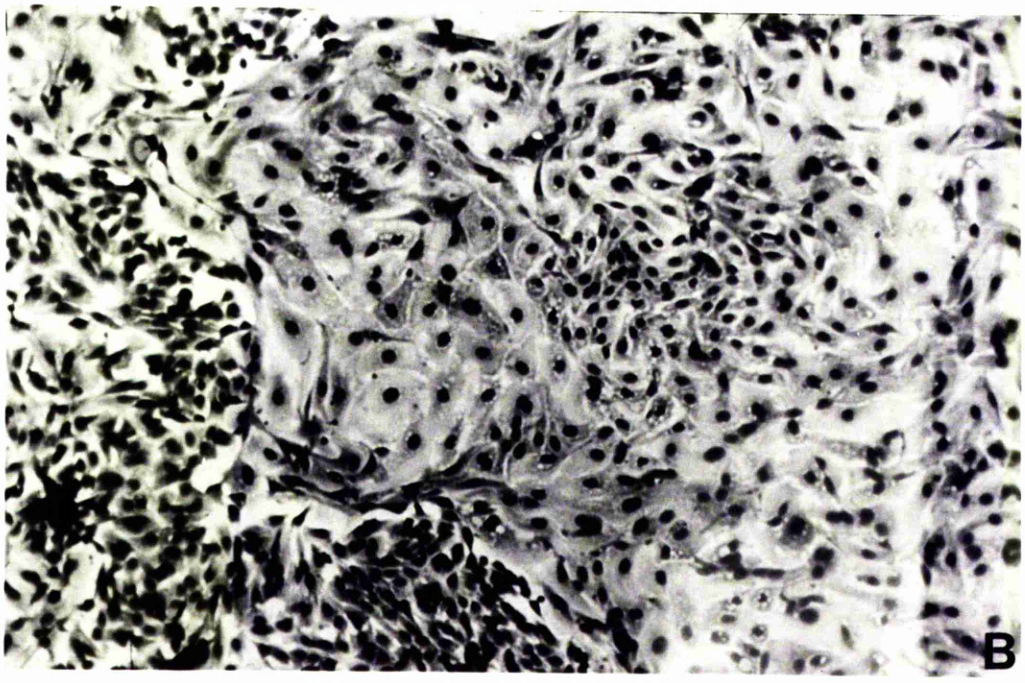
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epithelium consisted of colonies of tightly packed polygonal or 'tadpole' shaped cells that grew in a characteristic sworling appearance. In successful cultures heavy growth was seen by the end of a week and consisted of scattered fusiform cells together with confluent colonies of polygonal cells, characterised by prominent nuclei and a tendency to curl around each other. Colonies of normal epithelial cells continued to spread and grow for upto 10-12 days when degeneration occurred, whereas cancer cells showed a slow rate of growth and colonies survived at least 3-4 weeks. At the end of the second week, colonies of epithelial cells (Fig. 3.8a,b) were clearly seen. Again among cancer cell colonies, polyhederal cells appeared to contain more than one nucleus (Fig. 3.8c).

The percentage of cells recovered from cultures depended upon the density of plating, the time in culture, the physiological state of the cells and other conditions.

3.213 Subcultures

Mixed cell populations from rat uterus were subcultured for upto six passages. The cells maintained their fibroblastic morphology.

Pure epithelial cultures derived from rat uterus were found to be extremely difficult to subculture. Epithelial cell morphology was rarely seen after passaging (Fig. 3.9a). Human normal epithelial cells also became fibroblastic in appearance after subculturing (Fig. 3.9b). There was no suggestion that epithelioid morphology re-emerged even after a few passages. It is, however, not possible to say whether epithelial cells acquire fibroblastic morphology on passaging or small numbers of fibroblasts are carried through the primary culture and become very active after first passaging. Once again, a good biochemical marker exclusive to either fibroblasts or epithelial cells is needed.

3.220 GENERAL MORPHOLOGY

Two main cell types were studied. One was fibroblast-like,

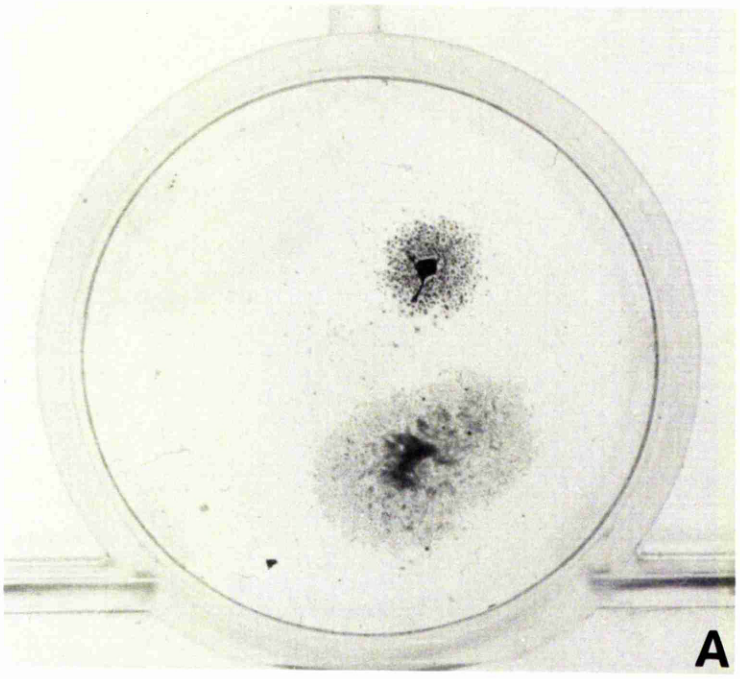
Figure 3.8: Human endometrial cancer cells in primary culture

a) Colonies of epithelial cells grown from small pieces of tissue x 4

b) Human endometrial cancer cells growing in monolayer. Cells prepared by the method (see Section 2.514) described for normal endometrium x 160

c) Cancer cells with different morphologies. Note the multinucleated cells within a colony x 250

Cells were fixed with methanol (see Section 2.541) and staind with Giemsa (see Section 2.550)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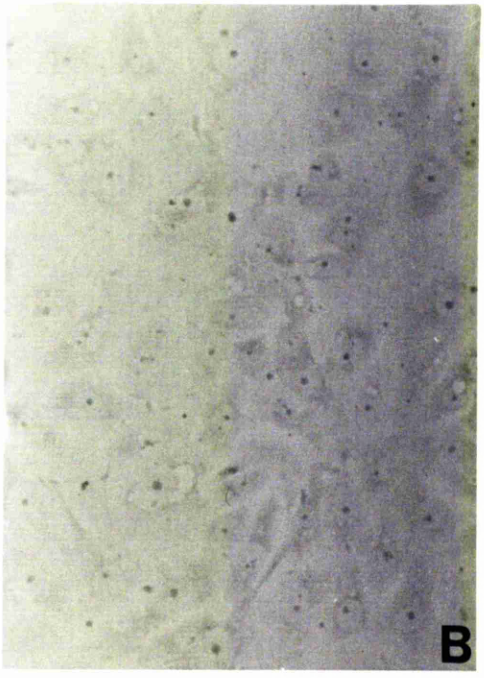
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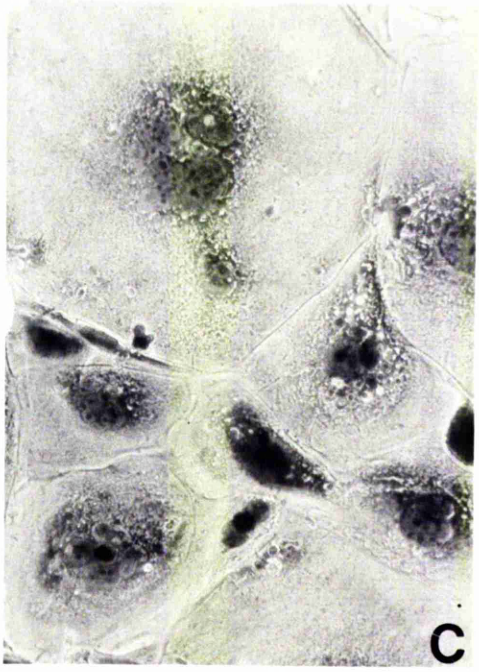
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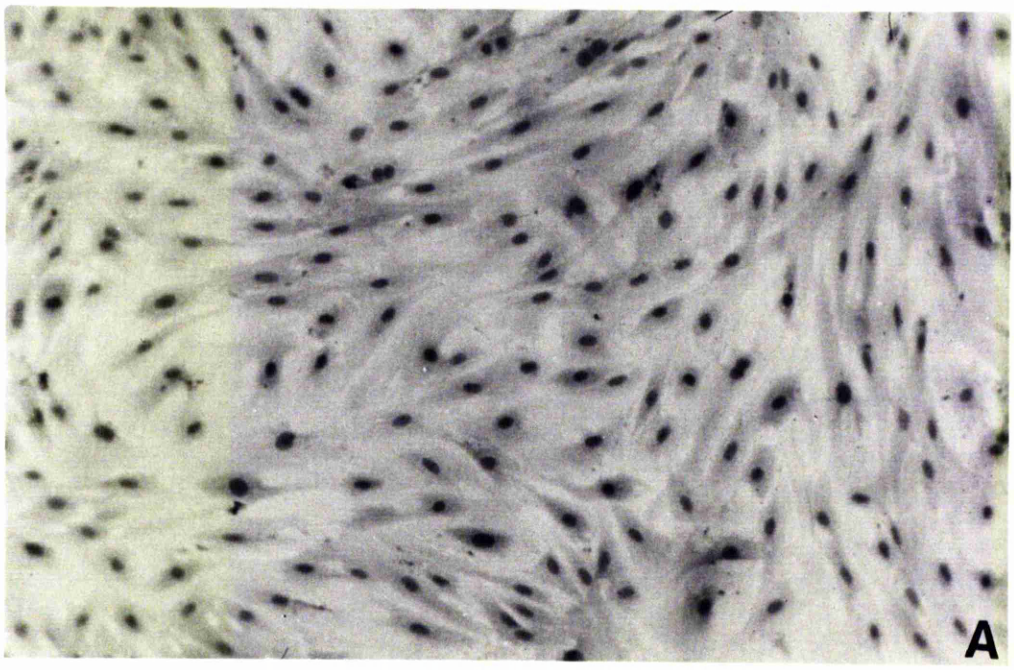
Figure 3.9: Endometrial cells in secondary culture

a) Rat endometrial epithelial cell population in second passage. Note fibroblastic appearance x 120

b) Human endometrial epithelial (normal) cells in second passage. Note again the fibroblastic shape x 120

Cells were fixed with methanol (see Section 2.541) and stained with Giemsa (see Section 2.550)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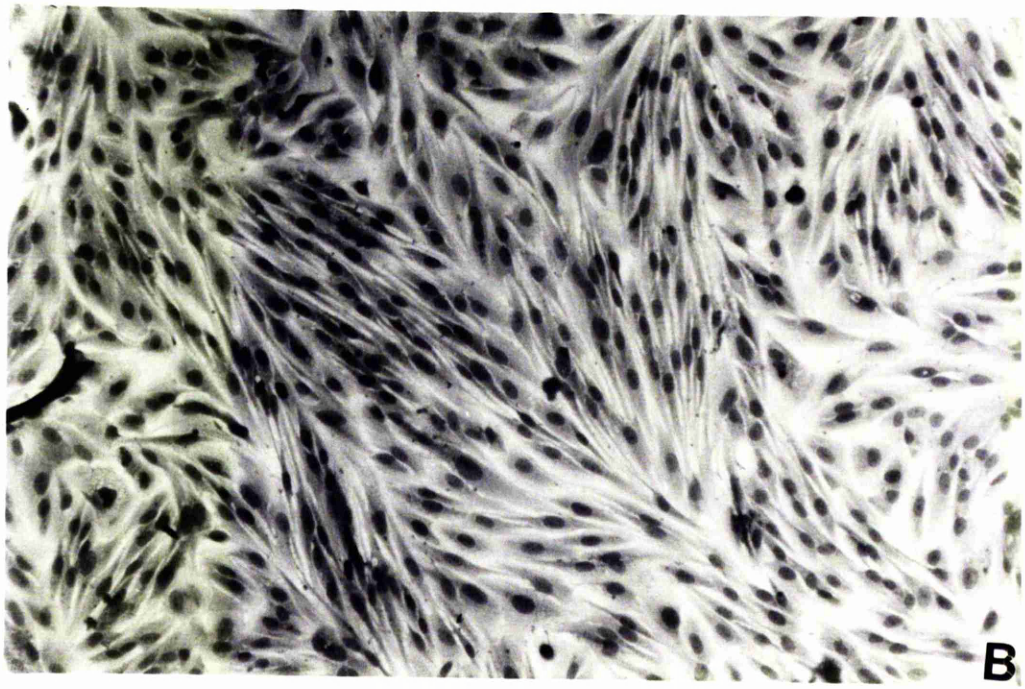
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large, elongated, spindle-shaped and with very little intercellular space (Fig. 3.4b). No contact inhibition was observed and several layers of cells were overlapping (Fig. 3.4c). These fibroblasts grew in an uncontrolled manner without clearly defined colony areas.

The second type, epithelial-like cells, are ovoid or cuboidal, some appeared vacuolated. They showed contact inhibition - growing in a defined colony. Abundant binucleated, sometimes multinucleated and multinucleolated cells were seen in both these types. Cell size in both these types varied enormously ranging between 1-15μm.

Maintenance of typical epithelial morphology during attempts to passage epithelial cells by replating after trypsinisation proved unsuccessful. Spindle-shaped fibroblastic morphology was retained for successive passages of cells grown from the initial mixed cell populations.

3.230 COLONY FORMATION

Immediately after attachment, clumps and groups of epithelial cells gave colonies of cells with prominent nucleoli, sworling around each other. Single cells were also noticed to initiate colony formation. A sequential development of a colony is shown in Fig. 3.3. The internal cells of the colony were polyhed ral in shape, but those situated in the periphery or lining the internal cavity possessed a columnar and elongated shape.

3.240 ULTRASTRUCTURE

Electron microscopy was used to study morphology of the cultured cells at ultrastructural level. The overall appearance of uterine cells in culture was consistent with morphological characteristics of the tissue in vivo (Noyes, 1973; Dallenbach-Hellweg, 1981).

3.241 Transmission Electron Microscopy (TEM)

Epithelial cultures, from both rat and human endometrium

possessed microvilli, scattered over most of the free surfaces of the cells (Figs. 3.10a; 3.13b). These microvilli contained a central core and surface glycoprotein strands (glycocalyx typical of epithelia; Fig. 3.11b). Epithelial type junctional complexes, with numerous desmosomes were well developed between adjacent cells, with the membranes interdigitating in complex patterns (Figs. 3.10a; 3.11a; 3.13a,b). Bundles of tonofilaments cross the cytoplasm (Fig. 3.10b). Epithelial cells had large nuclei with dispersed chromatin and one, or more, prominent nucleolus. Microtubules, mitochondria and lysosomes were abundant. A conspicuous Golgi apparatus was normally located in a perinuclear position. Lipid droplets were sparse in the cytoplasm. Free polyribosomes were numerous and rough endoplasmic reticulum (RER) was abundant. The well preserved nuclear membrane was frequently infolded and had many pores. Nucleoli were prominent and nuclear bodies were also sometimes seen.

The main difference between epithelial cells and fibroblasts was seen in the structure of the plasmalemma, which, in the fibroblastic cell lacked extensive microvilli (Fig. 3.12a) and in the type of junctional complexes between cells in contact. The fibroblast -like cells exhibited mesenchymal-type cell to cell junctions. Furthermore, the fibroblastic cells grew more often as multilayered sheaths on a thin basal lamina (Fig. 3.12a). Abundant collagen was also seen around the cells. Varying amounts of rough endoplasmic reticulum and numerous lysosomes occupied the cytoplasm. Unlike the epithelial cells that formed extensive contacts with the basal lamina, contact by fibroblasts was limited to a small area of the plasma membrane.

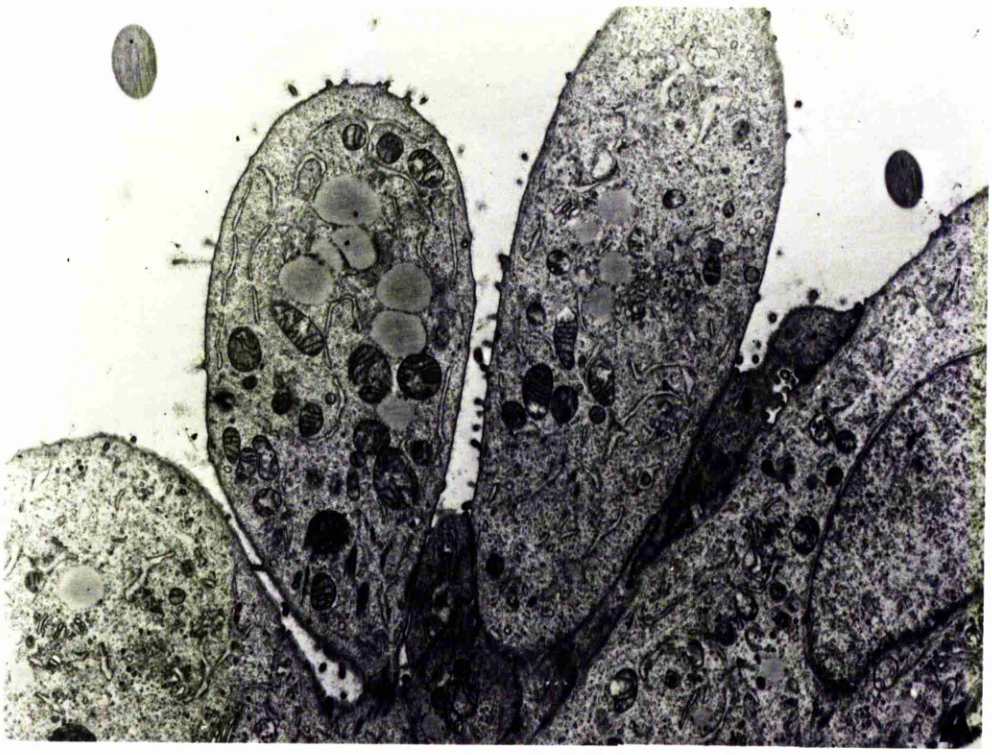
3.242 Scanning Electron Microscopy (SEM)

SEM of the migrating epithelial cells confirmed their flat morphology and also illustrated regular, well defined cell-to-cell

Figure 3.10: Transmission Electron Micorgraphs of rat endometrial epithelial cells

a) Epithelial cells in primary culture, showing microvilli on the free surfaces and junctions between two adjacent membranes x 7860

b) A cell from the same culture, showing tonofilaments running through the cytoplasm x 24400



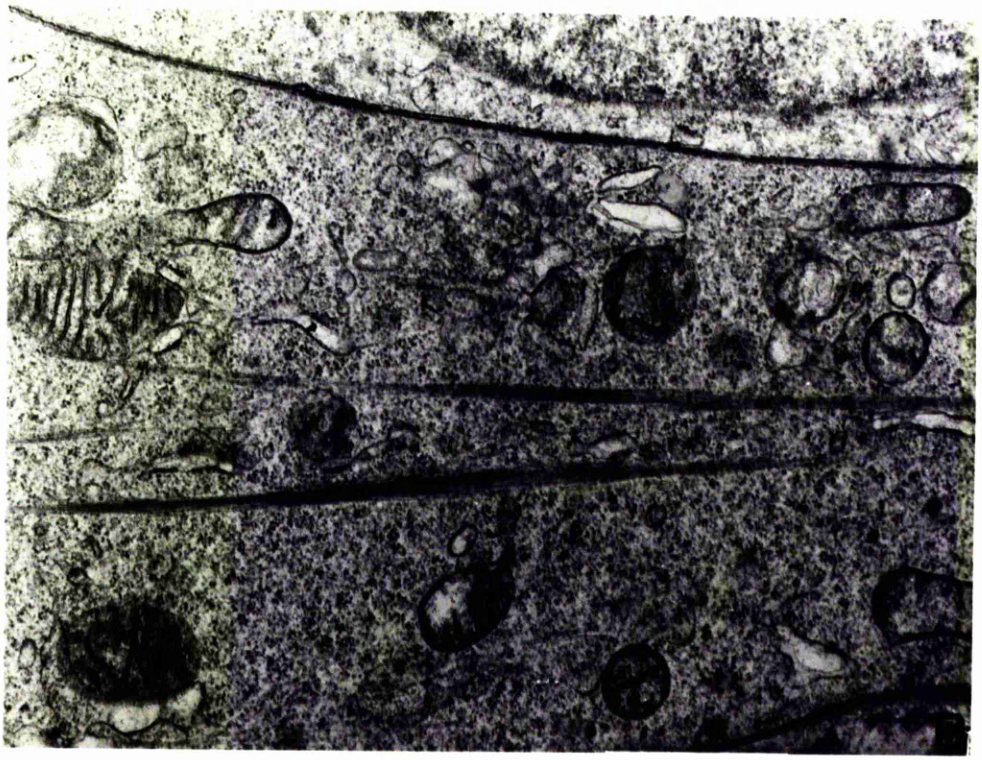
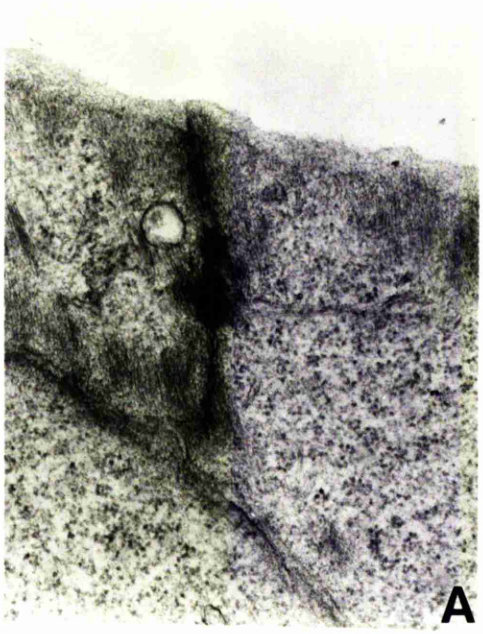


Figure 3.11: Electron Micrographs of immature rat endometrial epithelial cells in primary culture

a) Transmission electron micrograph, showing a typical desmosome between the two adjacent cells x 46000

b) Transmission electron micrograph showing microvilli, covered with glycocalyx each with a central core x 2800

c) Scanning Electron Micrograph showing epithelial cells covered with microvilli x 4580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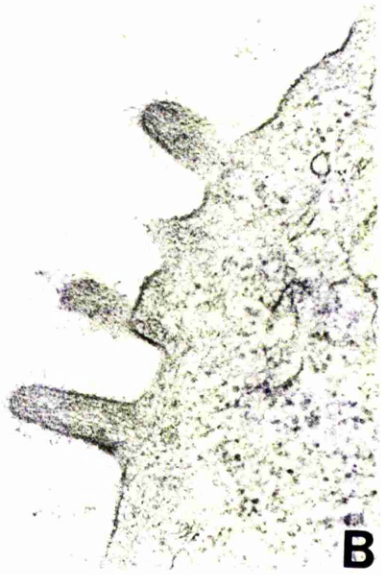
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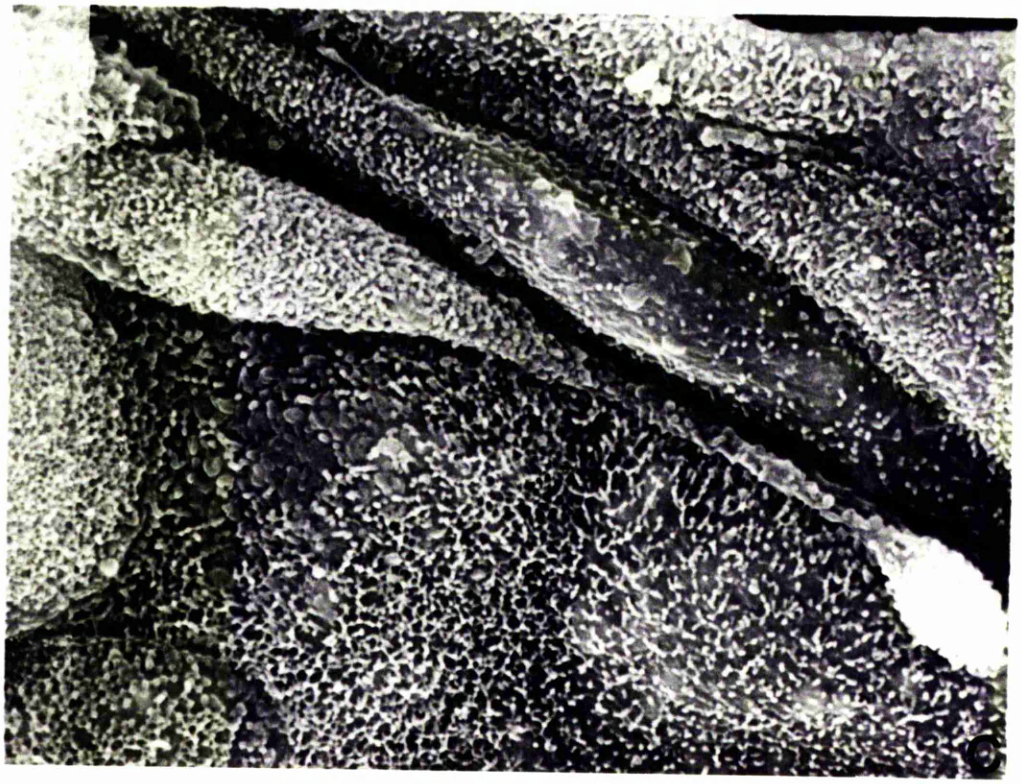
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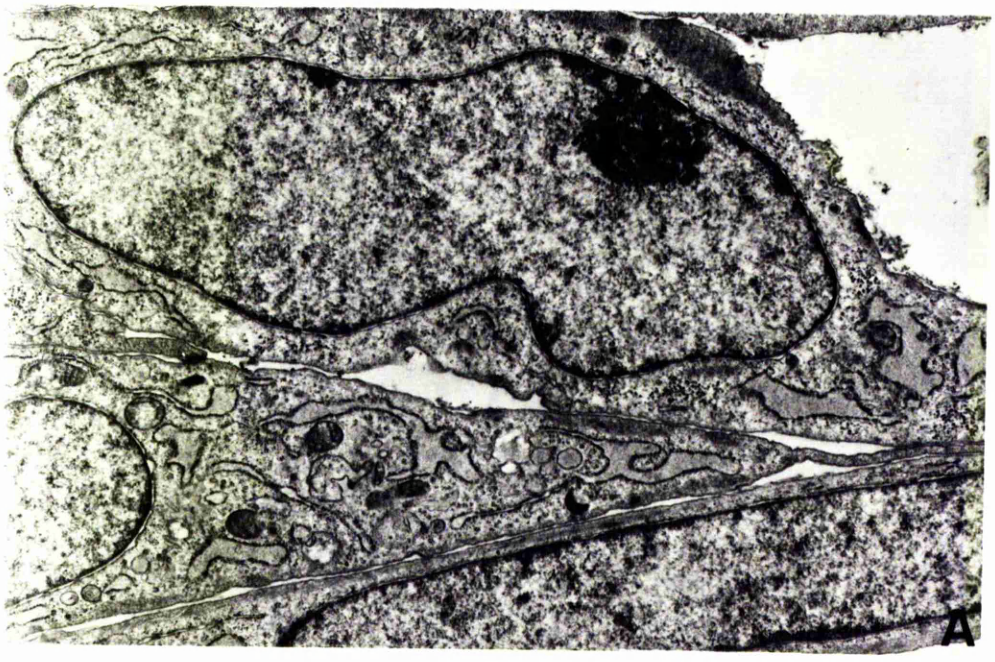
Figure 3.12: Electron micrographs of immature rat uterine mixed (fibroblastic) cells

a) Transmission Electron Micrograph perpendicular to the culture surface, showing overlapping of cells, lacking epithelial-type junctions or microvilli x 18800

b) Scanning Electron Micrograph showing cell surfaces without microvilli x 3100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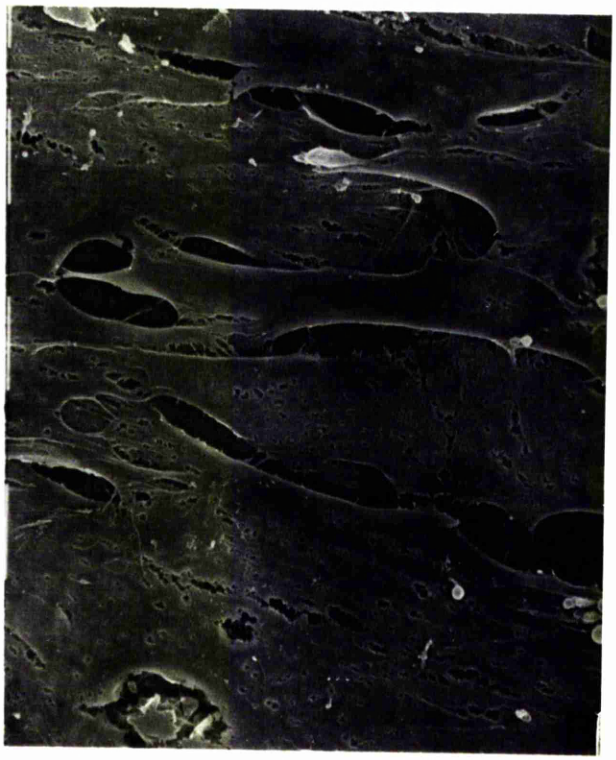
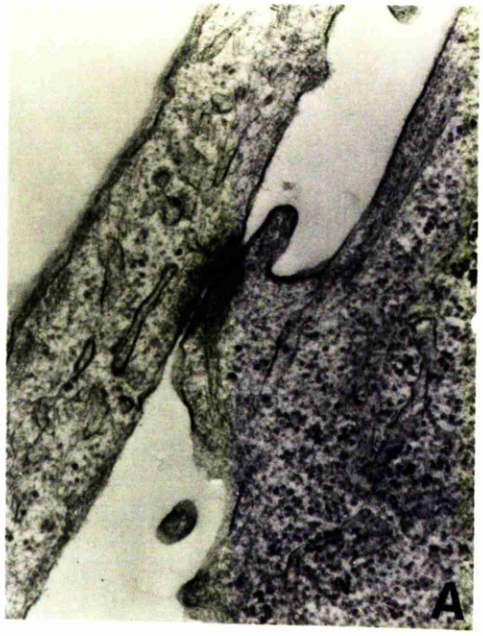


Figure 3.13: Transmission Electron Micrographs of human normal endometrial epithelial cells in primary culture

a) A typical epithelial type desmosome between two membranes x 96750

b) Epithelial cells showing abundant microvilli and junctional complex x 5900

c) A typical monolayered epithelial cell attached to substrate on one surface and with microvilli on free surfaces x 9800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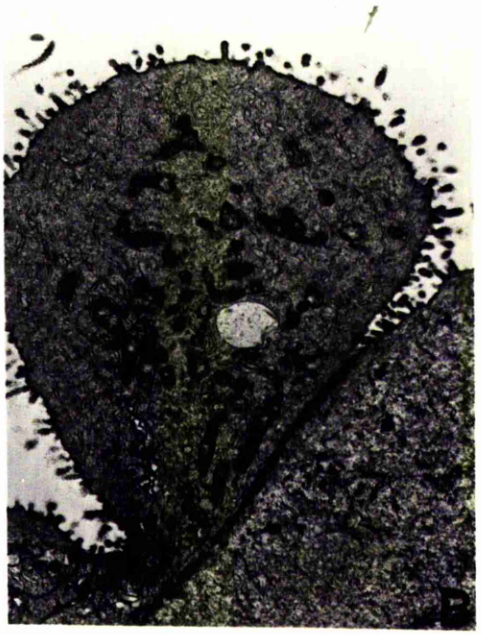
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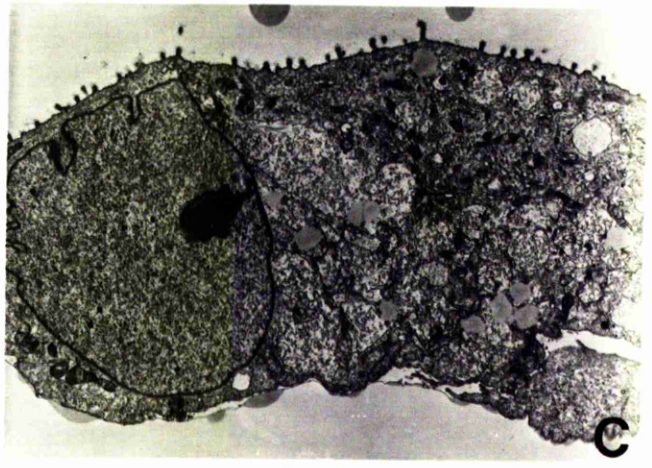
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borders typical of epithelium (Fig. 3.11c). SEM also confirmed the presence of abundant microvilli of reduced size on the free surfaces of cells in culture. In contrast, fibroblastic cultures showed irregular cell-to-cell margins (Fig. 3.4). A central nuclear lump was prominent in each cell. The cells had overlapping edges with no apparent junctions. The surface of the cells was smooth, lacking microvilli, some cells exhibiting small blebs (Fig. 3.12b).

3.250 IMMUNOSTAINING FOR TONOFILAMENTS

Immunofluorescent staining with antiserum against specific tonofilaments in epithelium (Section 2.560) gave a distinctive pattern in these cultured cells. In primary culture this immunostaining showed a typical cytoskeletal pattern in endometrial epithelial cells (Fig. 3.14 a,b), whereas fibroblastic cells were not stained. Interestingly, in mixed cell populations only epithelial cells were observed to be stained (Fig. 3.14 c,d).

3.300 DISCUSSION

3.310 CELL ISOLATION PROCEDURES

The procedure described in Section 2.511 for the preparation of free cell suspensions from whole immature rat uteri appears to satisfy the basic criteria required to justify the routine use of this method. Free cells in reasonable yields and virtually free of debris can be obtained in two hours, with a high degree (90%) of viability. The viability of such cell preparations has also been confirmed by determining the ability of the cell suspensions to convert glucose (^{14}C) to CO_2 and a lipid fraction at essentially constant rates for at least 10h of incubation (Williams and Gorski, 1973). They also demonstrated similar numbers of oestrogen binding sites per cell in the cell-free extract and in the intact cells, indicating that Oestrogen

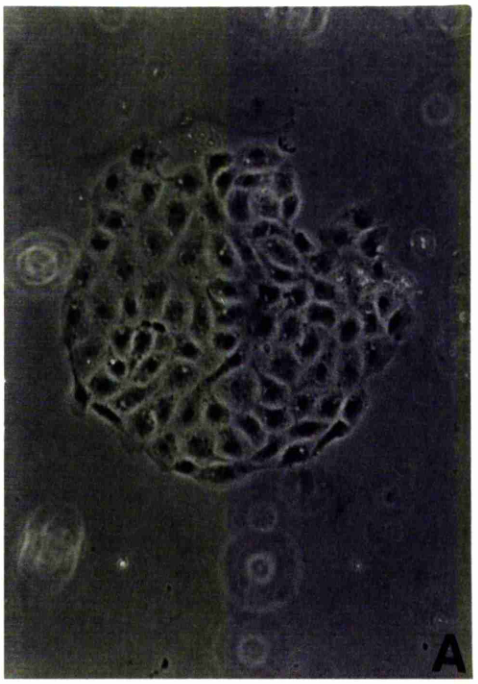
Figure 3.14: Immunostaining of tonofilaments in immature rat uterine cells in primary culture

a) A colony of epithelial cells (Phase contrast x 120

b) Same colony, showing fluorescent staining x 120

c) Mixed cell population (Phase contrast) showing a single epithelial cell x 350

d) Only the epithelial cell retains fluorescent staining x 350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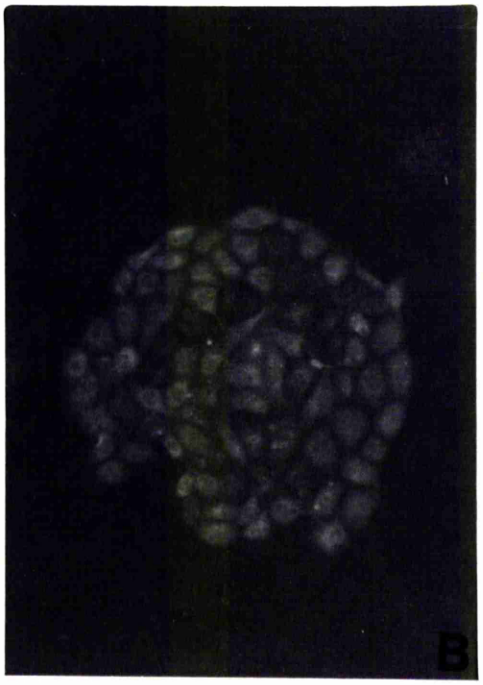
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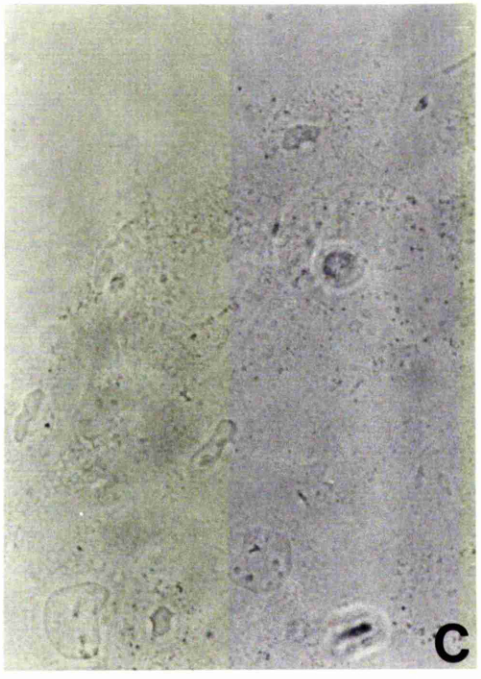
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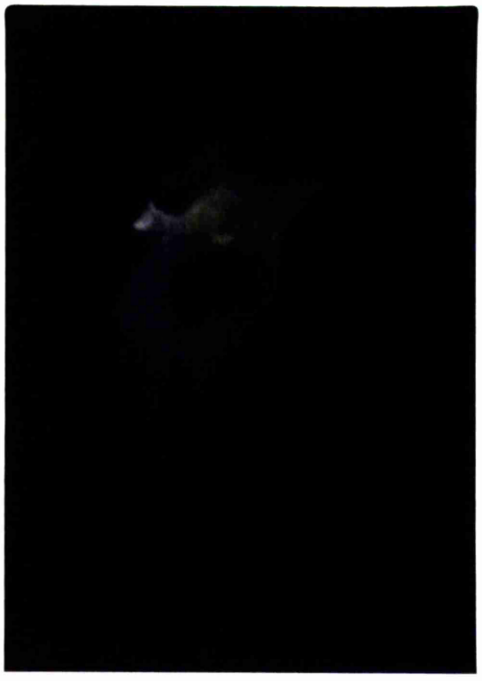


Receptor (ER) is not degraded during the collagenase treatment. Pietras and Szego (1979) described a trypsin method for preparation of endometrial cell suspensions both free and in small aggregates. They claimed it to be suitable for metabolic studies. Sananes et al. (1978) also used trypsin to prepare mixed cell populations from rat endometrium and showed their sensitivity towards an appropriate sequence of progesterone and oestrogen. The cells underwent a morphological transformation in vitro comparable to that observed in decidualisation in vivo. The latter two methods were also tried in this study but the procedure described in Section 2.511 was found to be the most suitable in terms of total output, viability, and ability of the cells to grow in culture.

Heald et al. (1975) reported a simple method for the preparation of luminal epithelial cells from rat endometrium. They adopted a mechanical method using the tissue squeezer. Bitton-Casimiri et al. (1977) also reported a preparation of epithelial cells from early pregnant rat uterus using a similar technique. Vazquez-Nin et al. (1979) presented the idea of combining the two techniques, first digestion with an enzyme mixture followed by a mechanical technique, to isolate pure epithelial cells from rat endometrium. The two techniques hypothesis was used in the present study, but with a different sequence (see Section 2.512). This method was found extremely satisfactory to study epithelial cells in culture.

The procedure for separation of epithelial cells from human endometrium, outlined in Section 2.513 was also found to be superior to alternative methods (Satyaswaroop et al., 1979; Varma et al., 1982), since it produced cleanly separated, recognisable glandular structures.

3.320 CELL CHARACTERISATION

Colonies of rat uterine cells that appeared epithelial like

through phase contrast microscopy were examined by electron microscopy. The method described in Section 2.621 allowed morphological investigations of cultured cells at electron microscopical level without changing the culture conditions or *in situ* situation during dehydration or embedding. In addition, differentiation between multilayer (Fig. 3.12a) and monolayer (Fig. 3.13c) was possible. The excellent preservation of cell structure is demonstrated by the retention of the fine microvilli (Figs. 3.10, 3.13). The separation of the culture layer from plastic dishes with propylene oxide was described earlier by Beesley *et al.* (1978). However, they separated whole culture layers and embedded them as clumps.

McCormack and Glasser (1980) used enzyme treated, pelleted cells for electron microscopy. A significant change in microvillous topography was reported on the surface of cultured cells by trypsinisation (Kinn and Allen, 1981). To avoid this trypsinisation artefact on cultured uterine cells, attempts were made to grow the cells on a variety of substrates, which were supposed to be less resistant to the release of intact layers of cells. Thermanox and polyester sheeting (Melinex-O) were particularly of interest. Trials to embed cells from plastic flasks, petri dishes and carbon-coated glass coverslips (Tolson and Broothroyd, 1982) were also done, but the method explained in Section 2.621 was found to be the best overall.

To observe the sample through SEM, a variety of methods have been used. Basically, the uterine cells have been fixed in glutaraldehyde and postfixed in osmium. Kirk *et al.* (1978) fixed human endometrial cells in glutaraldehyde at $4^{o}C$ and dehydrated straightaway. While McCormack and Glasser (1980) used room temperature to fix rat endometrial cells for SEM in glutaraldehyde and then Osmium Tetroxide (OsO_4). Dorman *et al.* (1982) used glutaraldehyde and OsO_4

for human endometrial cultured cells at room temperature. From the experiments described in this thesis, the endometrial cells were best preserved by fixing at 37°C for 1h with glutaraldehyde in a buffer very similar to the medium in which the cells were grown. Post-fixation with OsO_4 did not show any significant difference in morphology of these cells. Almost all studies (mentioned above) dealing with cultured uterine cells for SEM have used similar methods of dehydration and finally drying the samples in a critical pressure dryer (CPD) through liquid O_2. Concerning endometrial cells this study showed for the first time a significant difference between freeze dried and dehydrated samples. The dehydration techniques yielded cells which showed cracks and poor preservation.

3.321 Identification of Epithelial Cell Type

In the past (Berliner and Gerschenson, 1976; Bitton-Casimiri et al., 1977; Liszczak et al., 1977, Kirk et al., 1978; Satyaswaroop et al., 1979; McCormack and Glasser, 1980; Varma et al., 1982) the identification of endometrial epithelial cells in culture relied exclusively on morphological criteria. The distinction between single epithelial and mesenchymal cells in monolayer culture was found to be very difficult, if not impossible. Systematic identification of individual cells in monolayer has recently become possible with the recognition of specific markers. For example, the intermediate-sized filaments containing a pre-keratin-like protein (cytokeratin) are of widespread occurrence in epithelial cells both in intact tissue (Sun et al., 1979) and in cultured cells (Irwin, 1982). Moreover, monoclonal antibodies have been generated against detergent insoluble cytoskeletal extracts (the tonofilament-associated protein antigens) from cultured epithelium (Lane, 1982). As a major cytoplasmic component of epithelial cells, the tonofilaments appear in TEM as filaments of indeterminate

length, aggregated laterally into anastomosing bundles (Fig. 3.10a) and anchored at the cell periphery by looping into specialised intercellular junctions, the desmosomes (Fig. 3.11b). Immunostaining of tonofilaments has been used for the first time to identify epithelial cells in cultures of rat endometrium. By using this method a clear distinction between epithelial and mesenchymal single cells has been achieved (Fig. 3.14 a-d).

3.330 PROBLEMS

The major problems encountered in establishing the origin and identification of cultured cells from endometrial tissue, will be briefly summarised.

3.331 Cell Type Heterogeneity *in vivo*

The endometrium is an epithelio-mesenchymal structure comprising a complex array of cell types (see Section 1.2000). Both glandular and surface epithelium are composed of similar cells showing minor variations depending upon their topographical location. The stroma also contains a variety of cell types (Irwin, 1982).

3.332 Morphological identification *in vitro*

(a) Tissue dissociation: one of the main criteria used to identify cells in normal tissues is their anatomical location *in vivo* (Ham, 1974). The discrimination of different cell types *in vitro* is, therefore, complicated by the absence of neighbouring cell types. This constitutes an even greater problem when trying to distinguish between cells with morphological similarities such as the various cell types present in the endometrial epithelium.

(b) Changes induced by culture conditions: the general shape of the cultured cell provides little information, since the cell margins tend to be actively changing (Hechman, 1983). The same cell can change from a spindle cell to a squamous cell (Irwin, 1982) and then to a round cell, depending on the conditions imposed (Gerschenson *et al.*, 1979).

Phenotypic changes have been observed in some differentiated mesenchymal cells when put into culture (Irwin, 1982; Hechman, 1983). According to these reports, both cellular morphology and the synthesis of specific cellular products were affected by these changes. Changes in general morphology and growth pattern with time in culture have been studied by Irwin (1982) in primary cultures of human endometrial stromal cells. He observed a characteristic shift in cell shape at confluence. He also confirmed that these changes occur even after serial cultivation for at least 8 passages.

3.333 Overgrowth by fibroblasts

Epithelium mediates the functions of most organs. One possible means of studying these functions is to culture the cells in isolation from mesenchymal tissue constituents. The failure of isolated epithelial cells to grow for prolonged periods in culture has been attributed to the tendency for fibroblasts to grow more rapidly under routine conditions, thus overgrowing the cultures. Possible conditions for sustained growth of pure epithelial cells in culture are discussed in Chapter 4.

3.334 Other cell types

McCormack and Glasser (1980) divided uterine cultures into three main types, epithelial, stromal and myometrial. They proved these three types to be different in respect of both precursor incorporation and partition of oestrogen receptor between cytosol and nuclei after oestrogen administration. Echeverria et al. (1980) concluded from their culture experiments on rat endometrium that two sub-populations of epithelial cells existed. One set, polyhederal cells grouped in colonies, resembled luminal epithelial cells in situ. A second set appeared as flat cells which extended over the substrate and possessed large nuclei with dispersed chromatin and large nucleoli. They also

showed both epithelial cell types to be responsive to oestrogen. Gerschenson et al. (1979) presented data based on two sub-populations of epithelial cells from rabbit endometrial cultures. They named these as quiescent and dividing cells. Recently, Ricketts et al. (1983) described only two main types from rabbit endometrium, stromal which exhibited fibroblastic morphology and epithelial cells. In their opinion, epithelium differed only according to the stage of pregnancy with respect to the proportion of multinucleated cells observed.

Considering human endometrium, Kirk et al. (1978) emphasised that cultures contained both stromal and epithelial fractions. Satyaswaroop et al. (1979) sub-divided human endometrial glandular cells into polyhederal, spindle-shaped and spherical cells showing ultrastructural features of epithelium. Ziegler and Gurpide (1982) selected prolactin producing cells from endometrial epithelial cell population by taking advantage of the slowness of their attachment to the plastic dishes. They reported cultures of epithelial cells that did not attach during the first 48h after cell dispersion produced prolactin, after attachment.

The present study is consistent with the findings that rat endometrial epithelial cultures contained at least three (possibly more) cell populations. It may be possible that these various cell types merely represent various stages of differentiation. This would agree with Irwin's observation (1982) and it has also been shown earlier in this chapter that the change in cellular shape is related to position in the colony. Recently, Varma et al. (1982) concluded that three types of cultures from human endometrium, epithelial, stromal (polyhederal shaped), and fibroblastic cells. The latter two could be subcultured for upto 20 passages with retention of predominant morphology of the original cell types. Similarly, Irwin (1982) presented data on

two distinct morphologies, polygonal and fibroblastic in stromal cultures. He also proved that the morphological heterogeneity in these stromal explants disappeared after a few days in culture. These findings may explain why previous studies (Kirk et al., 1978; Trent et al., 1980) failed to detect morphological heterogeneity. The possibility of identifying particular types of cells individually in epithelial and stromal cultures using biochemical and histochemical markers, provides a new approach towards studying hormonal regulation of differentiation.

3.340 SUBCULTURE

Fibroblastic cells grown from a mixed cell population showed an increased uniformity in morphology in successive cultures. They looked more spindle shaped with elongated cytoplasm, growing parallel, but overlapping each other. It is either due to complete loss of epithelial cells by outgrowth of fibroblasts or due to changes in morphology of the epithelial cells. Halme et al. (1980) confirmed the possibility of subculturing mixed cell populations from rat uterus, but they have not been successful in preparing secondary cultures of these cells which retain the capacity to produce collagenase. One possible explanation is that the secondary cultures might be lacking a specific cell type, responsible for producing connecting substances. Vallet-Strouve et al. (1982) also managed to subculture ovine myometrial cells in culture. They observed a decline in 17-β steroid dehydrogenase activity in successive cultures, which paralleled the slowing of cell growth and overall protein synthesis.

Epithelial cultures from rat uterus were also subcultured but the retention of morphology of epithelial cells was impossible (Fig. 3.9a). Echeverria et al. (1980) managed to subculture flatter populations of rat endometrial epithelial cells without loss of

morphological characteristics. Liu and Tseng (1979) also provided data on subcultured human endometrial epithelial cells but, lacking any ultrastructural documentation. Kirk and Irwin (1980) showed the human fibroblastic stromal cells could be passaged. In contrast, epithelial cells survived only as a short-term primary culture, and could not be successfully subcultured. Irwin (1982) established cell lines from primary human endometrial stromal cells. He observed a series of morphological changes after the initial 5 days in culture. The stromal fibroblastic cells at confluency shifted into polygonal shape, which upon subculture reassumed fibroblastic morphology, undergoing the same sequence of morphological changes described in primary cultures.

Trent et al. (1980) claimed to have cultured human endometrial epithelial cells and passaged them up to 4 times. However, convincing ultrastructural evidence for the epithelial nature of these serially passaged cells was not presented.

3.350 PHYSIOLOGICAL RELEVANCE

The proportion of each cell type was found to vary in cultures from different endometria (see Section 3,121, and 3,220). A more extensive and detailed study is needed to establish a firm correlation between the phase of the reproductive cycle and the predominant morphology in primary cultures. However, a definite trend was observed for the elongated cells to predominate in cultures from human proliferative endometrium and the polygonal form in those from secretory endometrium. Another observation made was, that cells in secondary cultures were fibroblastic in morphology. At the same time, the cells in secondary cultures were found to be fast growing. Having ruled out the connection between the pre-ovulatory or cell dividing phase and the fibroblastic shape of the cells in cultures, it seemed reasonable to speculate that these two discrete morphologies in culture are related to

the degree of maturation acquired by the epithelial cells *in vivo*. If correct, it would appear that the differentiated epithelial phenotype reverts to an immature fibroblastic morphology under culture conditions. Analogous morphological changes have been observed in primary cultures of differentiated mesenchymal cells, such as smooth muscle cells (Heckman, 1983) have been interpreted as a result of de-differentiation under culture conditions.

Irwin (1982) showed polygonal human stromal cells in primary explants, changing to a more spread fibroblastic shape before proliferation, whereas in confluent culture, the change in cell shape was in the opposite direction. This study also supported this idea of change in cell shape in relation to proliferation. Studies with endothelial cells (Folkman and Moscona, 1978) and mouse fibroblasts (Folkman and Tucker, 1980) have shown that DNA synthesis and cell proliferation were related to the degree of cell spreading.

The possibility of a similar correlation between cell shape and growth control in endometrial cells seems an interesting prospect. Further work would be necessary to substantiate this hypothesis.

CHAPTER 4 - 'TOWARDS SERUM-FREE CELL CULTURE'

4.000 INTRODUCTION

The effects of the ovarian steroids on uterine cells depend both on the absolute and relative levels of these hormones (Gerschenson et al., 1974; Irwin, 1982). Therefore, the interpretation of results of experiments with steroid hormones will be greatly complicated unless the precise levels of each steroid available to the cells can be adequately controlled. However, the requirements of unspecified serum factors for the survival and proliferation of cultured cells is a major hindrance in reducing and controlling experimental variables due to the binding of extrinsic hormones and growth factors by serum components.

Recently, advances have been made in the development of chemically defined media for cell culture (Holley, 1975; Barnes & Sato, 1980a, b). However, the precise nutritional and hormonal requirements must be determined for each cell type as these vary from one cell type to another (Barnes & Sato, 1980a; Jozan et al., 1982; Bradshaw et al., 1983). Therefore, until such requirements are established serum-supplemented media must be used to support growth and viability of most cells in culture.

4.010 BINDING OF STEROIDS TO SERUM PROTEINS

The macromolecular components of serum include molecules with high-affinity binding sites for steroids (Westphal, 1971). Steroid hormones are present at unspecified levels in commercial serum in both free and protein-bound forms. The distribution between these two forms varies for each steroid (Cook et al., 1977; Sodergard et al., 1982). A variety of proteins have been characterised as major steroid binding components in the serum of various animal species. These include:

a) albumin

b) glycoprotein

c) corticosteroid binding globulin (CBG)

d) steroid hormone binding globulin (SHBG)

4.020 STEROID EXTRACTION FROM SERUM

Steroid hormones show increased dissociation from their carrier proteins at elevated temperatures. Based on this principle, dialysis at 37°C and gel filtration at 45°C have been employed to extract steroids from serum (Westphal, 1967). Another method involves the use of an adsorbent such as activated charcoal. Heyns et al. (1967) first reported the use of charcoal adsorption as a method to extract endogenous steroids from serum. Later, dextran coated charcoal (DCC) was used by others to obtain steroid depleted serum for the study of hormone sensitive cells in culture (Lippman & Bolan, 1975; Horwitz & McGuire, 1978; McCormack & Glasser, 1980; Irwin, 1982). The aim of this chapter is to describe experiments studying serum factors in cellular growth.

4.030 REPLACEMENT OF SERUM

It is generally accepted that the growth of virtually all types of cells in culture requires the presence of serum in the medium. Serum is a complex mixture, and many serum components are at best, poorly characterised. In addition, the concentrations of some serum components vary drastically among different batches. Investigators have long recognised the problems associated with the undefined nature of serum, and a number of different approaches have been taken to eliminate the requirement for a serum supplement in culture medium. Ham & McKeehan (1979) developed a potentially valuable method. Their approach is based on the careful adjustment of medium components to provide cells with optimal nutrients. Sato and colleagues developed another method based on the idea that one of the main functions of serum is to provide a mixture of hormones which is stimulatory for cell growth (Barnes & Sato, 1980a, b). This concept evolved from the observation that cell lines which were clearly hormone dependent in vivo did not show hormone dependent growth in vitro unless the serum added to the culture medium

was first depleted of certain hormones (Nishikawa et al., 1975). Thus, hormones in whole serum fully stimulated cell growth masking the effects of any exogenous hormones. It should be possible to replace the growth stimulating activities of serum with the proper choice of hormones and other growth factors at the right concentrations. In this study, various combinations of hormones and growth factors were defined for the replacement of the serum for culture of rat endometrial epithelial cells.

4.100 STEROID DEPLETED SERUM

The following experiments were set up to establish a suitable methodology for the effective extraction of endogenous steroids from foetal calf serum and to look at the effects of added hormones and factors on growth modulation.

4.110 DIALYSIS

The levels of various steroids were measured by radioimmunoassay (RIA) (Section 2.760) before and after dialysis for 48h at 4°C against de-ionised water (Section 2.352). This method was found to extract little amount of steroids from serum (Table 4.1b). Only 13% of the E_2 and 4% of P contained in FCS were removed after such treatment.

4.120 HEAT INACTIVATION

As can be seen from Table 4.1(b), no difference was observed in the levels of steroids in FCS before and after heating at 56°C (Section 2.353).

4.130 ADSORPTION WITH DCC

Extensive studies were done to assess the optimal conditions for the extraction of steroids from FCS (see Section 2,351). FCS was incubated with DCC for variable times (2-24h). Maximum removal of most steroids was reached within 2h.

4.131 Temperature

The extraction of steroids from FCS by (saturating concentration of) DCC was dependent on the incubation temperature. The

Table 4.1: Steroid hormone levels in foetal calf serum

a) The concentration of steroid hormones in a batch of foetal calf serum was measured by radioimmunoassay as described in Section 2.760. Steroid levels were determined in whole serum (FCS), and in serum charcoal stripped (see Section 2.351) at $4^{o}C$ (FCS-DCC(4)) and $56^{o}C$ (FCS-DCC(56)).

b) Steroid hormone levels measured in another batch of serum by RIA (see Section 2.760). Whole serum (FCS) was stripped with charcoal as mentioned in Section 2.351 at $4^{o}C$ (FCS-DCC(4)) and at $56^{o}C$ (FCS-DCC(56)). Heat-inactivated serum was prepared as described in Section 2.353 and dialysed serum by a method explained in Section 2.352.

A

SERUM	Cortisol (nmol/l)	Testosterone (nmol/l)	Oestradiol (pmol/l)	Progesterone (nmol/l)
FCS	30	0.19	254	0.2
FCS-DCC(4)	30	0.15	100	0.2
FCS-DCC(56)	30	0.15	100	0.2

B

SERUM	Cortisol nmol/l	Testosterone nmol/l	Oestradiol pmol/l	Progesterone nmol/l
FCS	20	0.5	321	0.2
FCS-DCC(4)	20	0.5	100	0.2
FCS-DCC(56)	20	0.5	100	0.2
FCS-Heat-inactivated	20	0.5	292	0.2
FCS-Dialysed	20	0.5	279	0.2

levels of oestrogen were reduced further by incubating FCS at 56°C, instead of 4°C (Table 4.1).

4.140 PROTEIN CONTENT OF SERUM

No significant effect was observed on total protein content of serum after the various treatments (see Table 4.2).

4.200 SERUM AND GROWTH PATTERNS

Experiments to investigate the ability of whole FCS and DCC-stripped FCS to promote cell attachment and growth were performed by measuring both cell number and rate of DNA synthesis.

4.210 CELL ATTACHMENT

A rat uterine mixed cell suspension was equally divided into a variable number of wells each containing a glass coverslip. The total number of cells attached and spread after 2h, 4h, 6h, 12h, 48h, was counted as described in Section 2.713. The number of cells which settled down had reached a maximum within the first 6h of incubation in medium without serum (Table 4.3). Over the same time period, the number of the cells attached in FCS-DCC(4) was greater than that in FCS. while the minimum number occurred in FCS-DCC(56). After 12h, the coverslip with FCS-DCC(4) had more cells and maximum spreading than any other medium. The attachment process continued until 24h, then cell division started (shown for FCS) and a partial monolayer was observed at the end of the second day of culture.

Rat endometrial epithelial cell attachment and spreading was also rapid in cultures started with FCS-DCC(4) (Table 4.4). The attachment process was completed in the first 6-8h of incubation. 5% serum was found to be the optimum concentration for initial attachment and early growth of epithelial cells (Table 4.5).

Table 4.2: Protein content in serum

Concentration of total protein was determined as described in Section 2.740, in whole serum (FCS), serum charcoal stripped (see Section 2.351) at $4^{o}C$ (FCS-DCC(4)) and at $56^{o}C$ (FCS-DCC(56)) and heat-inactivated serum (see Section 2.353). The values are the mean $\pm$ S.D. of the triplicate assays corrected for dilution.

SERUM	PROTEIN CONTENT (gms/100ml)
FCS	4.85 ± 0.12
FCS-DCC(4)	4.9 ± 0.105
FCS-DCC(56)	4.825 ± 0.090
FCS-Heat-inactivated	4.8 ± 0.19

Table 4.3: Attachment of rat uterine mixed cells

Rat uterine mixed cells were isolated as described in Section 2.511 and plated homogeneously in multiwell plates, containing a glass coverslip in each well. Medium contained 10% serum (FCS, FCS-DCC(4), FCS-DCC(56) or dialysed serum) or was serum-free. At the times indicated the coverslips were recovered and fixed in methanol (see Section 2.540), stained with Giemsa (see Section 2.550) and the cells on each coverslip were counted. Spread cells were taken as those which had comparatively elongated cytoplasm attached to the substrate.

RAT UTERINE MIXED CELL POPULATION

Time	Serum	Number of Cells Attached	Spread
2h	FCS	610	
	FCS-DCC $4^{o}C$	937	
	FCS-DCC $56^{o}C$	189	
	FCS-dialysed	461	
	No Serum + EGF + INS	2201	
4h	FCS	1296	109
	FCS-DCC $4^{o}C$	1900	360
	FCS-DCC $56^{o}C$	477	13
	FCS-dialysed	1355	99
	No Serum + EGF + INS	2809	42
6h	FCS	3916	1180
	FCS-DCC $4^{o}C$	5673	2233
	FCS-DCC $56^{o}C$	1897	313
	FCS-dialysed	2101	406
	No Serum + EGF + INS	7223	609
12h	FCS	5571	2410
	FCS-DCC $4^{o}C$	6147	3279
	FCS-DCC $56^{o}C$	2331	490
	FCS-dialysed	3783	961
	No Serum + EGF + INS	4298	739
24h	FCS	13078	8219
48h	FCS		3×10^{4} (Partial monolayer)

Average of two different experiments.

Total number of cells per cover slip.

Table 4.4: Attachment of rat endometrial epithelial cells

Epithelial cells were isolated from immature rat endometrium (see Section 2.512) and plated as described in Table 4.3. After certain times, attached and spread cells were counted. Clumps of 3 or more cells were observed to be settling on the glass surface and colonies growing were counted as described in Section 2.711. Each value is the average of two separate experiments.

Time after Plating	Serum fed	Number of Attached cells	Spread cells	Clumps	Colonies
2 hours	FCS	946	-		
	FCS-DCC (4°C)	1407	151		
	FCS-DCC (56°C)	1132	-		
	No serum + EGF + Ins + Cortisol	308	-		
4 hours	FCS	1652	127	✓	
	FCS-DCC (4°C)	2607	709	✓	
	FCS-DCC (56°C)	1349	106		
	No serum + EGF + Ins + Cortisol	442	33		
6 hours	FCS	3206	1401	✓	
	FCS-DCC (4°C)	5062	2911	✓	
	FCS-DCC (56°C)	2119	486		
	No serum + EGF + Ins + Cortisol	904	170		
12 hours	FCS	5054	3981	✓	43
	FCS-DCC (4°C)	7619	6130	✓	49
	FCS-DCC (56°C)	2440	698	very few	4
	No serum + EGF + Ins + Cortisol	1229	200		
24 hours	FCS				105
	FCS-DCC (56°C)				22
48 hours	FCS				136
	FCS-DCC (56°C)				31

Table 4.5: Effect of serum concentration on attachment and growth of endometrial epithelial cells

Immature rat endometrial epithelial cells were prepared and cultured as described in Table 4.3. Cells were plated in different concentrations of whole serum (FCS) for the first 12h, then the medium was replaced with a fresh one, containing various serum levels. After a further 48h, cells were counted as described in Section 2.710. Each value is the mean $\pm$ S.D. of triplicate experiments.

Initial plating with	At 12h medium replaced with	Cell No. at 48h. ($x10^4$)
1% FCS	1% FCS	2.0 ± 0.2
	5%	4.5 ± 0.4
	10%	6.0 ± 0.9
5% FCS	1% FCS	3.0 ± 0.5
	5%	11.0 ± 1.6
	10%	14.0 ± 2.4
10% FCS	1% FCS	2.5 ± 0.5
	5%	9.0 ± 2.0
	10%	9.5 ± 2.9

4.220 CELL PROLIFERATION

Table 4.6 shows the comparative growth promoting ability of FCS and FCS-DCC(56) in terms of total cell number, DNA and protein contents of mixed cell populations in both first and second passage. DNA content dropped significantly on day-3 of the first passage particularly in FCS-DCC(56). Neither total cell number nor protein content differ significantly between FCS and FCS-DCC(56). During the second passage no significant differences were observed in growth efficiency of cells plated in FCS and FCS-DCC(56).

Rat endometrial epithelial cells exhibited their best growth with FCS-DCC(4) (Fig. 4.1). Significantly reduced growth with dialysed serum was seen. No difference was observed with FCS or heat-inactivated serum.

FCS-DCC(56) enhanced the growth of human endometrial cancer cells over the first 2 (Fig.4.6) and 3 (Fig. 4.14) days.

4.221 Colony Formation

FCS-DCC(4) exhibited the maximum ability to develop a colony by 12h from a piece of gland (Table 4.4). FCS-DCC(56) failed to promote colony formation from normal rat endometrial glands.

4.222 Change of Medium

Epithelial cells settled down and formed colonies in their original medium when left for 24h. Replacement with a fresh medium after 6h resulted in decreased number of colonies after 48 or 72h (Fig. 4.2a). In contrast, cellular growth, in terms of the rate of DNA synthesis (see Section 2.713), was much reduced in the cultures without any change of medium (Fig. 4.2b). Overall, the optimum effect on growth of cultures over 48h was best observed by changing the medium after 24h of incubation (Fig. 4.1).

Figure 4.1: Effect of modification of serum on growth

Rat endometrial epithelial cells were prepared as described in Section 2.512 and plated with 10% FCS. Medium was replaced with medium containing the indicated serum at 6h ▨, 12h□, and 24h ■. Cells were counted at 48h (see Section 2.710). Each bar represents the mean ± S.D. of triplicate experiments.

* $P < 0.2$

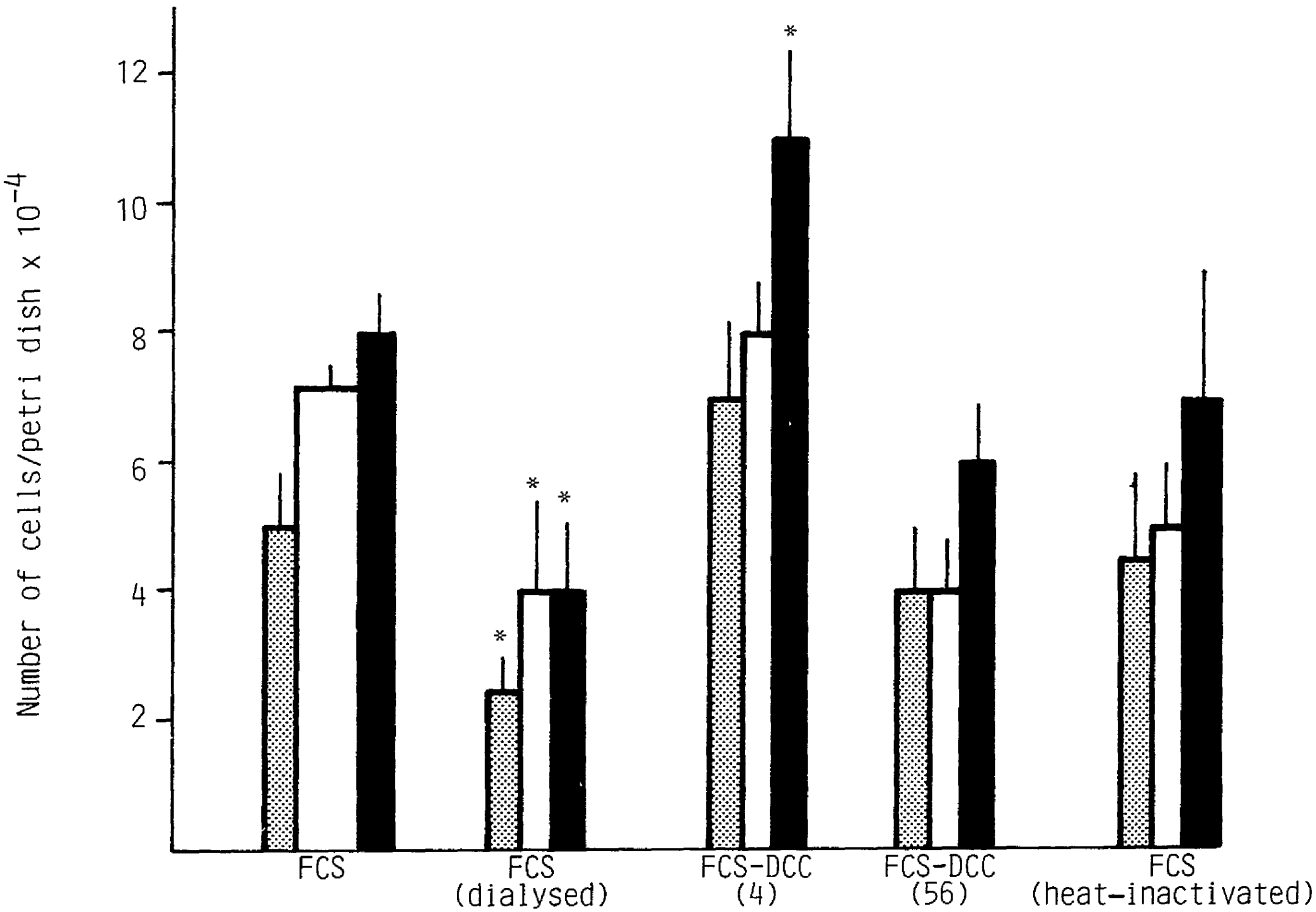
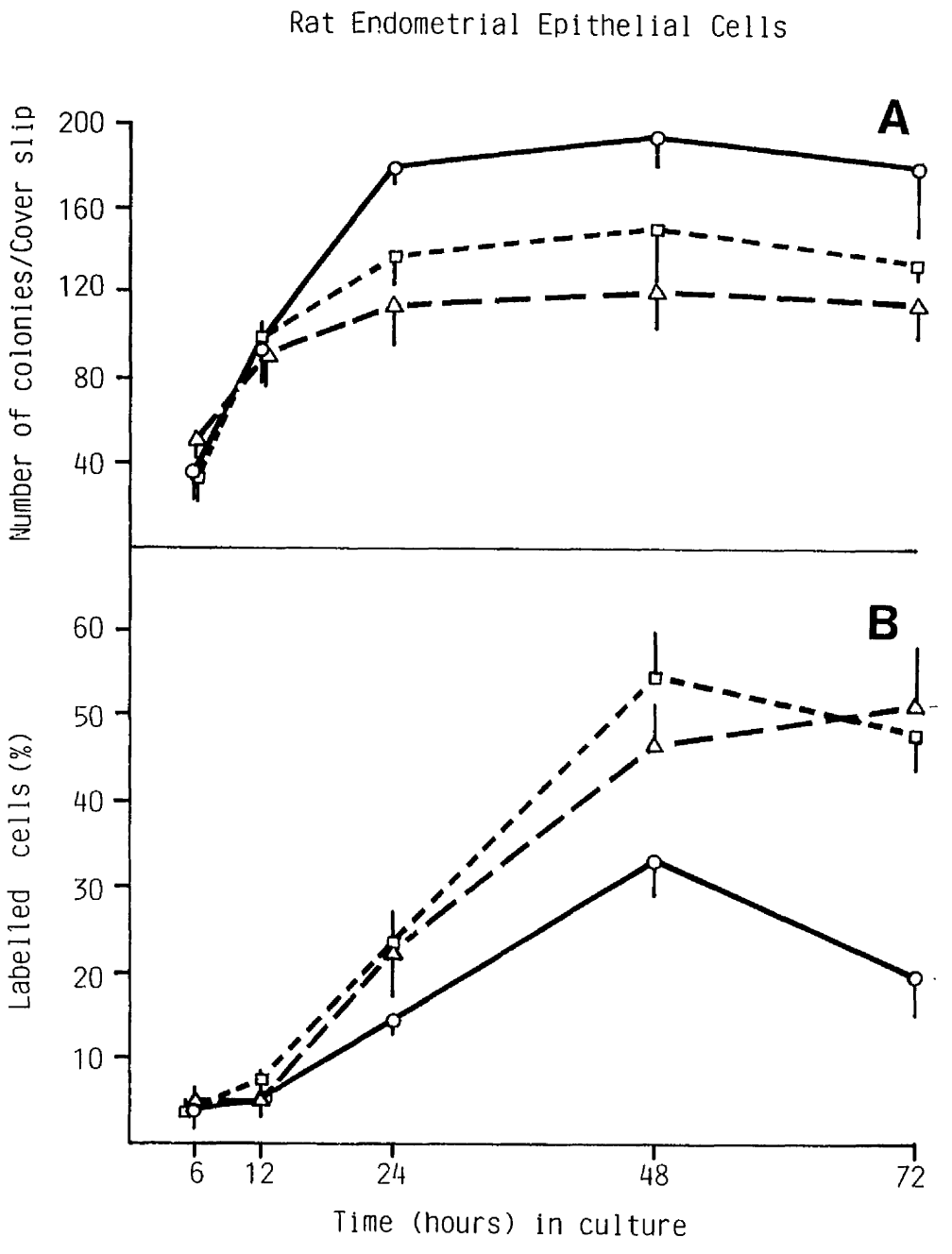

Number of cells/petri dish x 10^{-4}
12
10
8
6
4
2
*
*
*
*
FCS
FCS (dialysed)
FCS-DCC (4)
FCS-DCC (56)
FCS (heat-inactivated)

Figure 4.2: Effect of change of medium on the growth of rat endometrial epithelial cells

Cells were prepared as described in Section 2.512 and cultured in 10% FCS. Medium was replaced with a similar one after 6h (—·—) or 12h (----). Alternatively medium was left unchanged throughout the experiment (——). Cells on coverslips were recovered from the cultures at each indicated time and processed (as described in Section 2.731) for ^{3}H-dT labelling index.

a) Total number of colonies growing on each coverslip was counted (see Section 2.711)

b) Percentage of labelled cells was determined by the method described in Section 2.711.


Rat Endometrial Epithelial Cells
A
B
Number of colonies/Cover slip
200
160
120
80
40
Labelled cells (%)
60
50
40
30
20
10
6
12
24
48
72
Time (hours) in culture

4.230 INCORPORATION OF ^{3}H-THYMIDINE

Equilibrium with the intracellular (acid soluble) pool of thymidylate (Fig. 4.3a) was completed after 40 min and incorporation of radioactivity into the acid insoluble material (Fig. 4.3b) increased linearly after that time.

The incorporation of ^{3}H-thymidine into the acid insoluble material increased when charcoal stripped medium was fed to the mixed cell population (Fig. 4.4a), while heat-inactivated serum was not different from FCS. Pure epithelial cell populations showed similar responses, being maximally stimulated by FCS-DCC(56) (Fig. 4.4b). Autoradiographic results also confirmed maximal stimulation of labelling indices by FCS-DCC(56) (Fig. 4.5). The peak depended on time, culture conditions and cell density.

High temperatures (56^{o}C) and charcoal treatment of serum, both together and individually, accelerated thymidine incorporation into human endometrial cancer cells (Fig. 4.6). Dialysed serum again reduced the incorporation.

4.300 DEVELOPMENT OF SERUM-FREE MEDIUM

Serum is the usual source of growth promoting substances for cells in culture. It contains a variety of identified and unidentified growth factors (Holley & Kiernan, 1974; Gospodarowicz & Moran, 1976; Barnes & Sato, 1980a,b; Bradshaw et al., 1983). Two purified polypeptides (insulin and epidermal growth factor, EGF) were used in the present study. The precise mechanisms by which these peptides stimulate cell proliferation are not knwon. The available data show that binding of EGF to specific cell surface receptors is obligatory for stimulation of DNA synthesis (Carpenter, 1981; Fox et al., 1982). However, not all cells having receptors for EGF proliferate in response to this growth

Figure 4.3: ^{3}H-thymidine incorporation by endometrial epithelial cells

Rat endometrial epithelial cells were prepared (see Section 2.512) and cultured in medium supplemented with 10% FCS. 48h later cultures were pulse-labelled for ^{3}H-dT (see Section 2.730) for the indicated times and the radioactivity incorporated into acid-soluble (a) and acid-insoluble (b) material was determined (see Section 2.732). Each point is the mean $\pm$ S.D. (bars) of triplicate cultures.

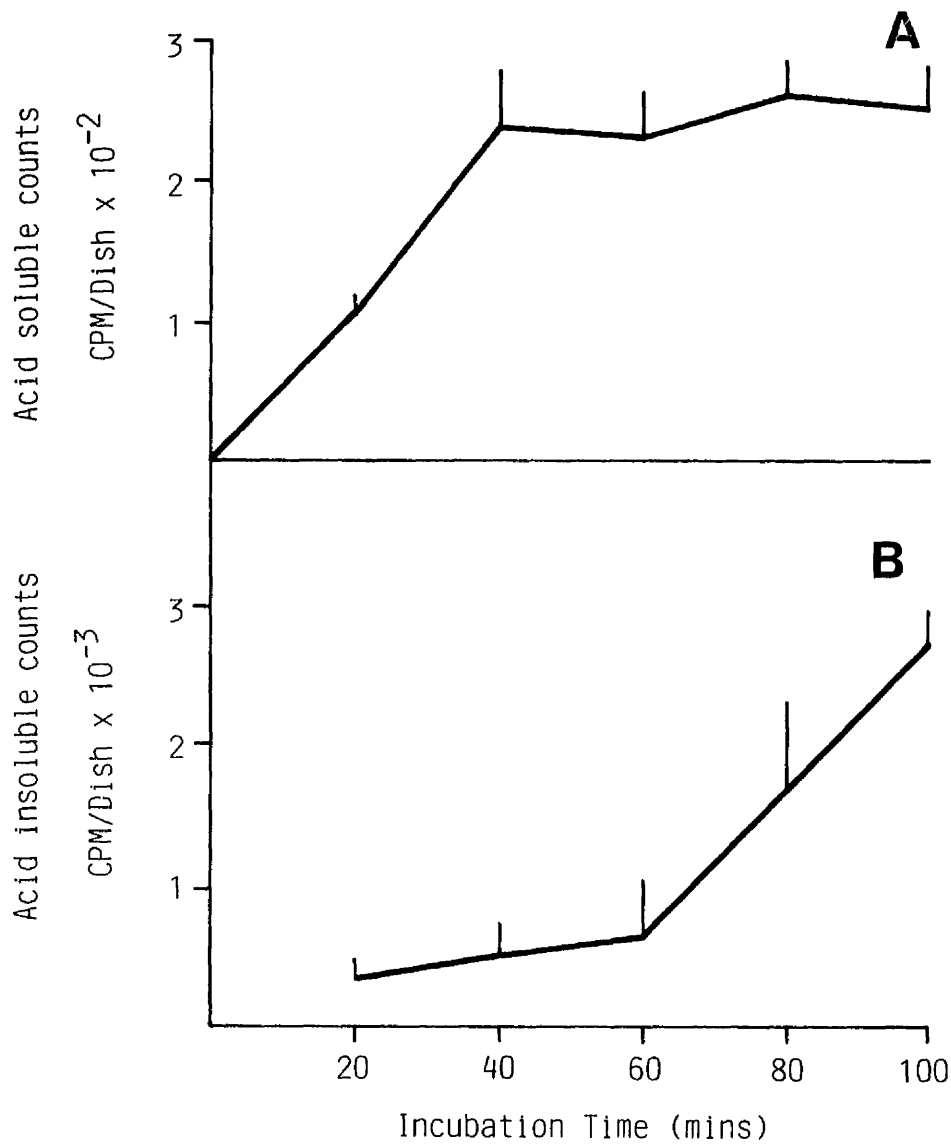

A
B
Acid soluble counts
CPM/Dish x 10^{-2}
Acid insoluble counts
CPM/Dish x 10^{-3}
3
2
1
3
2
1
20
40
60
80
100
Incubation Time (mins)

Figure 4.4: Effect of serum on ^{3}H-dT incorporation by rat uterine cells

Cells were cultured as described previously (see Section 2.520). After 12h, medium was replaced with fresh media containing 10% FCS, FCS-heat-inactivated, FCS-DCC(4), or FCS-DCC(56). After 6 days, cells were labelled with ^{3}H-dT for the last 3h. Radioactivity incorporated was measured as described in Section 2.732.

a) Immature rat uterine mixed cells

b) Immature rat endometrial epithelial cells

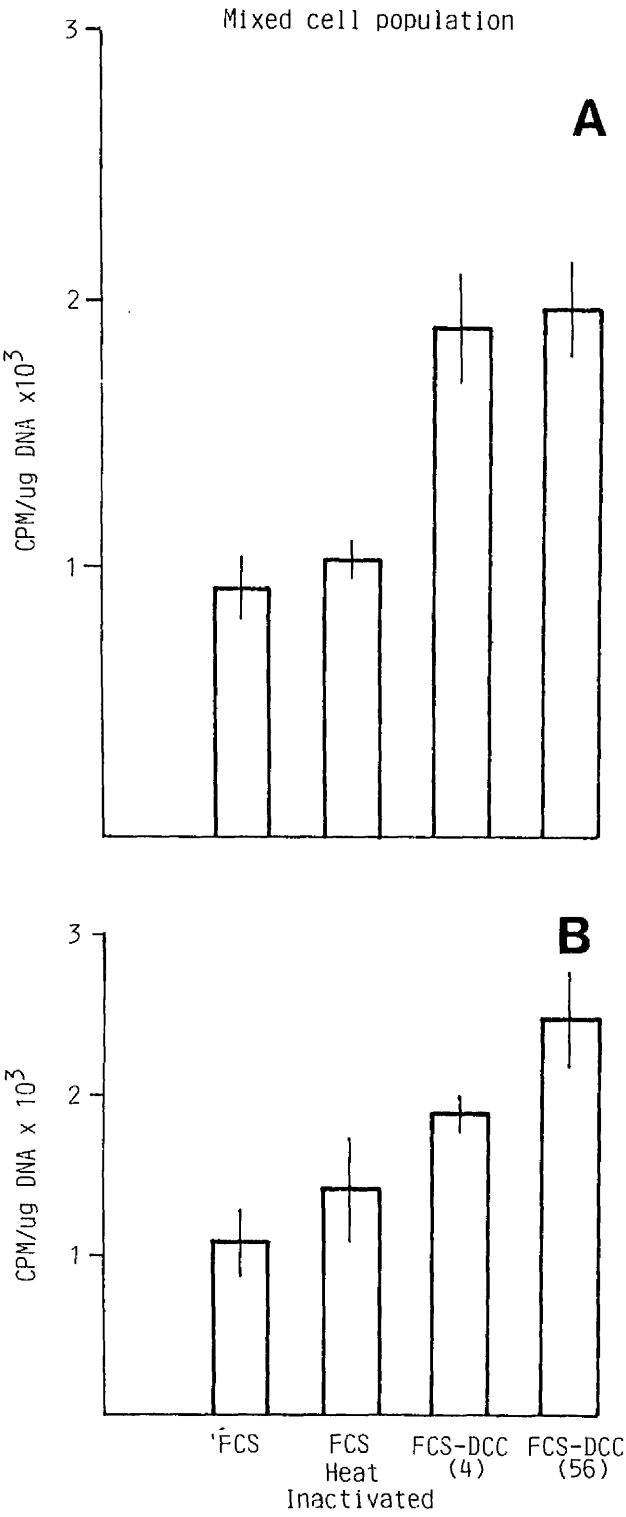

Mixed cell population
A
B
CPM/ug DNA x10^3
CPM/ug DNA x 10^3
3
2
1
FCS
FCS Heat Inactivated
FCS-DCC (4)
FCS-DCC (56)

Figure 4.5: Effect of serum and time in culture on thymidine labelling index

Rat endometrial epithelial cells were plated in 10% FCS as described in Figure 4.4. After 12h, medium was changed and appropriate serum (10%) was added. At the end of each day, cultures were pulsed with ^{3}H-dT for 3h (see Section 2.730). Autoradiographs were processed as described in Section 2.731 and percentage of labelled cells were determined as explained in Section 2.711.

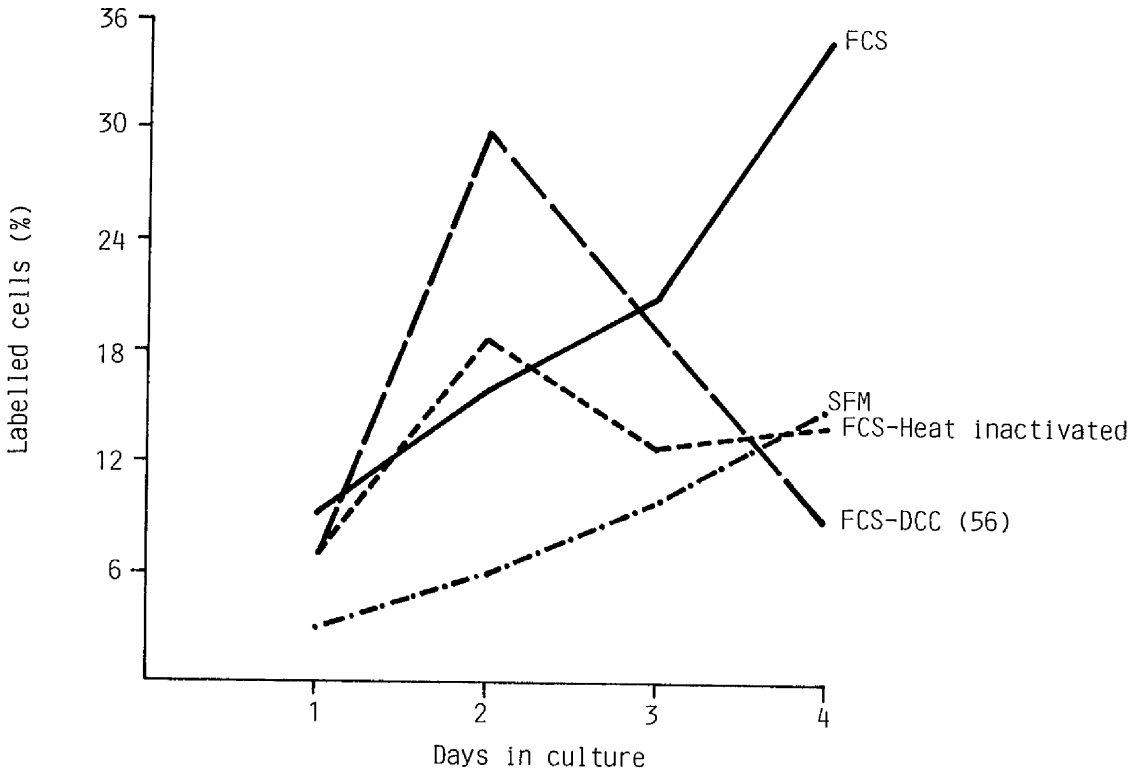
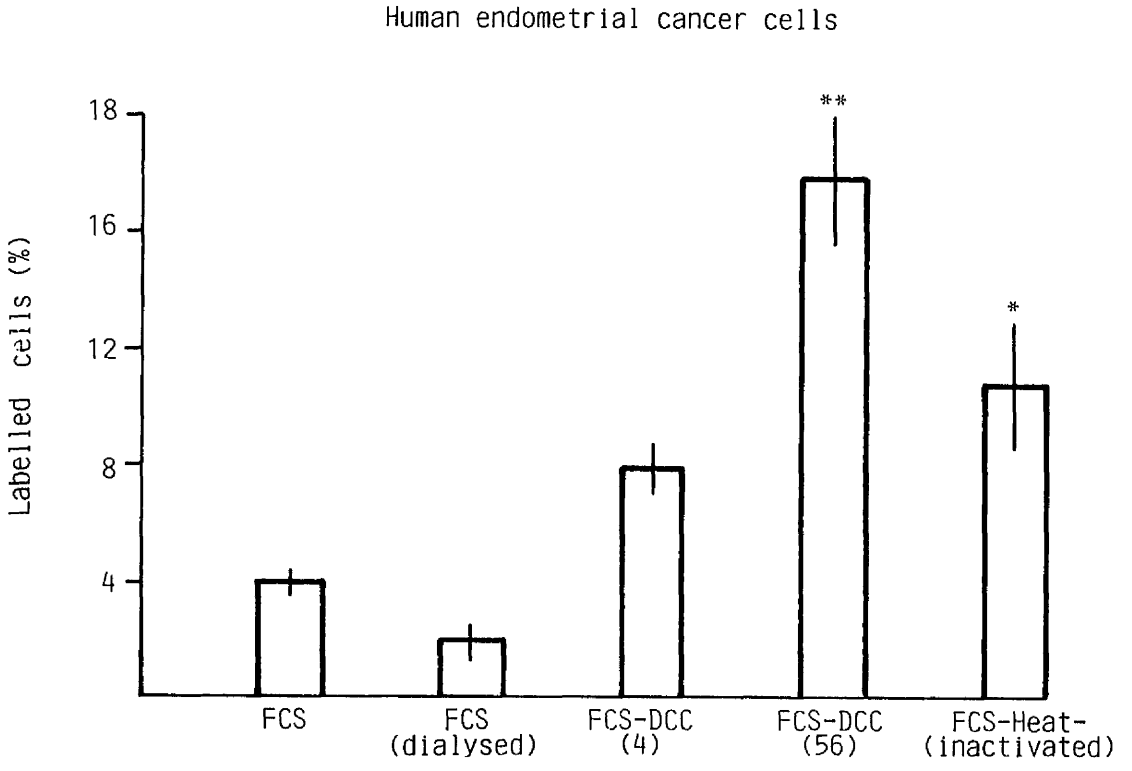

36
30
24
18
12
6
Labelled cells (%)
1
2
3
4
Days in culture
FCS
SFM
FCS-Heat inactivated
FCS-DCC (56)

Figure 4.6: Effect of serum on ^{3}H-thymidine incorporation in human endometrial cancer cells

Cells were isolated (see Section 2.514) and cultured with 5% FCS-DCC(4) for 24h. Then medium was changed and appropriate sera (5%) were added. Cells were cultured for another 48h. ^{3}H-dT was pulsed for the last 3h and the cells were processed as described in Section 2.731 and counted as described in Section 2.711. Each point is the average of duplicate experiments.


Human endometrial cancer cells
Labelled cells (%)
18
16
12
8
4
**
*
FCS
FCS (dialysed)
FCS-DCC (4)
FCS-DCC (56)
FCS-Heat-(inactivated)

factor (Carpenter & Cohen, 1979; Cohen, 1983). Insulin exerts its biological effects by binding to specific membrane receptors in target cells (Levine, 1982). In most cells studied, insulin at physiological concentrations has no mitogenic effects on its own, but can synergise with other growth factors (Rudland & De Asua, 1979). On the other hand, at supraphysiological concentrations - insulin alone is mitogenic for a variety of cell types (Gospodarwicz & Moran, 1976; Levine, 1982). The need for such high insulin concentrations has been partly explained by a rapid loss of its biological activity in cysteine-containing medium (Hayashi et al., 1978; Barnes & Sato, 1980a) or by its binding to the culture dishes (Cecil & Robinson, 1975). However, at present, most of the evidence indicates that the mitogenic effect of supraphysiological concentrations of insulin is mediated through interaction with a receptor for insulin-like growth factors (Bettger et al., 1981; Lenoir & Honegger, 1983).

Glucocorticoids have been shown to affect the proliferation of cultured cells from a variety of animal species (Cristofalo & Rosner, 1981). The growth response to glucocorticoids will vary depending on the cell type, species, tissue of origin, culture conditions and state of differentiation of the cells. To exert their proliferative effects glucocorticoids may require the presence of serum (Irwin, 1982) or serum-free medium (Bettger et al., 1981) or other growth factors (Rudland & De Asua, 1979).

Another molecule, the iron binding protein, transferrin, is reported to be important for cell proliferation (Barnes & Sato, 1980b) and for cell differentiation (Ekblom et al., 1983). Since it is also an abundant component of serum, the dynamics of transferrin receptor, rather than the presence of the ligand itself appears to be crucial in these events. A distinct cell surface glycoprotein, known to be associated

with proliferation, was recently shown to be identical to the transferrin receptor (Ekblom et al., 1983).

Another important question is whether different growth promoting molecules stimulate proliferation via similar mechanisms or each has its characteristic mode of action. Studies using quiescent mouse fibroblasts (De Asua, 1980) suggest that the stimulation of DNA synthesis by different growth factors may involve different sets of events. Synergistic effects of two growth factors used together are reported to suggest independent mechanisms of action. A search for synergistic effects and for independent actions of three growth factors at once were used to assess, in this study, whether different hormones affect endometrial epithelial cell proliferation by different mechanisms.

4.310 CELL ATTACHMENT

Serum-free medium containing EGF and insulin permitted the mixed cell population to settle down at a faster rate (Table 4.3). In fact the maximum number of cells were noted to attach to the plastic substrate with this medium during the first 6h of cultures (Table 4.3), but only a minimum number of cells managed to spread and survive. Some of the rat endometrial epithelial cells were also observed to be attached (Table 4.4) but they required serum to spread and start growth.

4.320 CELL PROLIFERATION

EGF enhanced cell proliferation significantly in serum-free medium (Figs. 4.7, 4.8, 4.9). Insulin alone did not induce mitogenic changes over 2 days but, in conjunction with other growth factors, did increase proliferation (Figs. 4.7-4.9). Transferrin, alone and in combination, increased cellular growth (Fig. 4.9). Stimulation of growth by the optimal concentration of transferrin was further promoted by EGF and dexamethasone but was unaffected by insulin. Dexamethasone or cortisol, in combination with EGF, insulin and/or transferrin promoted cell proliferation.

Figure 4.7: Effect of growth factors on the growth of rat endometrial epithelial cells in serum-free medium (SFM)

Cells were prepared (see Section 2.512) and cultured (see Section 2.520) with 5% FCS-DCC(4) for 12h. Medium was, then, changed and appropriate growth factors were added in the complete absence of serum. After another 48h, cells were counted (see Section 2.710). Each point is the mean $\pm$ S.D. of triplicate experiments. Concentrations of the hormones used were as follows:

EGF - Epidermal growth factor (10ng/ml)

I - Insulin (5µg/ml)

C - Cortisol (10^{-7}M)

D - Dexamethasone (10^{-7}M)

The statistical significance of the observations is:

* $P < 0.01$

** $P < 0.005$

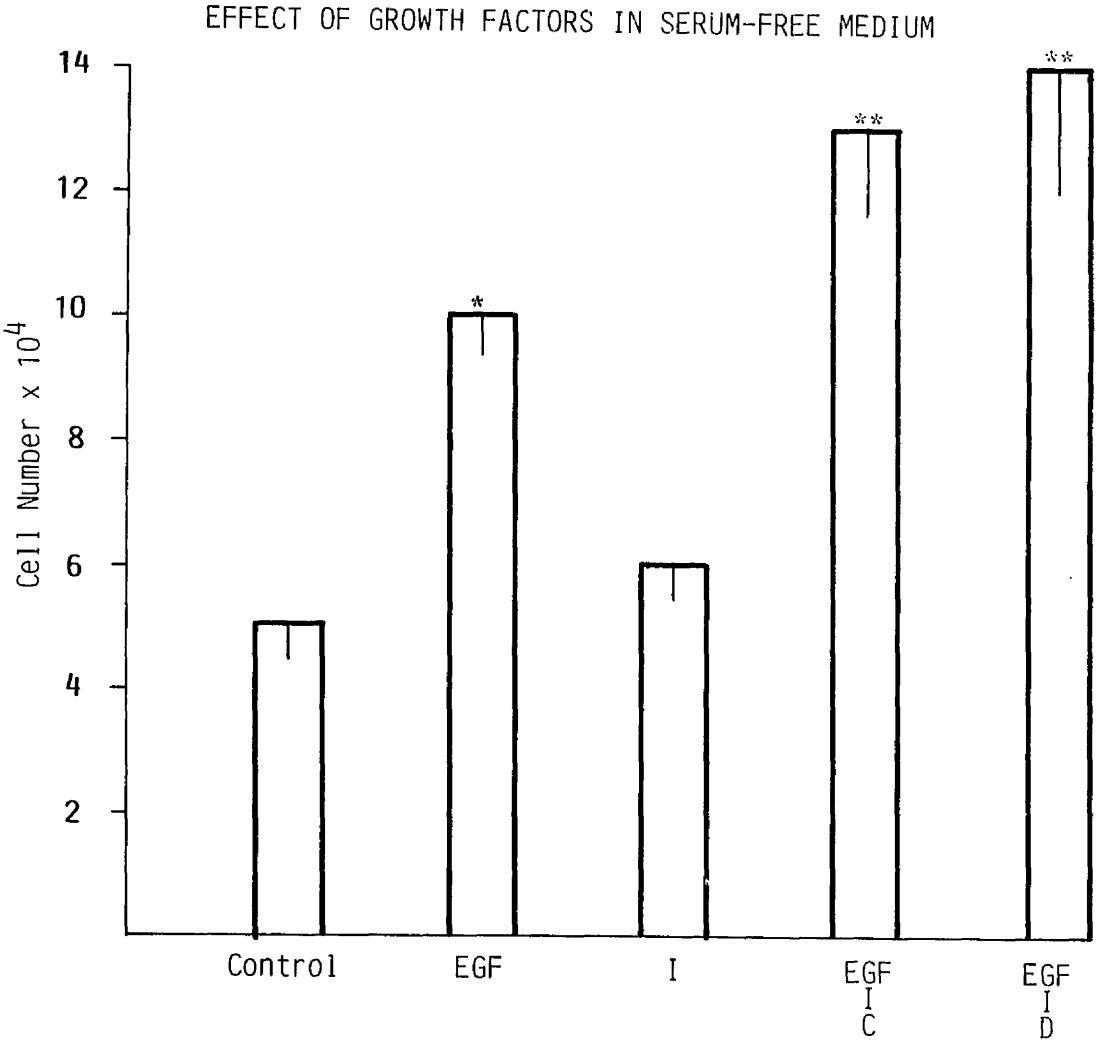

EFFECT OF GROWTH FACTORS IN SERUM-FREE MEDIUM
Cell Number x 10^4
14
12
10
8
6
4
2
*
**
**
Control
EGF
I
EGF I C
EGF I D

Figure 4.8: Effect of growth factors, alone and in combination, on the growth of rat endometrial epithelial cells in serum-free medium (SFM)

Cells were prepared, cultured and processed as described in Figure 4.7. Concentrations of various growth factors used were as follows:

EGF - Epidermal growth factor (10ng/ml)

I - Insulin (5μg/ml)

C - Cortisol (10^{-7}M)

D - Dexamethasone (10^{-7}M)

Each point is the mean ± S.D. of triplicate experiments.

* $P < 0.01$

** $P < 0.005$

*** $P < 0.001$

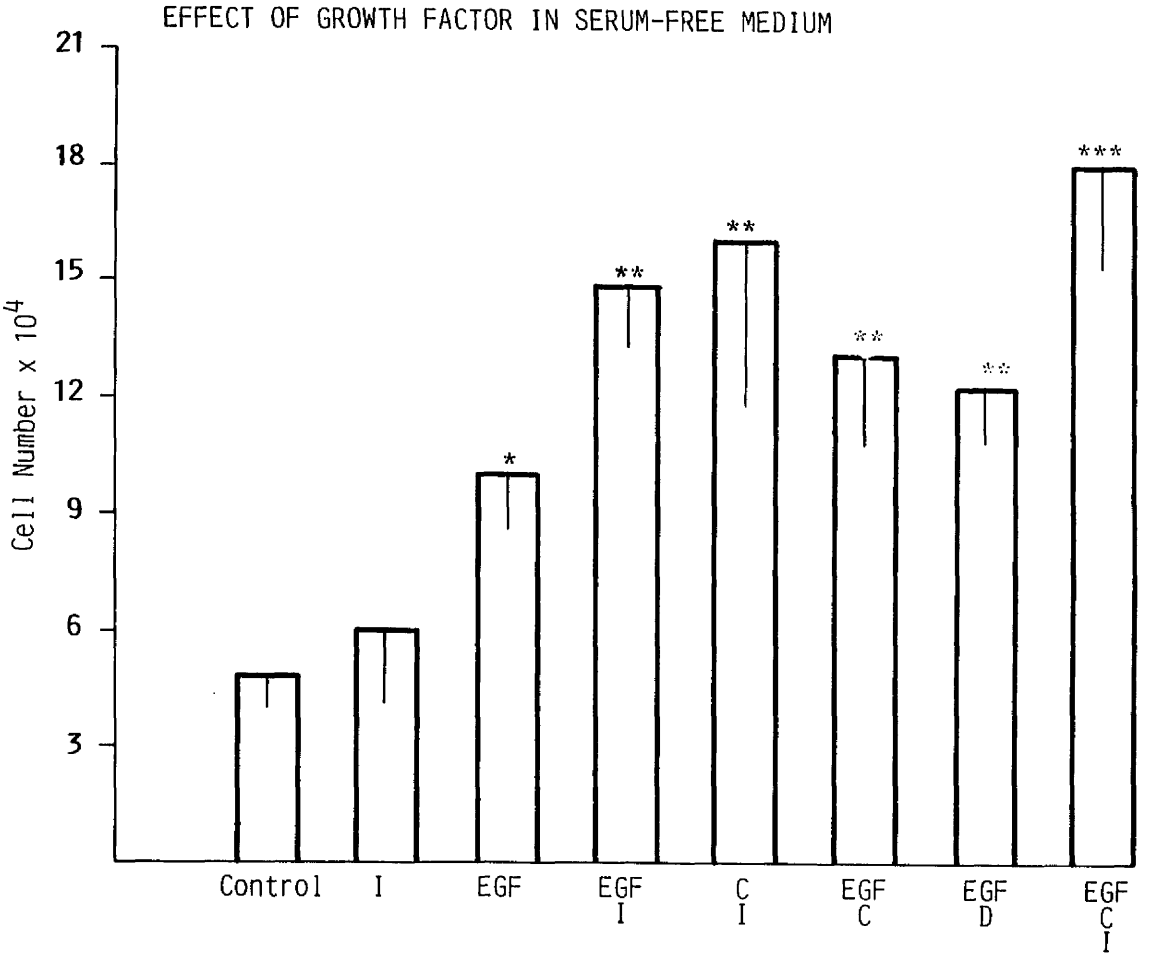

EFFECT OF GROWTH FACTOR IN SERUM-FREE MEDIUM
Cell Number x 10^4
21
18
15
12
9
6
3
*
**
**
**
**
***
Control
I
EGF
EGF I
C I
EGF C
EGF D
EGF C I

Figure 4.9: Effect of growth factors on the growth of rat endometrial epithelial cells in SFM

Epithelial cells were processed as described in Figure 4.7.

Concentrations of growth factors were as follows:

EGF - Epidermal growth factor (10ng/ml)

I - Insulin (5μg/ml)

D - Dexamethasone (10^{-7}M)

T - Transferrin (5μg/ml)

Each point is the mean ± S.D. of triplicate cultures.

* $P < 0.005$

** $P < 0.001$

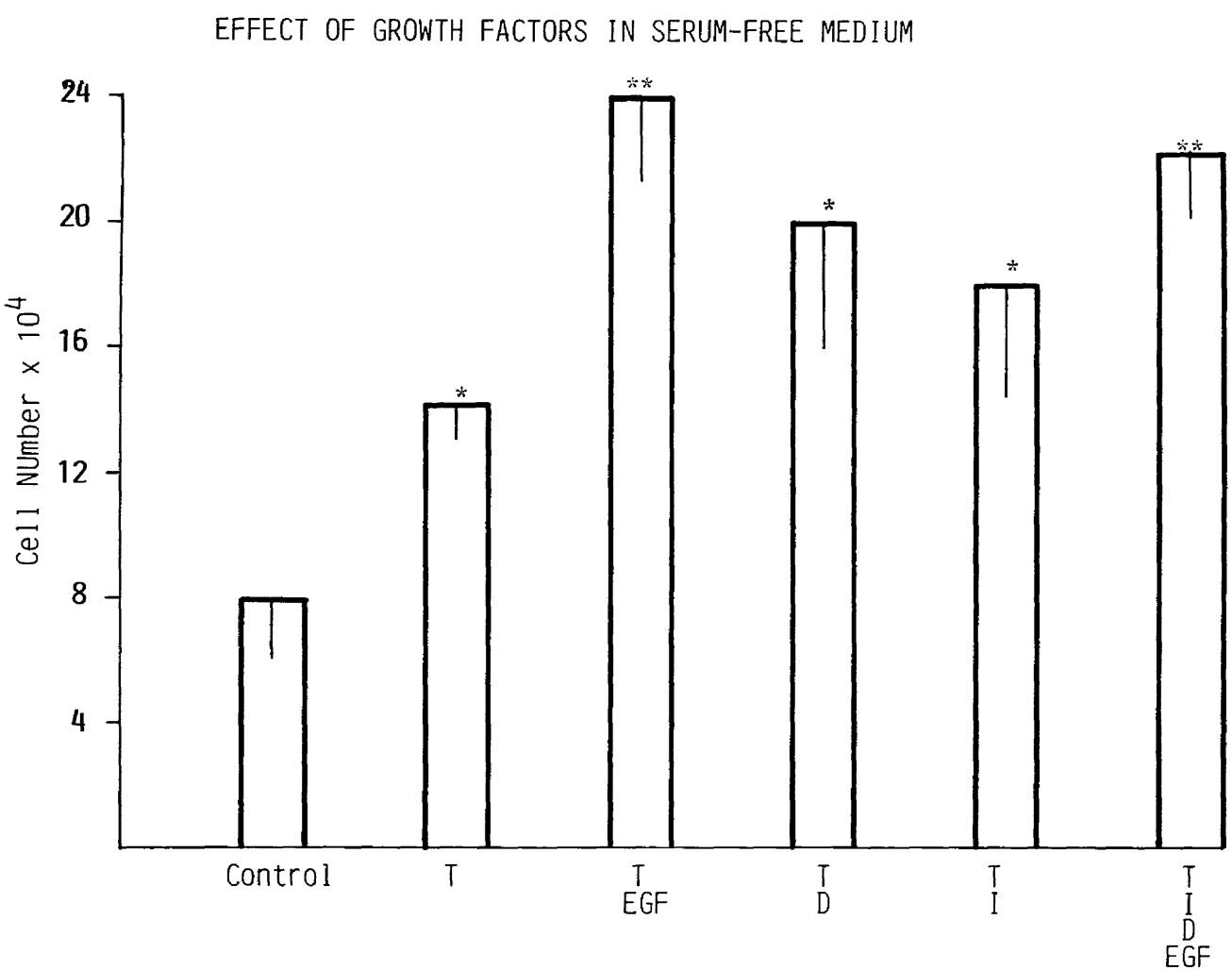

EFFECT OF GROWTH FACTORS IN SERUM-FREE MEDIUM
Cell NUmber x 10^4
24
20
16
12
8
4
**
*
*
*
**
Control
T
T EGF
T D
T I
T I D EGF

Table 4.6: Effect of whole serum(FCS) and charcoal stripped serum (FCS-DCC(56)) on the growth of the mixed cell populations

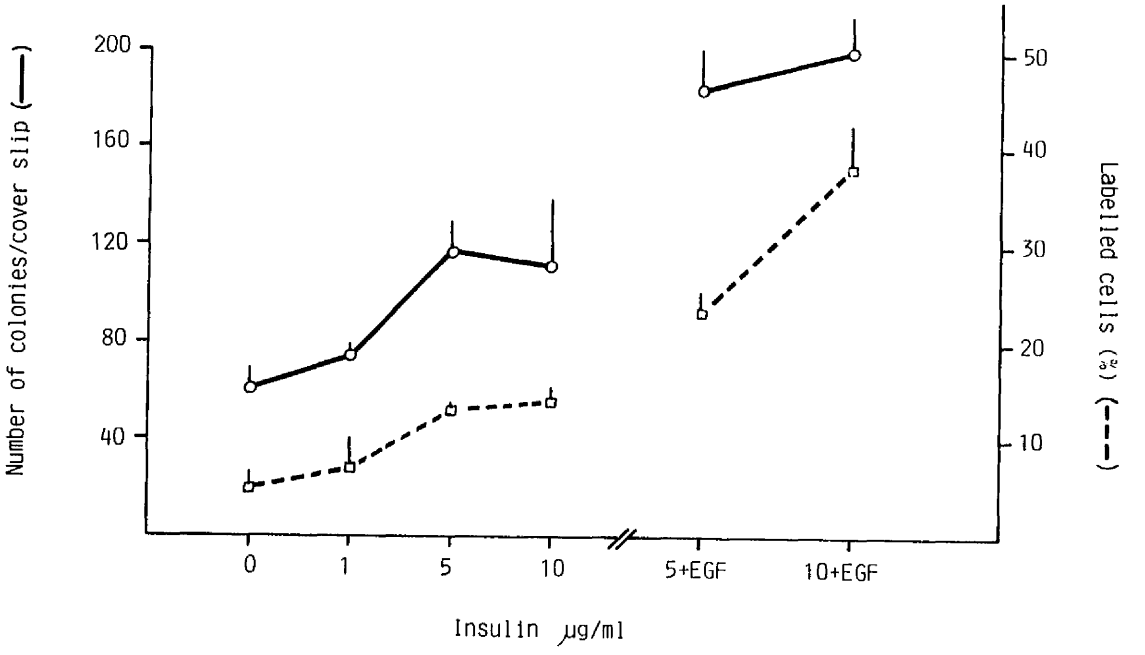
Cells were prepared (see Section 2.511) and cultured as described in Section 2.520. Cells were plated down with 10% whole serum (FCS) for the first 12h of each passage, after which medium was replaced with a fresh one containing 10% FCS or charcoal stripped serum (FCS-DCC(56). At the end of each day cells were harvested (see Section 2.530) and counted as described in Section 2.710. DNA (see Section 2.750) and protein (see Section 2.740) contents were determined in each cell suspension.

Days in culture	Serum	Cell Number ($x10^5$)	DNA (ug)	Protein (ug)
		1ST PASSAGE		
1	FCS	14.3	14.8	290
	FCS-DCC(56)	14.7	11.2	310
2	FCS	21.0	12.6	475
	FCS-DCC(56)	19.0	10.5	520
3	FCS	18.9	10.0	510
	FCS-DCC(56)	15.0	4.0	450
4	FCS	17.0	8.8	500
	FCS-DCC(56)	16.2	10.5	530
		2ND PASSAGE		
1	FCS	3.3	5.7	93
	FCS-DCC(56)	3.6	5.8	91
2	FCS	2.6	7.0	200
	FCS-DCC(56)	3.3	7.7	206
3	FCS	3.8	1.04	183
	FCS-DCC(56)	3.9	1.37	193
4	FCS	1.2	1.44	175
	FCS-DCC(56)	2.5	1.28	230

Figure 4.10: Effect of Insulin on growth of rat endometrial epithelial cells

Epithelial cells were prepared and cultured in 5% FCS-DCC(4) for 12h as described in Figure 4.7. Medium was replaced with SFM and various concentrations of insulin, alone and combined with EGF (10ng/ml) were added. Cells were cultured for another 4 days. Then cells were counted (see Section 2.710) or pulsed with ^{3}H-dT for 3h and processed as described in Section 2.731.

Each point is the mean $\pm$ S.D. of triplicate experiments.


Number of colonies/cover slip (——)
200
160
120
80
40
50
40
30
20
10
Labelled cells (%) (– – –)
0
1
5
10
5+EGF
10+EGF
Insulin µg/ml

4.330 DNA SYNTHESIS

A dose dependent response to insulin was observed in terms of thymidine utilisation and total number of colonies per unit area after 4 days (Fig. 4.10). This stimulation was further enhanced by the addition of EGF. Thymidine incorporation was observed to be stimulated by insulin at the end of the third day of culture (Table 4.7) as compared to the control level. Increased DNA synthesis was noted in medium containing EGF plus insulin with time (Table 4.7).

4.400 EFFECTS OF GROWTH FACTORS IN THE PRESENCE OF SERUM

EGF alone and with dexamethasone promoted cell growth in the presence of FCS (Fig. 4.11), whereas insulin, cortisol and transferrin induced no effect in rat endometrial epithelial cells over the two days. Various concentrations of FCS-DCC(4) were tried with added growth factors (Table 4.8). EGF alone seemed to be most effective at the highest serum concentration (10%). Cortisol and insulin also showed stimulating effects in the presence of FCS-DCC(4). FCS-DCC(56) enhanced growth in the presence of EGF, insulin and transferrin (Fig. 4.12). Cortisol also increased the cell number in the medium containing FCS-DCC(56), but insulin and transferrin alone did not show any significant effect.

In human endometrial cancer cells, increased growth was achieved in medium containing FCS-DCC(56) compared to FCS (Figure 4.13, 4.14). Insulin and EGF each stimulated growth in Serum-free Medium (SFM). EGF caused an increase in the number and size of colonies (Figure 4.13) relative to insulin. Insulin and EGF also promoted growth in presence of FCS but not FCS-DCC(56), whereas a cortisol response was seen in medium containing FCS-DCC(56) (Figure 4.14).

4.410 OTHER MEDIA

Rat endometrial epithelial cells were grown in different media. The best growth response was observed by Eagle's MEM (Figs.

Table 4.7: Effect of serum and growth factors on thymidine labelling index

Rat endometrial epithelial cells were prepared (see Section 2.512) and cultured in whole serum (FCS) for the first 12h. Then medium was replaced with appropriate media. At the end of each day, cells were incubated with ^{3}H-thymidine for 3h and processed as mentioned in Section 2.731. Each value of labelling indices is the average of two independent experiments.

	DAY IN CULTURE		
	1	2	3
Foetal calf serum (FCS)	17%	41%	25%
Charcoal stripped serum (FCS-DCC)	27%	37%	31%
Without serum		2%	3.5%
No serum + insulin		2%	7%
No serum + insulin + EGF		4.5%	10%

Table 4.8: Effect of serum and growth factors on the growth of endometrial epithelial cells

Rat endometrial epithelial cells were prepared and cultured as described in Table 4.7. After the first 12h incubation with 10% FCS, medium was replaced with a fresh one, containing various concentrations of serum and different growth factors. After another 48h, cells were recovered and counted (see Section 2.710). Each value is the average of two independent experiments.

	NUMBER OF CELLS (10^4)			
Serum(%)	Control	+EGF	EGF + INS	EGF + INS + C
10	5.1	9.68	10.3	12.2
5	2.6	4.0	5.6	7.0
3	2.2	2.5	3.5	5.6
2	2.0	2.6	3.0	5.0
1	1.3	2.0	3.3	6.0
0	0.4	1.0	1.7	2.0

Figure 4.11: Effect of growth factors on the growth of rat endometrial epithelial cells in the presence of FCS

Cells were isolated (see Section 2.512) and cultured in 10% FCS for 12h. Medium was then replaced with fresh medium containing FCS (10%) and appropriate growth factors. Cells were counted (see Section 2.710) after another 48h. Concentrations of the growth factors used were:

EGF - Epidermal growth factor (10ng/ml)

I - Insulin (5µg/ml)

C - Cortisol (10^{-7}M)

T - Transferrin (5µg/ml)

D - Dexamethasone (10^{-7}M)

Each point is the mean ± S.D. of triplicate cultures.

* $P < 0.02$

** $P < 0.005$

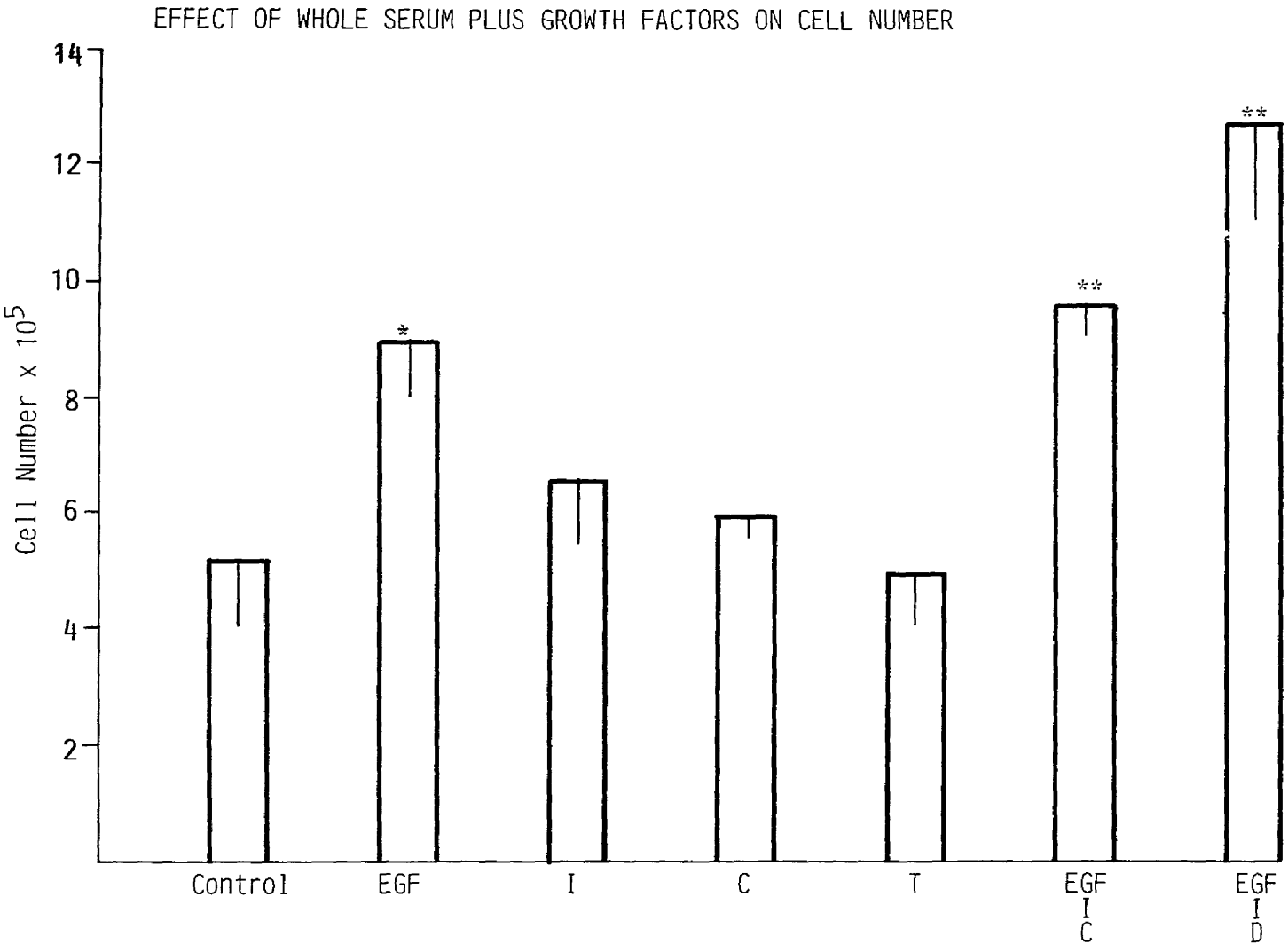

EFFECT OF WHOLE SERUM PLUS GROWTH FACTORS ON CELL NUMBER
Cell Number x 10^5
14
12
10
8
6
4
2
*
**
**
Control
EGF
I
C
T
EGF I C
EGF I D

Table 4.9: Effect of substrate on attachment of epithelial cells

Rat endometrial epithelial cells were isolated (see Section 2.512) and cultured on uncoated plastic surfaces or on surfaces coated with different substrates (see Section 2.526). Seven parallel cultures were prepared for each substrate. Cells were counted after 6, 12, 24 or 48h or medium was changed at 6, 12 or 24h and cells counted at 48h. All cultures were fed with 10% FCS.

Cell suspension was replaced with a fresh medium at

	6h		12h		24h	

Cells were counted after total time of

	6h	48h	**12h**	48h	**24h**	48h	**48h**
Control	**0.8×10^3**	5×10^4	**0.2×10^4**	6×10^4	**2.0×10^4**	9×10^4	**8.2×10^4**
Fibronectin	**1.7×10^3**	7×10^4	**0.7×10^4**	9×10^4	**2.2×10^4**	8×10^4	**8×10^4**
Gelatin	**3.5×10^3**	4×10^4	**1×10^4**	11×10^4	**2.5×10^4**	8×10^4	**8.1×10^4**
Fibronectin + Gelatin	**4×10^3**	8×10^4	**0.8×10^4**	8×10^4	**1.8×10^4**	7×10^4	**7.3×10^4**

Figures represent total number of cells at the time indicated.

All cultures were fed with whole serum (FCS)

Figure 4.12: Effect of growth factors on the growth of rat endometrial epithelial cells in the presence of FCS-DCC(56)

Cells were prepared (see Section 2.512) and cultured in 10% FCS-DCC(4) for 12h. Medium was then replaced with one containing 10% FCS-DCC(56) plus appropriate growth factors. Cells were counted after 48h. Concentrations of growth factors used were the same as in Figure 4.11. Each point is the mean ± S.D. of triplicate cultures.

* $P < 0.02$

** $P < 0.002$

EFFECT OF CHARCOAL STRIPPED SERUM PLUS GROWTH FACTORS ON CELL NUMBER

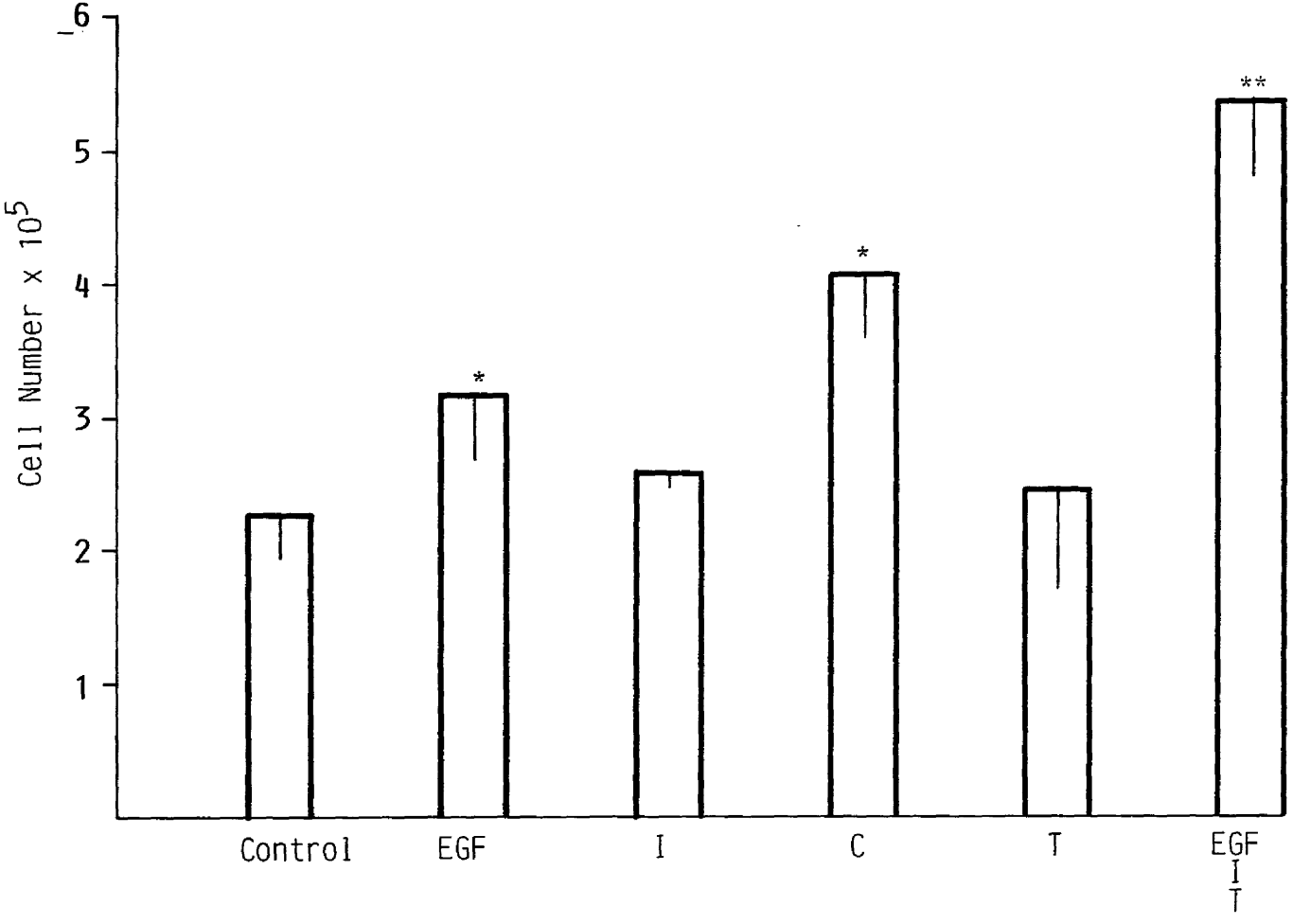


Table 4.10: Effect of serum and substrates on the growth of epithelial cells

Rat endometrial epithelial cells were prepared and cultured as described in Table 4.9. Cells were counted (see Section 2.710) after 48h, from two parallel sets, one being cultured with 10% FCS and the other with 10% FCS-DCC(56). Each value is the average of two experiments.

SUBSTRATE COATING	Whole Serum (FES)	FCS-DCC (56°C)
None	3.9×10^4	2×10^4
Fibronectin	4.5×10^4	5×10^4
Gelatin	5×10^4	4×10^4
Fibronectin/ Gelatin	6.5×10^4	3×10^4
Rat tail Collagen	4.5×10^4	5.5×10^4

Figure 4.13: Effect of serum and growth factors on the growth of human endometrial cancer cells

Endometrial cancer cells were prepared (see Section 2.514) and cultured in 5% FCS-DCC(4) for the first 24h. Medium was then replaced with one containing the following:

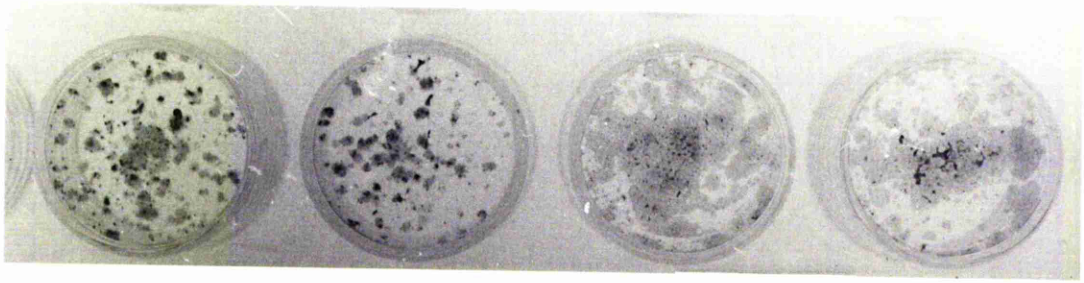
a) SFM + EGF (10ng/ml) x 1.4

b) SFM + insulin (5µg/ml) x 1.4

c) 5% FCS-DCC(56) x 1.4

d) 5% FCS x 1.4

Cells were grown for another 2 days, then fixed with methanol (see Section 2.541) and stained with Giemsa (see Section 2.550).



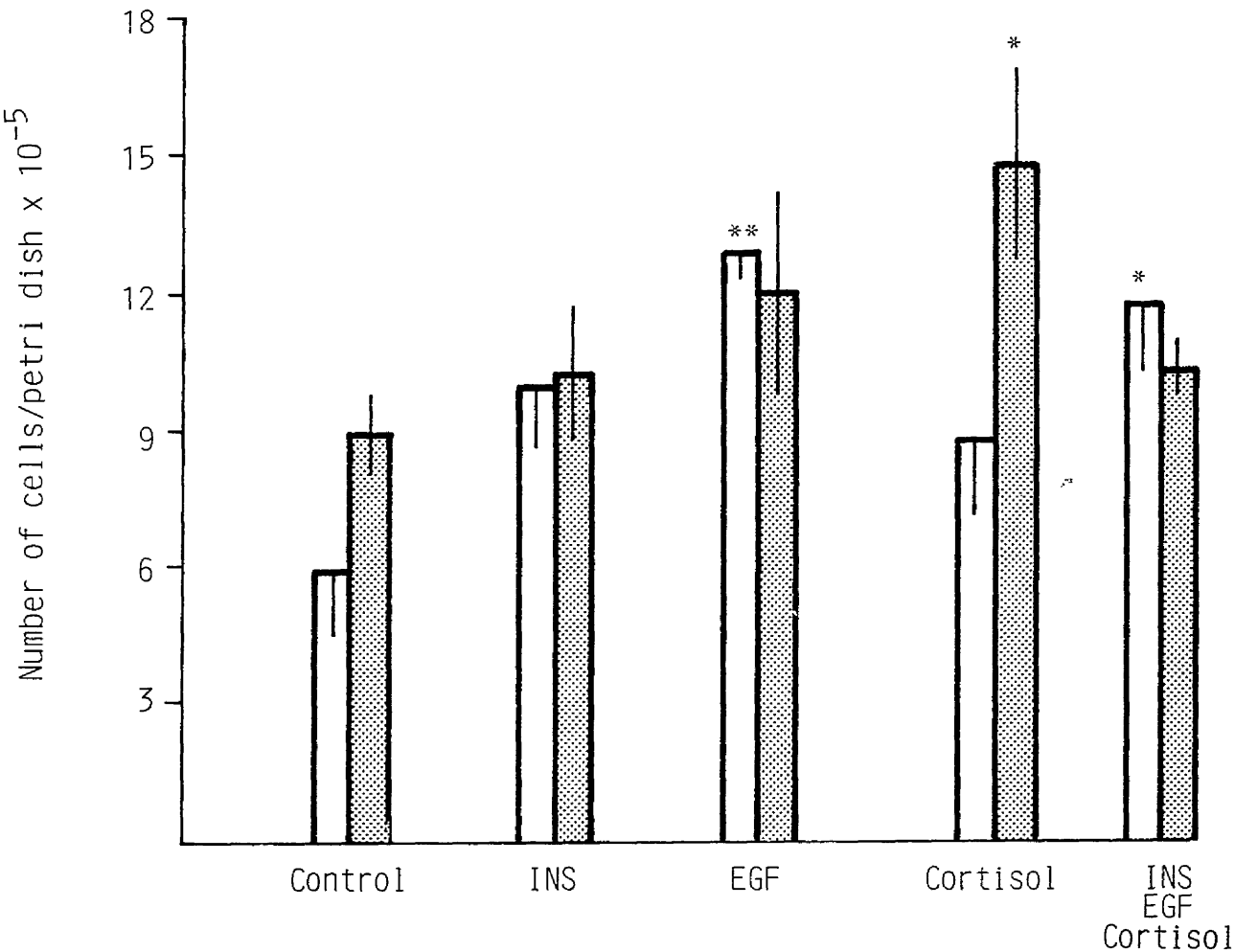
A B C D

Figure 4.14: Effect of serum plus growth factors on the growth of human endometrial cancer cells

Endometrial cancer cells were prepared and cultured as described in Figure 4.13. After 24h medium was replaced with 5% FCS or FCS-DCC(56) plus growth factors (as indicated). After another 72h, cells were counted (see Section 2.710). Each point is the average of duplicate experiments

* $P < 0.05$

** $P < 0.02$


Number of cells/petri dish x 10^{-5}
18
15
12
9
6
3
**
*
*
Control
INS
EGF
Cortisol
INS
EGF
Cortisol

4.15a, 4.16) in the presence of FCS. In the absence of serum, MCDB-104 increased cell proliferation tremendously with added factors (Figure 4.15b).

4.500 CELLULAR MORPHOLOGY

The structural characteristics of endometrial epithelial cells in primary cultures have been described in Chapter 3. These cellular structures varied widely with changed culture conditions. These structural modifications were studied by culturing the cells in SFM.

4.510 LIGHT MICROSCOPY

The selective growth of epithelial cells is shown in Figure 4.17. Proliferating fibroblastic cells can be observed in cultures growing with serum containing medium. The growth was restricted to colonies of epithelial cells in the absence of serum but in this case the presence of EGF was required. During the first 24h in FCS, fibroblasts do not divide but only complete the process of attachment and spreading (Fig. 4.17a). After 24h of incubation, fibroblasts surrounding the epithelial colonies start dividing (Fig. 4.17c), whereas, in cultures set up in SFM there are no traces of fibroblastic growth (Fig. 4.17b,d).

Another interesting observation was made concerning the behaviour of epithelial cells towards thymidine labelling. The loose cells outside the colonies or at the edges of colonies were heavily labelled in cultures grown in serum (Fig. 4.18a), whereas in SFM the central cells and inner parts of the colonies were comparatively more highly labelled (Fig. 4.18b).

After 4 days, cells in serum-enriched cultures were observed to be more closely packed in colonies and particularly of spindle shaped morphology (Fig. 4.18c). SFM provided the conditions where most of the cells were flattened and polyhedral in shape (Fig. 4.18d).

Figure 4.15: Effect of different media and growth factors on the growth of rat endometrial epithelial cells

Epithelial cells were isolated as described in Section 2.512 and cultured with 10% FCS in MEM (Eagle's medium) for 12h. Medium was then changed to:

a) appropriate media plus 10% FCS

b) MCDB 104 without serum but with added growth factors (EGF 10ng/ml, insulin 5μg/ml, cortisol 10^{-7}M).

Cells were further grown for 48h and counted (see Section 2.710). Each point is the mean ± S.D. of triplicate cultures.

* $P < 0.02$

** $P < 0.005$

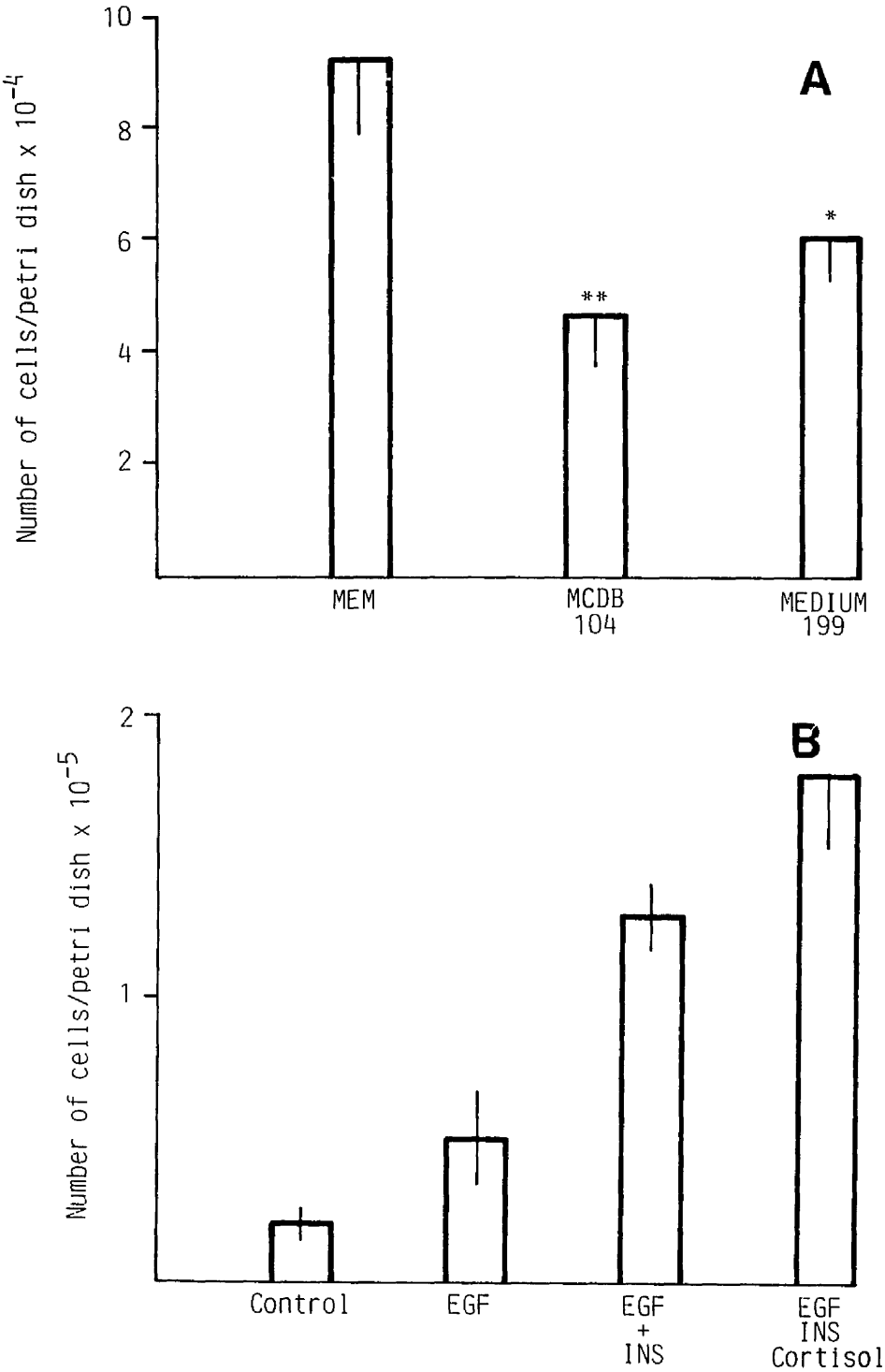

A
Number of cells/petri dish x 10^{-4}
10
8
6
4
2
**
*
MEM
MCDB 104
MEDIUM 199
B
Number of cells/petri dish x 10^{-5}
2
1
Control
EGF
EGF + INS
EGF INS Cortisol

Figure 4.16: Effect of media on the growth of rat endometrial epithelial cells

Cells were prepared and plated as described in Figure 4.15. After the first 12h, medium was replaced with:

a) MCDB 104 x 4

b) MEM (Eagle's medium)

with 10% FCS. After a further 48h, cells were fixed with methanol (see Section 2.541) and stained with Giemsa (see Section 2.550).

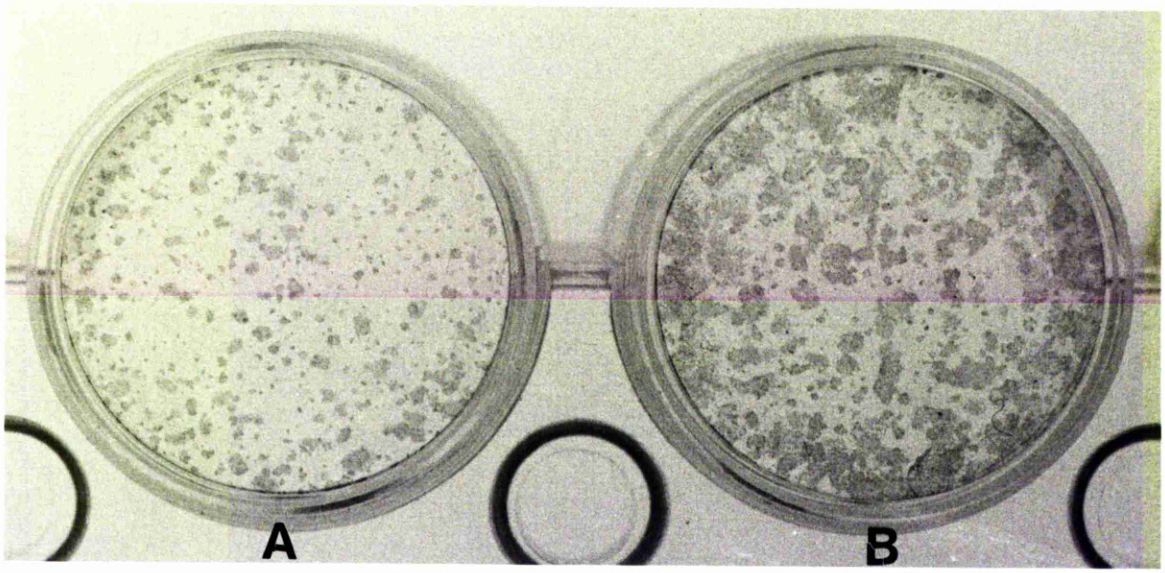

A
B

Figure 4.17: Effect of charcoal stripping of serum on the growth pattern of rat endometrial epithelial cells

Cells were isolated as described in Section 2.512 and plated in 10% FCS for 12h. Then medium was replaced with:

(a & c) 10% FCS

(b & d) 10% FCS-DCC(56)

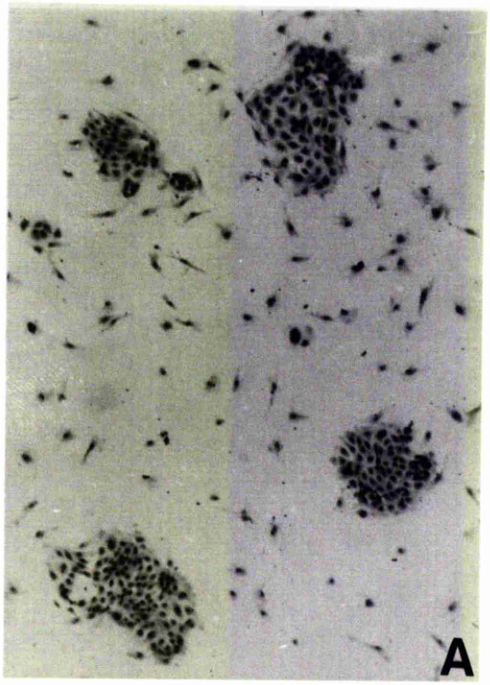
Cells were further cultured for 24h (a & b) and 48h (c & d). Note the lack of fibroblastic cells from the cultures grown with FCS-DCC(56)

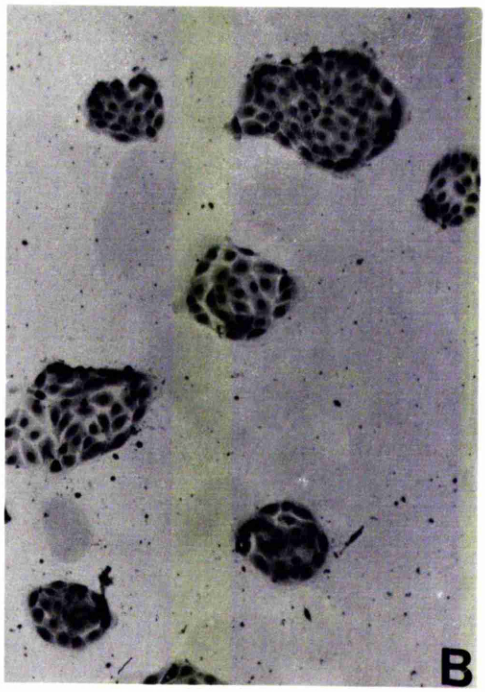
a) x 30

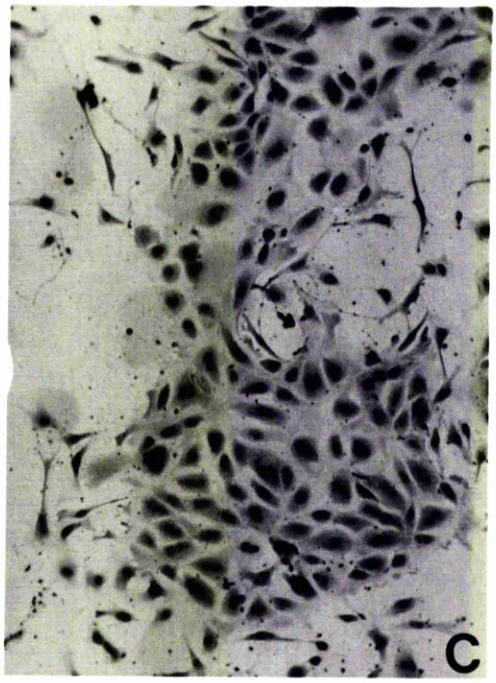
b) x 80

c) x 160

d) x 80


A


B


C

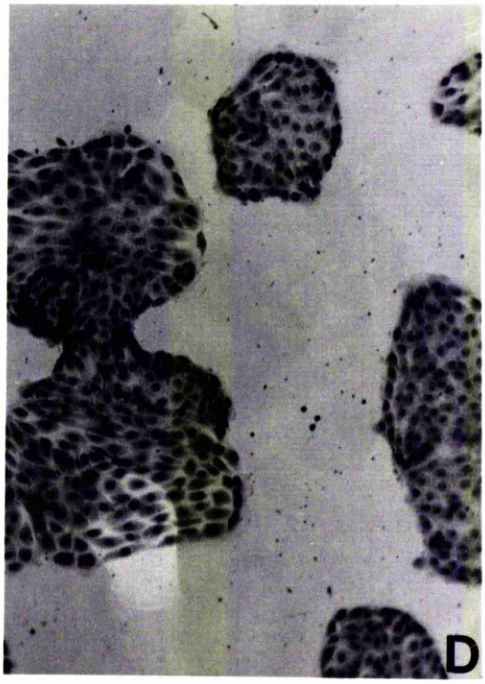

D

Figure 4.18: Effect of serum and SFM on growth patterns of rat endometrial epithelial cells

Cells were prepared (see Section 2.512) and cultured with 10% FCS for 12h. Medium was then replaced with:

(a & c) 10% FCS

(b & d) SFM

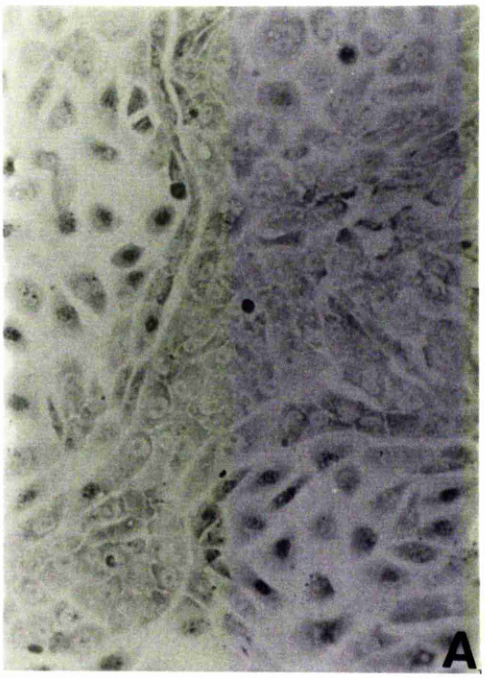
After a further 48h cells in a & b were processed for ^{3}H-thymidine labelling index (see Section 2.731) and c & d were fixed after 4 days with methanol (see Section 2.541) and stained with Giemsa (see Section 2.550).

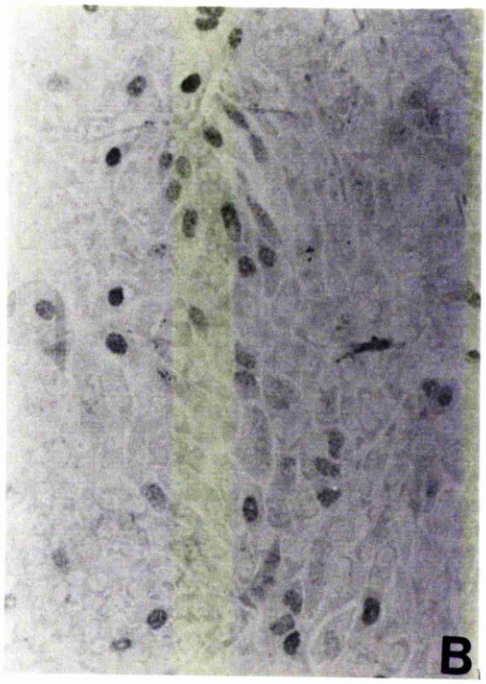
a) x 80

(b) x 80

c) x 80

d) x 80


A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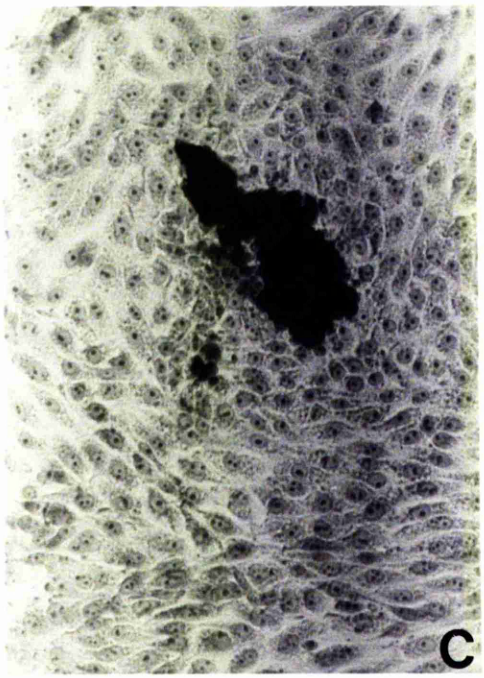
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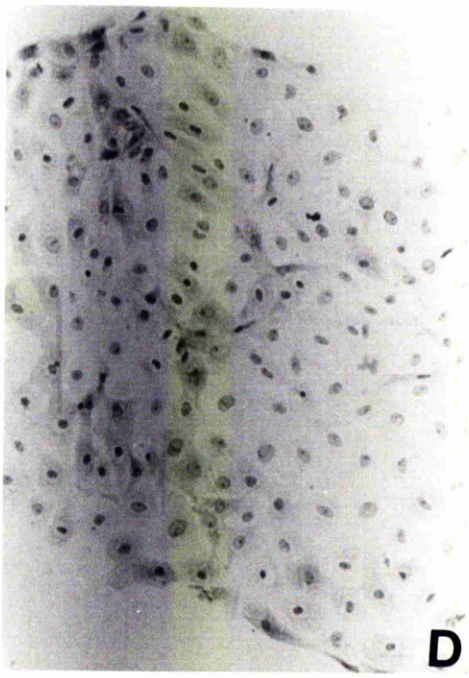

D

4.511 Cytoskeleton

Fluorescent staining patterns were observed after incubating the cells with antibody against tonofilaments. Staining was equally distributed in cells throughout the colony with SFM (Fig. 4.19a). In medium containing serum peripheral cells in colonies showed heavy staining compared to the next layer of cells (Fig. 4.19b).

The distribution of actin cytoskeleton was observed using NBD-Phallacidin, a fluorescent marker for cellular actin (see Section 2.580), in cultures fed with serum and in SFM. As is seen in Figure 4.20, actin filaments were equally distributed throughout the cytoplasm of the cells growing in serum added medium (Fig. 4.20a). In the case of SFM, fluoresence accumulated specifically around the nucleus (Fig. 4.20b).

4.520 ULTRASTRUCTURE

When viewed by SEM, the epithelial cells from serum-enriched cultures displayed conspicuous surface features. Microvilli were generally more numerous, thin, long and interconnected (Fig. 4.21a). The surface of some of the cells appeared somewhat lumpy, being covered by 'carved up' microvilli. A few of the microvilli were found connecting adjacent cells (Fig. 4.21a). In contrast, the cells cultured in SFM exhibited thick, comparatively small, stubby microvilli on the free surface (Fig. 4.22a,b). Peripheral cells of the colonies, in both cases were lacking microvillous structures (Figs. 4.21c; 4.23b). Intercellular junctions were found more convoluted, reflecting perhaps tighter binding between adjacent cells in cultures with SFM (Fig. 4.23a) than between serum provided cells (Fig. 4.21b). Another rounded structure made by small blebs was observed on the surface of the cells, usually peripheral cells, grown in serum added medium, (Fig. 4.21c).

The study of cell morphology at ultrastructural level by TEM confirmed the observation made by SEM. The epithelial cells grown in SFM were joined by desmosomes at frequent intervals (Fig. 4.24a) and

Figure 4.19: Effect of serum and SFM on the cytoskeleton of rat endometrial epithelial cells

Cells were prepared as described in Section 2.512 and cultured with 10% FCS for 12h. Medium was then changed to:-

a) SFM x 630

b) medium containing 10% FCS x 630

Cells were grown for a further 48h then labelled with an antibody against tonofilaments (see Section 2.560) and photographed through a fluorescent microscope.

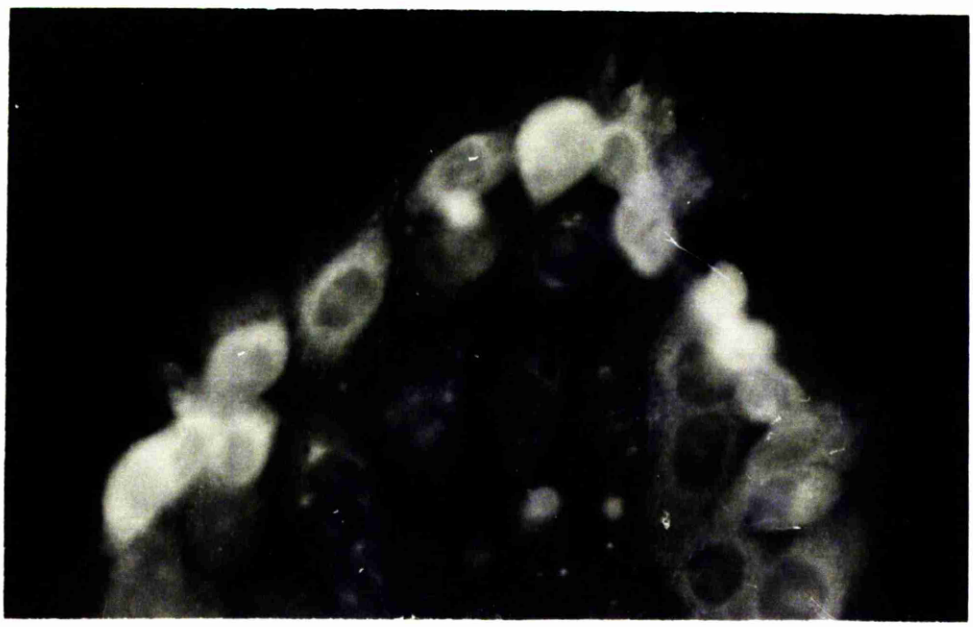
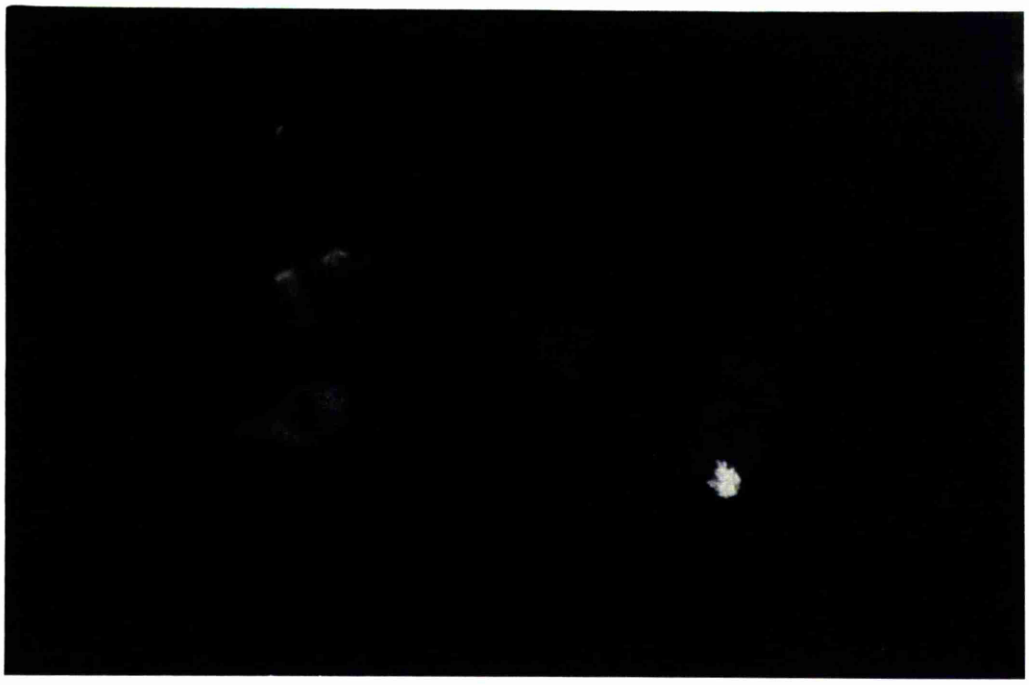


Figure 4.20: Effect of serum and SFM on the cytoskeleton of rat endometrial epithelial cells

Cells were prepared and cultured as described in Figure 4.20 and stained with NBD Phallacidin, a fluorescent stain for actin (see Section 2.580).

a) 10% FCS x 280

b) SFM x 280



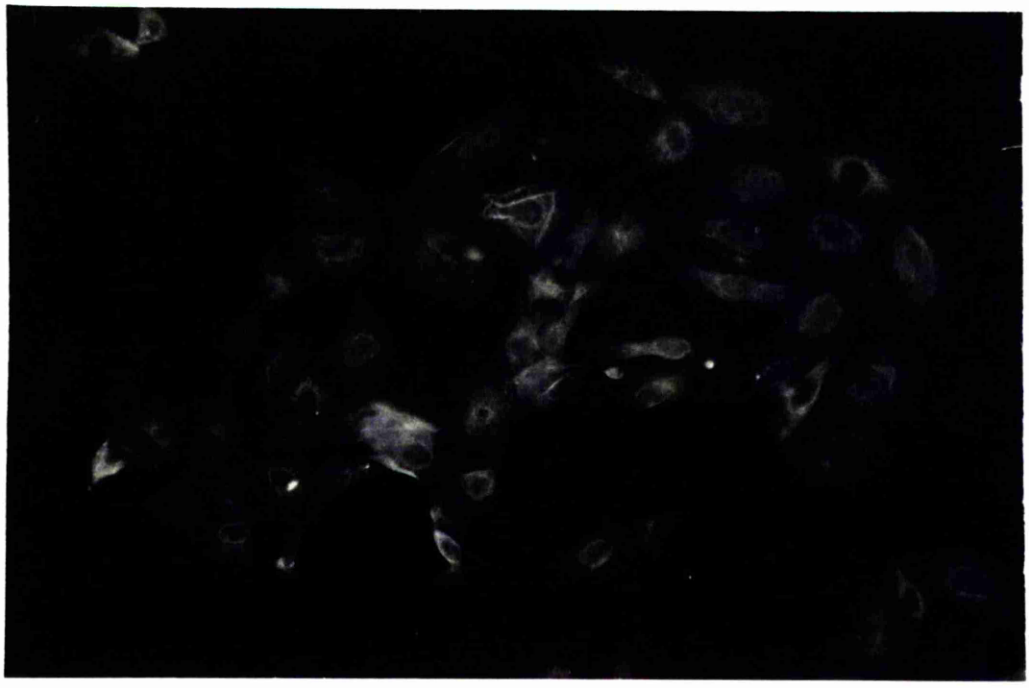


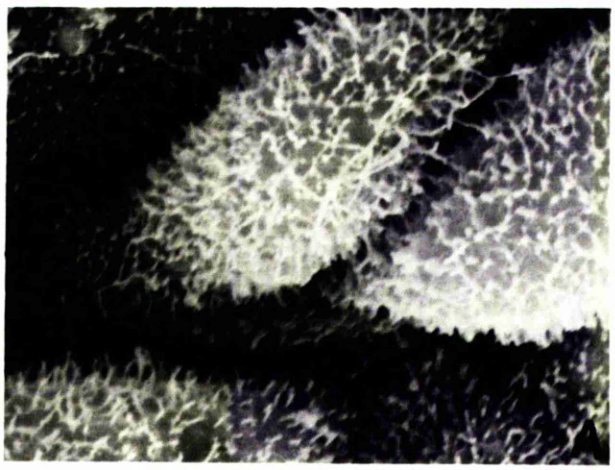
Figure 4.21: Effect of serum on the surface morphology

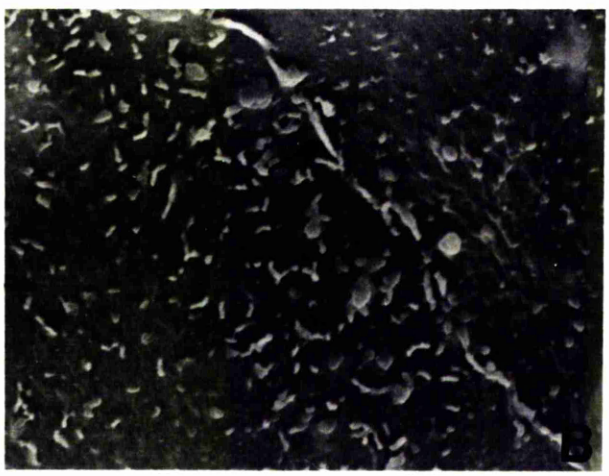
Scanning electron micrographs (SEM) of rat endometrial epithelial cells (see Section 2.512) cultured in 10% FCS for 12h + 2 days

a) Thin long microvilli on the surface of the cells, interconnecting with each other x 2500

b) Intercellular junctions between neighbouring cells (arrow). Note that these are uniformly linear x 5000

c) A peripheral cell from an epithelial cell colony. Note the lack of abundant microvilli and the presence of a rounded structure (arrow) on the surface of the cell x 5000





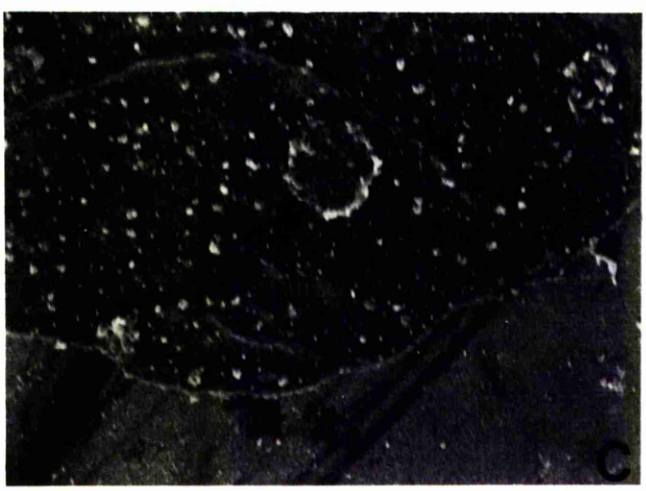
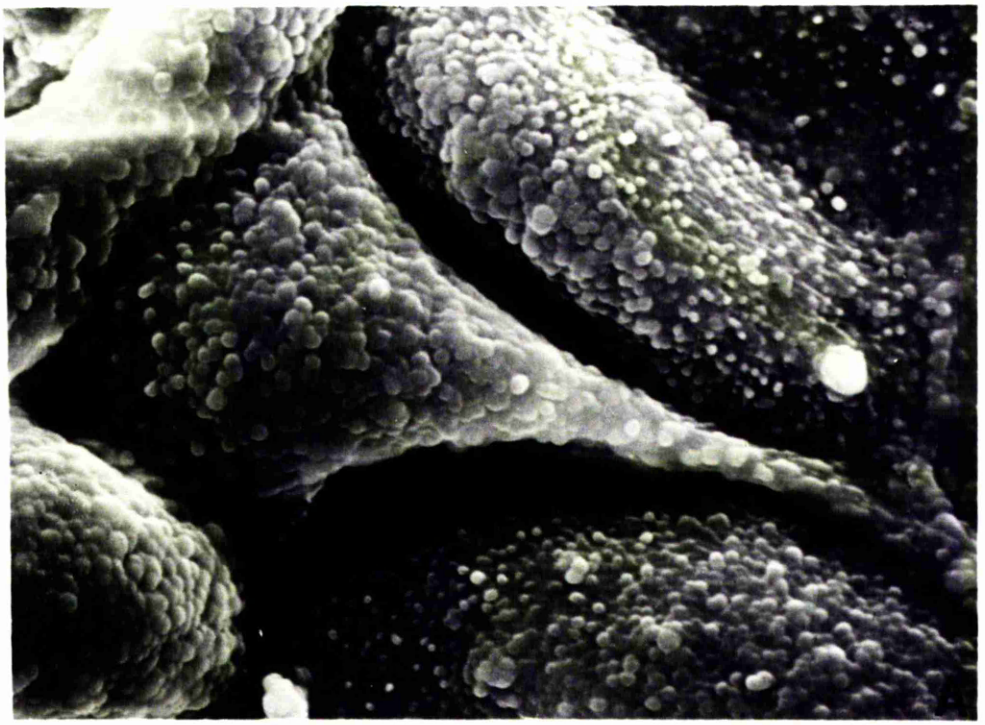


Figure 4.22: Effect of SFM on the surface morphology

Rat endometrial epithelial cells were prepared (see Section 2.512) and cultured for 12h with 10% FCS. Medium was then changed and cells were cultured in SFM for another 2 days and processed for SEM (see Section 2.622).
Note the decreased length and thickness of microvilli compared to cells cultured in whole serum (Figure 4.21).

a) x 4400

b) x 17900



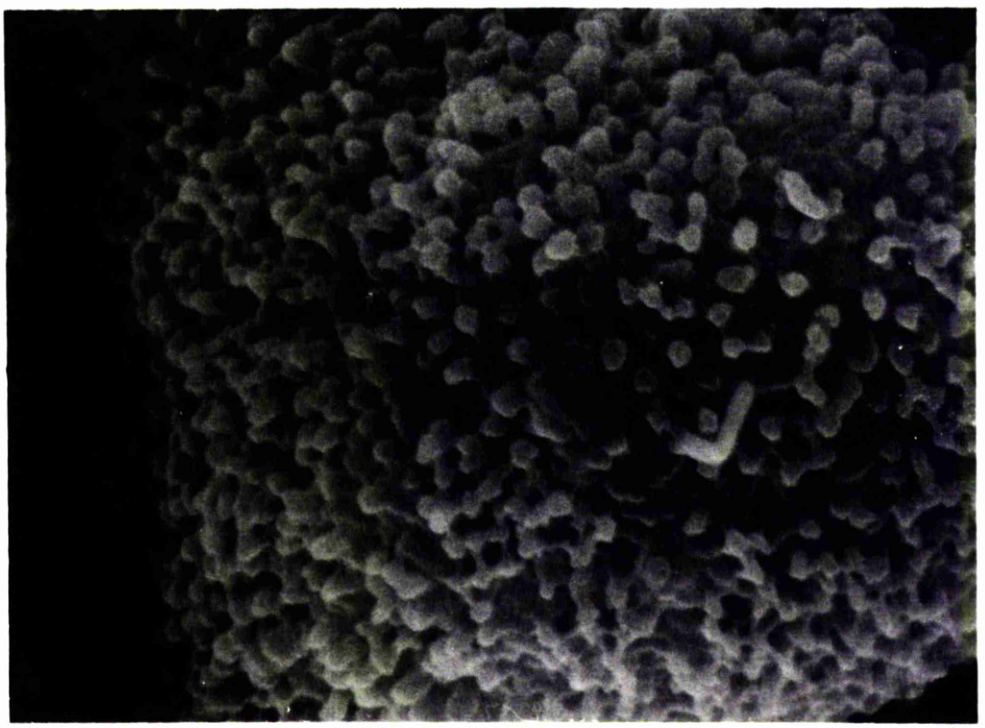
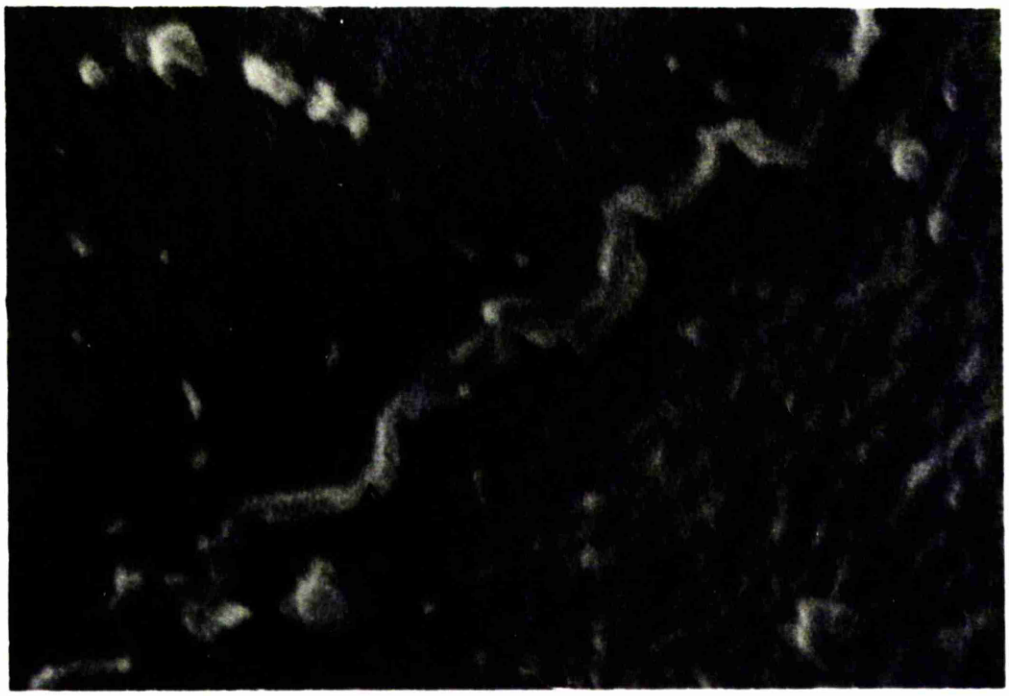


Figure 4.23: Effect of SFM on the surface morphology

SEM of rat endometrial epithelial cells prepared as described in Figure 4.22.

a) Intercellular junctions (arrow) note that these are convoluted x 35300

b) Peripheral cells of the epithelial cell colony, again with very few microvilli on the surface x 8800



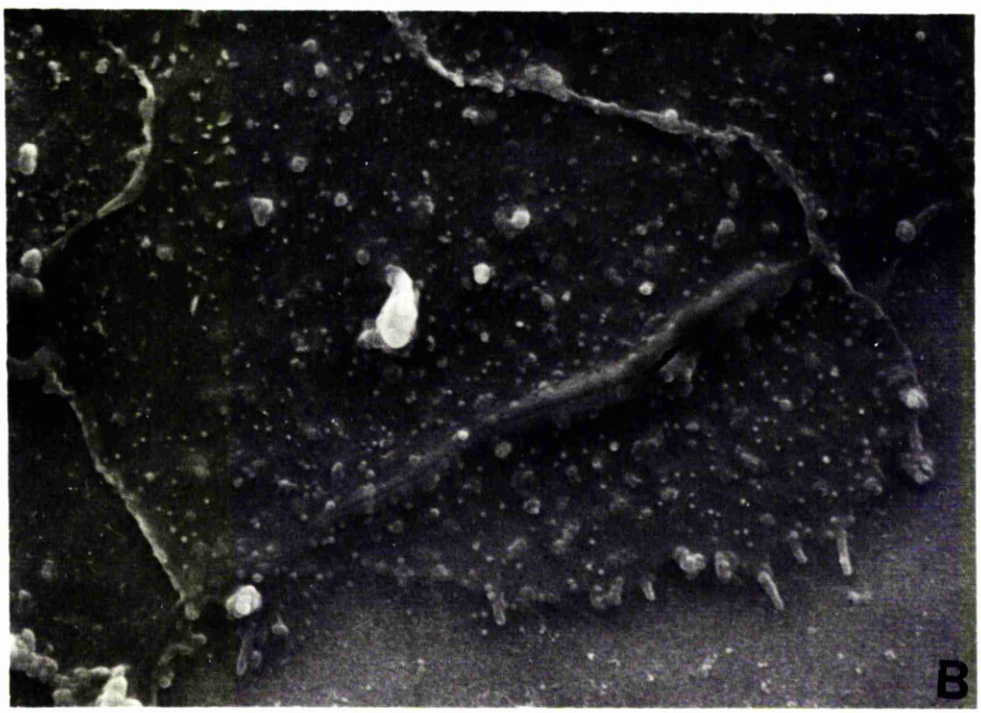
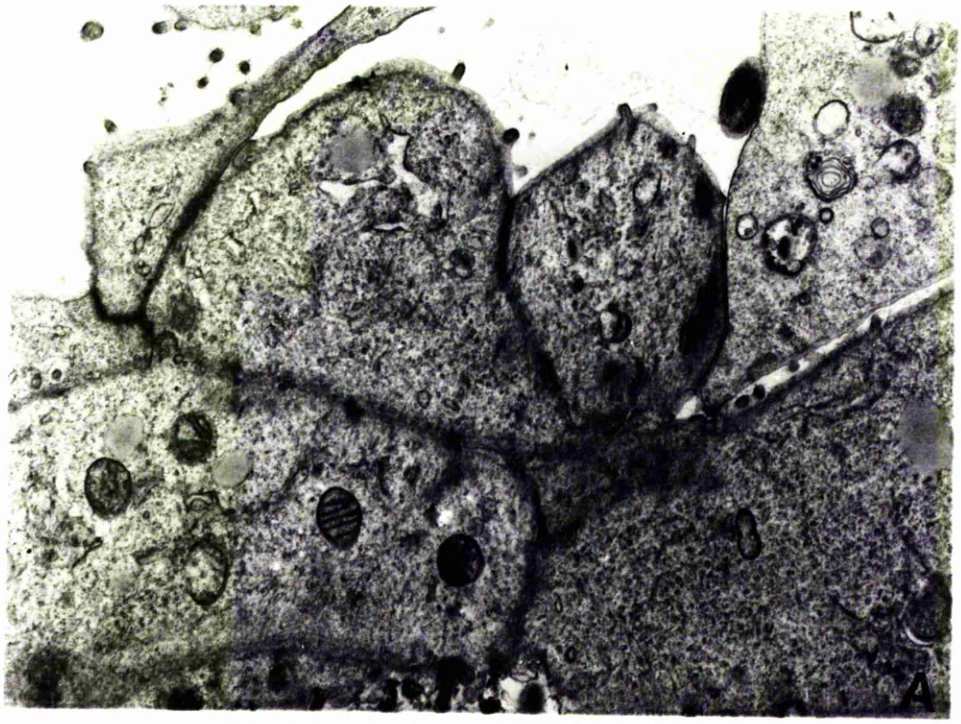

B

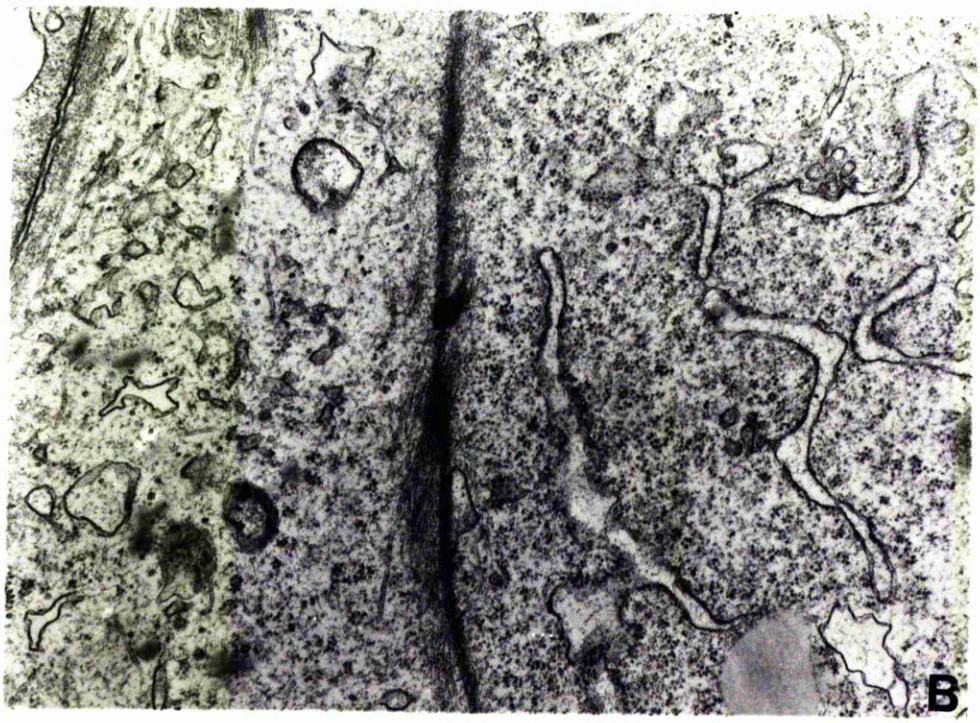
Figure 4.24: Effect of SFM on cellular morphology

Rat endometrial epithelial cells were prepared as described in Figure 4.22 and processed for TEM as explained in Section 2.621.

a) Note the frequency of desmosomes between the adjacent cells x 18344

b) Note the bundles of tonofilaments running through intercellular junctions and increased amount of RER x 31044


A


B

contained bundles of keratin filaments (Fig. 4.24b). Convoluted types of junctional complexes were also observed (Fig. 4.25a). SFM caused an increase in Rough Endoplasmic Reticulum (RER) and lysosomal materials (Figs. 4.24; 4.25). The size and quantity of lipid droplets remained variable from cell to cell, but an overall increase was noticed in cultures maintained by SFM (Fig. 4.25a).

4.600 EFFECT OF SUBSTRATES

In an effort to elucidate the nature of the serum factors involved in attachment and proliferation of the cells, analysis of the interactions of endometrial mixed and epithelial cells with various substrates, was included in this study. Figure 4.26 shows the effect of fibronectin and gelatin on attachment and growth of a rat uterine mixed cell population.

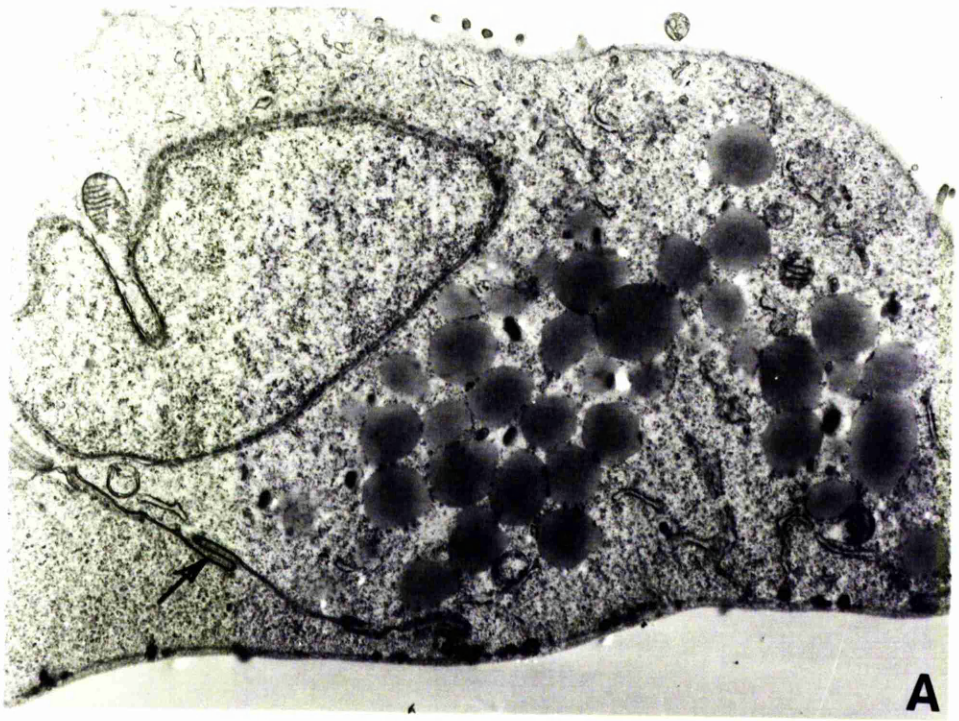
Attachment was observed to be rapid on the plastic dishes coated with fibronectin either alone or with gelatin. Cell numbers were elevated by 6h and remained above control for at least 48h. Little, if any, change was observed when cultures were exposed to gelatin alone. In the case of rat endometrial epithelial cells, fibronectin and gelatin, alone and in combination, enhanced attachment, but this was apparent relative to control levels only during the first 12h of culture. After 48h the number of cells in control and experimental dishes was equal (Table 4.9). Both of these substrates increased attach ment as well as growth over a period of 48h in the presence of FCS-DCC(56). Even more growth was seen in FCS-DCC(56) when the substrate was coated with rat tail collagen (Table 4.10). Figure 4.27 shows the morphology of epithelial cells on these substrates. A fibronectin-coated dish showed the cells scattered in an elongated form, with a more spread and flattened morphology (Fig. 4.27b). Gelatin coated dishes presented more

Figure 4.25: Effect of SFM on cellular morphology

TEM of rat endometrial epithelial cells as described in Figure 4.24.

a) Note the convoluted junctional complex (arrow) and abundant amount of lipid x 10580

b) Note the increased amount of lysosomes and RER x 25000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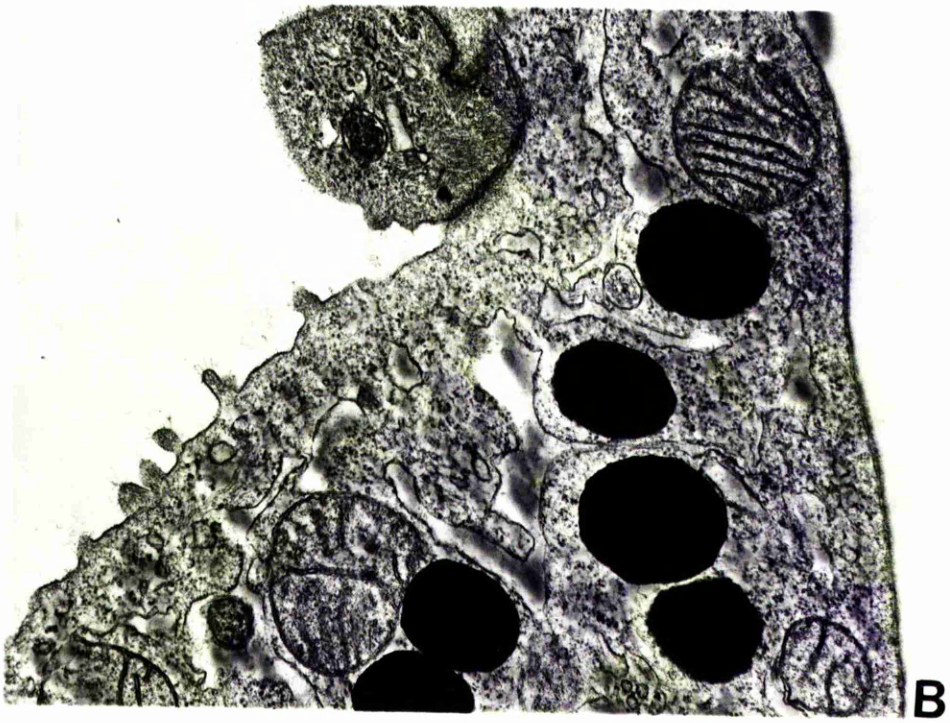

B

Figure 4.26: Effect of substrates on attachment and growth of the rat uterine mixed cell population

Rat uterine mixed cells were prepared (see Section 2.511) and cultured in 10% FCS. The plastic surface was coated with various substrates (see Section 2.526). Cells were counted (see Section 2.710) at each time indicated. Each point is the average of duplicate cultures.

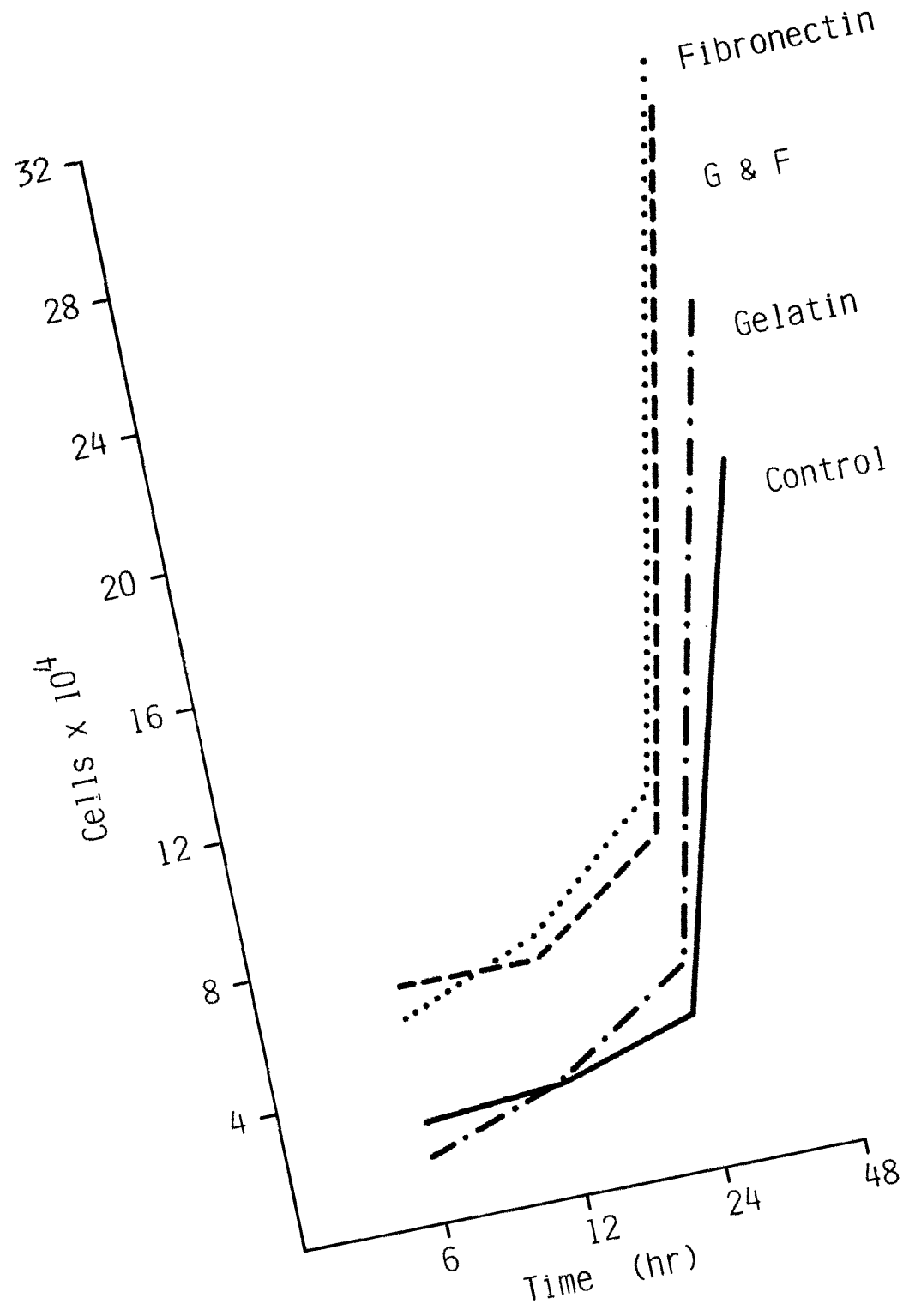

Fibronectin
G & F
Gelatin
Control
32
28
24
20
16
12
8
4
Cells x 10⁴
6
12
24
48
Time (hr)

Figure 4.27: Effect of substrate on growth and morphology of rat endometrial epithelial cells

Cells were prepared (see Section 2.512) and cultured with 10% FCS on the following substrates:

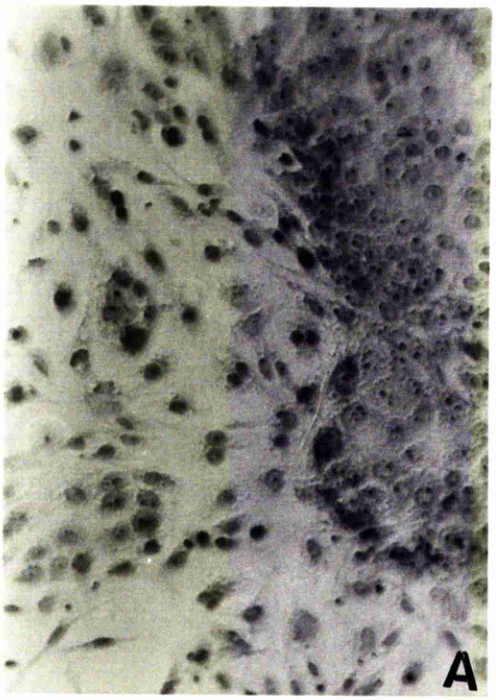
a) Control, plastic surface x 120

b) Fibronectin-coated dish x 120

c) Fibronectin/gelatin-coated dish x 120

d) Gelatin-coated dish x 120

e) SEM of epithelial cells grown on rat tail collagen (see Section 2.526). Note the elongated outgrowths from the cell surfaces x 5000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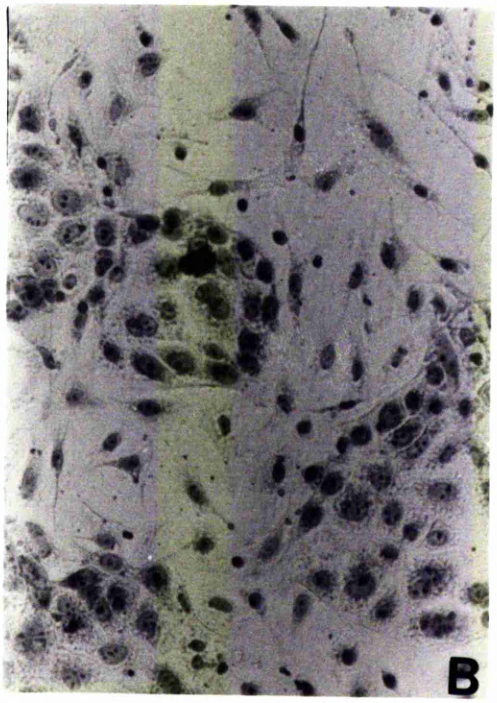
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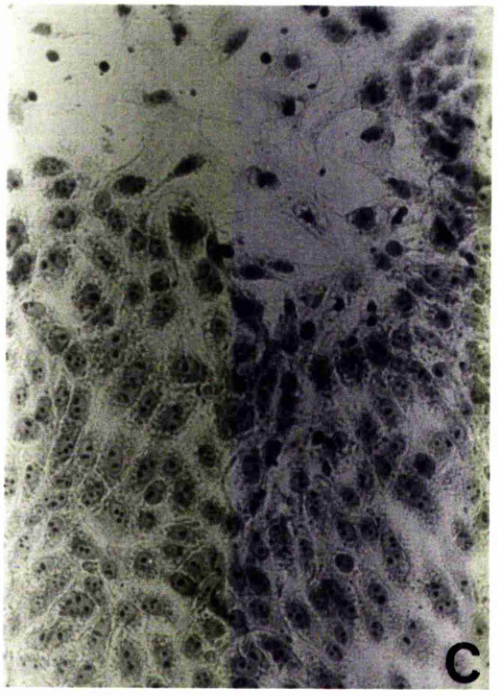
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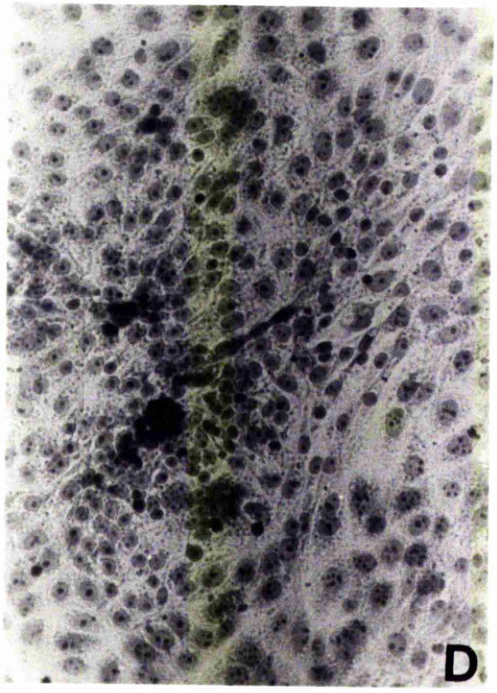
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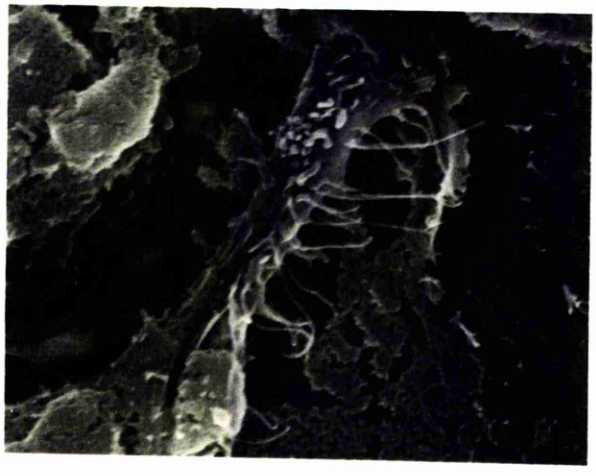


congested, and more often polygonal cells, as observed at light microscopical levels (Figs. 4,27c,d). Cells grown on the surface coated with rat tail collagen produced elongated Cilia-like outgrowths (Fig. 4.27e), which presumably tighten the binding of cells to collagen fibres.

4.700 DISCUSSION

Dextran coated charcoal (DCC) was used to extract endogenous steroids from serum. As a routine procedure it has the advantage of speed and practical simplicity. The studies described have shown that this method fulfils two important conditions:

a) effective depletion of endogenous steroid hormones

b) it renders serum biologically active for the cell type under study

4.710 EFFECT OF DCC ON SERUM COMPONENTS

The conditions for effective extraction should coincide with those for enhanced dissociation of the steroid-protein complexes. The results described in this chapter suggest that under the conditions used, the limiting factor for the extraction by DCC adsorption was the high affinity binding of steroids to serum proteins (see Section 4.010).

The efficiency of extraction increased with increasing incubation temperatures. This can not be attributed to a change in the steroid-adsorptive properties of charcoal, as they are not affected by temperature (Murphy, 1967). Nor can an increased dissociation of steroid-albumin complexes explain the data since the binding of steroids to albumin shows very little temperature dependence. Interestingly, Milgrom and Baulieu (1969) have shown that upon incubation with DCC, cortisol-albumin complexes dissociate rapidly in a temperature-independent manner, whereas cortisol-CBG complexes have a lower dissociation rate which increases with increasing temperature.

Previous studies have reported the extraction of steroids from serum by DCC adsorption. A fairly good correlation is found between the results presented in this chapter and those of Armelin (1973), Lippman and Bolan (1975) and Irwin (1982). Kirkland et al. (1976) found unusually high levels of E_2 in FCS after pre-incubation at 56°C and subsequent adsorption with DCC, suggesting a strong association of the steroid with some serum component(s). Since SHBG is heat-labile and shows decreased binding activity at elevated temperatures (Westphal, 1971), it is unlikely that binding of E_2 to SHBG could explain this finding. However, a more recent study (Agarwal & Philippe, 1978) has shown that incubation of BSA with some glucocorticoids at 60°C leads to the formation of steroid-protein complexes which are resistant to dissociation by charcoal. A similar mechanism may explain the findings of Kirkland et al. (1976) in view of the extensive association of E_2 with serum albumin (Sodergard et al., 1982).

The results reported here clearly disagree with those of Klevjer-Anderson and Buehring (1980), who showed removal of more than 99% of E_2 and P from serum by DCC treatment at 4°C.

The results in Section 4.140 showed no measurable change in the protein content of FCS after effective removal of endogenous steroids by adsorption with DCC (see Section 2.351). These results are in agreement with earlier studies by Heyns (1967) and Irwin (1982). Some reports indicate removal of up to 3% (Armelin, 1973) or 5-10% (Kirkland et al., 1976) of serum proteins after DCC adsorption. This difference may be explained on the basis of the charcoal concentration used.

4.720 EFFECT OF DCC ADSORPTION ON THE GROWTH PROMOTING ACTIVITY OF FCS

The following findings are consistent with this study:

a) DCC adsorption extracts steroids and other growth factors

b) FCS-DCC does not reduce the incorporation of ^{3}H-dT

c) heat-inactivation of FCS does not induce any significant change in growth of endometrial cells

d) DCC treatment at 56^{o}C removes components of serum which provide attachment factors to the cells

e) both DCC adsorption and heat-inactivation, simultaneously remove or inactivate growth inhibitory or limiting compounds.

Rat endometrial cells cultured with FCS-DCC exhibited increased rates of DNA synthesis at certain stages, which could be explained by considering specific differentiation stages of the cells (Heckman, 1983). This may be explained by interactions of the cells with components from medium or serum promoted by the absence of certain serum factors and possibly also by some secretory products of the cells. Some discrepancies are evident on comparing the present data with previous reports (Irwin, 1982). Some of these differences could be related to the cell type studied (Heckman, 1983). Variability between different serum batches could also account for the difference between present and previous reports.

Increased activity of thymidine kinase may also occur and, be related to an increased proportion of cells undergoing DNA synthesis. Further work is required to clarify the questions arising from the present study. The results presented here do, however, indicate that FCS constitutes an important source of variability in studies measuring the incorporation of a radioactive precursor by cultured cells. This problem may be overcome, in part, by the use of charcoal stripped serum.

4.730 DEVELOPMENT OF SERUM-FREE MEDIUM

The use of serum-free culture medium permits control of the chemical composition of the cellular environment. In addition, recent evidence indicates that a number of cell types express differentiated functions only in specifically designed serum-free media. Of particular

interest among these are rat ovarian cells (Orley et al., 1980) which only express their hormone-responsive character in serum-free culture.

To that end, we have followed the innovative approach of Gordon Sato and his colleagues (Barnes & Sato, 1980a,b) who have shown that the serum or plasma requirements for growth of a number of cell lines can be satisfied by the addition of specific hormones and growth factors to synthetic media.

In serum-free media it is possible to carry out exact nutritional studies without a contribution from the serum component (Rizzino et al., 1979). Interestingly, several laboratories have found that manipulation of the nutritional balance in serum-free or low-serum medium can alter the responses of the cells to hormones (Holley, 1975; Barnes & Sato, 1980a,b; Bettger et al., 1981; Kaighn et al., 1981; Jozan et al., 1982; Umans et al., 1982; Bradshaw et al., 1983).

In agreement with the observation of Gerschenson et al. (1974) and Irwin (1982), EGF and insulin are clearly important components of the defined medium able to sustain growth of the endometrial epithelial cells. EGF has been reported to be the most essential factor for survival and growth of various epithelial cells in SFM (Holley, 1975; Hayashi & Sato, 1979; Bradshaw et al., 1983). Insulin in combination with EGF and dexamethasone, showed stimulatory effects. These data are at variance with the observations of Jozan et al. (1982), on the effects of these growth factors on MCF-7 cells. It may be that these differences result from the use of different types of serum (Darbre et al., 1983; Page et al., 1983). It was also found, in this study, that the requirement of endometrial epithelial cells for insulin, EGF, and/or cortisol for optimal growth is different depending on the basal nutrient medium used (Wu & Sato, 1978). Similarly, McKeehan & McKeehan (1980) have reported that the ability of cells to respond to growth factors can

be changed by adjusting the concentration of some nutrients. The changes reported in this study clearly demonstrate that serum (whole and charcoal stripped) can alter or modify the effectiveness of various growth factors.

Another reason for not seeing expected responses, may be connected with cell density. Kaighn et al. (1981) showed that growth of prostate cells in a chemically defined medium is critically dependent on population density. Holley (1975) also mentioned that crowded normal cells need a higher concentration of growth factors. This can be explained on the basis of evidence that increasing the cell density probably increases the rate of destruction of the factors (Gerschenson et al., 1974).

Transferrin has also been described as an effective component of medium for avariety of cell lines (Barnes & Sato, 1980a; Irwin, 1982; Ekblom et al., 1983). In this study, it was observed to be extremely supportive for cell proliferation, but only in the complete absence of serum. Most of the stimulating activity of transferrin is presumably related to its iron binding properties (Ekblom et al., 1983). It may also be due to binding by the molecule of other metal ions which may be present in the medium at concentrations which are toxic (Iscove & Melchers, 1978). Unresponsiveness of transferrin in the presence of serum can be explained on the basis of the presence of complementary molecules in serum (Barnes & Sato, 1980a).

4.731 Selective growth of epithelial cells

A systematic investigation of the selective growth of epithelial cells in vitro has become possible only recently due to the identification of culture conditions that inhibit fibroblast proliferation (Munir & Leake, 1982; Pigott et al., 1982; Hecḱman, 1983). The studies reported here demonstrate that the growth rate of

fibroblasts from the rat endometrium can be slowed by a factor of three to four fold. This decrease is achieved by the inclusion of charcoal stripped serum. Hechman (1983) suggested a requirement for fatty acids for fibroblasts to proliferate. He also reported delipidated serum as an agent to selectively culture epithelial cells from rat trachea. Perhaps factors like fatty acids are being removed from the serum by charcoal stripping. Similarly, fractionation of serum by gel filtration yielded a fraction, which did not support the proliferation of glia and contaminating fibroblasts in rat central neurons in monolayer culture (Kaufman & Barnett, 1983). These studies confirm the observations presented here of differential effects of serum on individual cell types.

Often in serum-containing medium, survival and growth of unwanted cells make quantitative experiments on primary or early passage cultures impossible and in some cases results in the ultimate loss of the culture due to outgrowth of such cells. Ideal conditions require both complete absence of serum and provision of nutrients necessary for growth and differentiation of specific cell types. The combination of supplements found to support growth in the absence of serum is unique for each cell type (Barnes & Sato, 1980a). Our results are consistent with their findings. The ideal medium formulated for a particular cell type will allow little or no growth of other cell types.

Selective inhibition of fibroblasts by polyamines in a variety of primary cultures has also been shown (Stoner et al., 1980; Swanson & Gibbs, 1980; Jensen & Therkelsen, 1982). Jensen and Therkelsen (1982) concluded that, in general, polyamines have a cytotoxic effect when present in sufficiently high concentrations and that skin fibroblasts and epithelial cells are equally sensitive to this effect. However, according to them spermine and spermidine, but not of putreseine, are markedly more cytotoxic and fibroblasts exhibit a significantly higher sensitivity to the inhibitory effect.

Another approach reported in the literature, for avoidance of fibroblastic growth in epithelial cultures, is usage of feeder layers. Various cell lines are being used as confluent feeder layers to inhibit growth of unwanted cell types (Yang et al., 1981; Freshney et al., 1983). It has considerable value as both a selective technique for exclusion of stromal cells in the propagation of epithelial cells (Laing, 1980; Love, 1982) and may also promote survival and cell proliferation over that obtained on uncoated substrates (Freshney et al., 1983).

Interestingly, in variance with the idea of selective growth of epithelium, Chan & Haschke, (1983) suggested a supportive attitude of fibroblasts towards epithelial growth. They reported diffusible substance(s) released by fibroblasts in conditioned medium, that stimulated the growth of cultured rabbit corneal epithelial cells in culture.

4.740 CELL ATTACHMENT

Attachment of isolated cells to substrate is a critical step in the establishment of primary cultures. Serum is a main source of factors providing attachment to cells in culture (Klebe et al., 1977). The present study confirms this and further demonstrates that the attachment factors present in serum are resistant to charcoal treatment but not heat inactivation. Attachment was observed to be enhanced by using FCS-DCC(4). Perhaps charcoal stripping also removes some substance(s), which inactivates attachment factor(s) present in serum (Armelin, 1973; Lippman & Bolan, 1975; Irwin, 1982; Munir & Leake, 1982). Similar charcoal treatment at 56^{o}C results in the loss of attachment factors. Two lines of evidence support this possibility. First, the addition of FCS-DCC(56) to medium results in short-lived, loosely attached cells in culture. Attachment is greatly improved after coating the substrates. Secondly, as shown in this chapter, human

endometrial cancer cells fared much better in FCS-DCC(56) than whole serum both in terms of attachment and growth of the cells. Decreased requirements of attachment factors by cancer cells have already been described (Yamada & Olden, 1978). In the present study, analysis of the interactions of rat uterine mixed (fibroblastic) and epithelial cells with substrates was made as part of an attempt to define serum-free culture conditions. The efficient attachment of the fibroblastic cell population (see Table 4.3) in SFM indicated that attachment factor(s) from serum (Hook et al., 1977) are not required by these cells (Curtis, 1983). Viability and spreading of these cells was, unfortunately, observed to be poor, whereas, epithelial cells required attachment as well as spreading factors from the serum (see Table 4.4). On the basis of selective attachment, a specific cell population can be isolated (Ziegler & Gurpide, 1982).

The coating of the substrate surface plays an active role in the control of cell attachment, growth and differentiation, possibly via a modulation of cell shape and orientation. Such changes are thought to modify responses to naturally occurring hormones and growth factors (Wiesel et al., 1983). It has been shown (Grinnell & Feld, 1979, 1980) that the spreading of human skin fibroblasts in SFM results from the deposition by these cells of fibronectin locally onto the surface of the culture dish. In the present study, a similar effect of fibronectin was observed with the fibroblastic cell population. Epithelial cells attached more readily on gelatin and collagen, which is again in agreement with previous reports (Dorman, 1982; Flynn et al., 1982. Gospodarwicz et al., 1983). Plastic dishes coated with fibronectin and/or gelatin supported rapid attachment and flattening of cells during the first 12h of incubation (Table 4.9). Surprisingly, after 48h, the number of cells growing in uncoated dishes reached the same as that in

coated dishes. This suggests a limit on the ability of endometrial epithelial cells to attach to any provided substrate (Munir & Leake, 1984). Coating of the dishes permitted an increase in the speed of attachment but cell proliferation was not greatly altered. Orley and Sato (1979) reported fibronectin as a suitable candidate for promoting the optimal adhesion and, consequently, enhancement of the growth of mammalian epithelial cells in serum-free medium.

Considerable evidence has accumulated on the possible relationship between the substrate and serum. Love (1982) showed reduced attachment and survival of colonies of human breast cancer cells on collagen-coated substrates, compared with a feeder layer, in the presence of FCS. The present data showed a similar reduction in attachment to collagen-coated plastic in the presence of whole FCS for immature rat endometrial epithelial cells. Interestingly, the same plastic surfaces coated with different substrates enhanced both attachment and growth in the presence of FCS-DCC(56), even after 48h. The present results suggest that each substrate interacts differently with growth factors to modulate cell growth and differentiation.

4.750 CELL MORPHOLOGY AND GROWTH

Endometrial epithelial cells in culture, undergo both biochemical and morphological differentiation. The structural changes were studied while culturing the cells in serum-free medium (SFM). Hormonal induction of morphological and biochemical markers characteristic of functional differentiation were assessed. The application of parallel TEM, SEM and light microscopy provides a three dimensional approach to the study of changes in the surface architecture of endometrial cells. The data presented in this study collectively point to the conclusion that cell shape may be important to the induction of functional differentiation in endometrial cells in vitro. The

changes reported suggest that differentiation in culture requires the assumption of a compact shape, but that shape change is not sufficient to induce differentiation. Recent work by Emerman (1979) has suggested that cell shape may have a major influence on the ability of mammary epithelial cells to differentiate functionally *in vitro*. Further support comes from the reports by other investigators indicating that the cell shape is important in regulation of proliferation (Folkman & Moscona, 1978). Folkman and Moscona (1978) have also shown for several cell types that cell shape is coupled to DNA synthesis, where Lifshitz *et al*. (1983) suggested that growth and morphologic responses arise from distinct processes. Assumption of a preferred shape, however, has not until now been proposed as a requirement for, rather than a consequence of, individual epithelial cell differentiation in culture (Emerman *et al*., 1979).

Our observation of the fine structure of endometrial epithelial cells cultured under standard conditions have confirmed earlier results which indicated that most *in vivo* epithelial markers are observed *in vitro* (Dallenbach-Hellweg, 1981; Irwin, 1982; Heckman, 1983). New findings include the ultrastructural features of epithelial cells grown under SFM conditions. SEM revealed that endometrial epithelial cells differ widely in apical topography in confluent cultures on feeding serum-supplemented medium and SFM. Surface morphology was observed to be relatively uniform in each condition. It is not clear whether the ultrastructural differences on the surfaces of these cells in the two conditions are due to the absence of serum or to specific effects of growth factors present in the medium. Another reason could be the various stages of the cell cycle. During the cell cycle, cells undergo a variety of surface changes (Wheatley, 1982). However, a clear difference between SFM and serum-supplemented media is the change of the

blobbed surface morphology into a surface covered with elongated microvilli. This was thought to have been brought about by trypsin (Kinn & Allen, 1981), but the present study shows that it is a result of growth conditions.

The epithelial cells in each colony varied in shape, being flattened at the periphery and becoming increasingly cuboidal towards the centre of the colony. Peripheral cells showed very few microvilli as compared to central cells. Additionally the multinucleated cells were more often seen in the centre of the colony. Gerschenson et al. (1974) studied rabbit endometrial cells and showed that only the multinucleated cells were responsive to hormones. Since central parts of the colony contained more differentiated cells, it can be concluded that highly differentiated cells are more responsive. According to another study, it is likely that microvillous cells are actively engaged in the secretory processes (Barberini et al., 1978). Observations of several cell types by others have led to the proposal that the formation of microvilli requires the movement of filamentous material from beneath the plasma membrane into the microinjections (Madison et al., 1979). There is a highly organised network of cytoskeleton beneath the membrane, which could be identified through the surface. Mooseker and Tilney (1975) proposed that the core filament bundles consist of polarised molecules of filamentous actin that are attached to the membranes at the tips of the microvilli by molecules of α-actinin. The observation concerning the loss of elongated microvilli, tonofilament and actin fibres (see Section 4.500) suggests that the hormones in SFM influence cells to return to a less differentiated state. Strum (1978) also related the loss of myofilaments to the return of cells to a less differentiated state. This appears to be analogous to the changes observed in the present study. Hull and Staehelin (1979) concluded that tonofilaments surround

the bases of the bundles of core microfilaments and loop through the network of actin-like filaments in the adherent zone. These tonofilaments are, therefore, in a position to prevent the sliding of the splayed core microfilaments towards the microvilli, thus constraining the actin-like filaments to move within the plane of the adhering zone. The restraining action of the tonofilament bundles could ultimately ensure that the sliding action of the actin-like filaments results in a shortening of the microvilli.

Epidermal Growth Factor changes cellular morphology and cytoskeletal organisation (Carpenter & Cohen, 1979). These morphological changes require both cellular energy metabolism and actin containing microfilaments (Chinkers et al., 1981). The results in this study clearly establish that the onset of increased thickness of microvilli in response to SFM is coupled with a loss of stress fibres at the cell periphery (see Section 4.500). The changes observed in cell periphery may reflect the uptake of certain metabolites from the medium (Mattson & Kowal, 1980).

Glucocorticoids have been reported to cause modifications on the cell surface of hepatoma and glioma cells (Armelin & Armelin, 1983). Glucocorticoids have also been shown to induce fibronectin deposition on a variety of cells in culture (Orley & Sato, 1979; Armelin & Armelin, 1983).

The effect of insulin in the present study may be explained by an increased availability of fatty acids for esterification and storage (Dich et al., 1983). Insulin is known to increase fatty acid synthesis in cultured hepatocytes (Topping & Mayer, 1982). More recently, Amatruda and Chang (1983) also showed an increase in lipid synthesis by high levels of insulin in hepatocytes.

These structural responses can be correlated with an increasing sensitivity of the epithelial cells to cell-cell contact when exposed to hormones (Strum, 1978). The cells in culture form colonies with junctional complexes composed of desmosomes and areas which appear to be tight and gap junctions. The increased density of junctional complexes in response to growth factors in SFM, suggests mRNA being stimulated to synthesise protein necessary for junction formation (Griepp et al., 1983). Burghardt and Anderson (1979) showed regression in gap junctions in ovarian interstitial cells after hypophysectomy. They also revealed that administration of exogenous Human Chorionic Gonadotrophin resulted in the restoration of the normal interstitial cell morphology.

4.800 SUMMARY

The overall picture that emerges is that growth of normal endometrial cells is probably controlled by interactions of the cells with various polypeptide and steroid hormones or hormone-like growth factors present in the surrounding fluids. As normal cells become more and more crowded they require higher and higher concentrations of the growth factors in order to grow. This increased requirement for growth factors is probably due, in part, to the diminished surface area of individual cells when they are packed in among other normal cells and in part to cellular destruction of the factors.

Malignant cells escape from normal growth controls by requiring less of hormones or growth f actors. They probably destroy the factors less rapidly, and they probably maintain more surface area exposed to the surrounding fluids. The changes in transformed cells that lead to lowered requirements for the growth factors may be the result of direct internal loss of control of DNA synthesis or to membrane changes that modify the requirements for hormone interaction with the membrane. The

results in this chapter confirm that SFM is capable of modulating surface composition and cytoskeletal architecture and, therefore, the functioning of endometrial cells. The rate of growth of endometrial cells in SFM is lower than that obtained in medium supplemented with serum. It is not known whether SFM will support the cell growth upon serial serum-free cultivation. Further improvements of the present formulation will be necessary to support optimal growth.

CHAPTER 5 - STEROID-CELL INTERACTIONS

5.000 INTRODUCTION

Despite advances in the knowledge of how steroids regulate protein synthesis, elucidation of the mechanisms by which steroids regulate growth has not been achieved. Both direct and indirect mechanisms have been proposed for steroid-stimulated growth. In the former type of mechanism, steroid hormone would interact directly with target cells to affect their proliferation. An example of this may be the oestrogen-induced protein from rat uterus, which promotes mitotic activity (King, 1978). Alternatively, steroids could act as modulating agents changing the sensitivity of target cells to external stimuli, which affect cell external proliferation (King & Yates, 1980; Yates & King, 1980). Steroids can also induce synthesis and secretion of substances which affect the growth of homologous cells (Veith et al., 1983).

Indirect mechanisms have been proposed for oestrogen stimulated growth in particular. One hypothesis sustains that oestrogens stimulate growth in target cells by inducing the production of specific growth factors by other tissues (Sirbasku & Benson, 1979). This model has been recently modified (Ikeda et al., 1982; Sirbasku & Leland, 1982) to include the possibility of these oestrogen-induced growth factors being produced by the same cells whose growth is stimulated. Alternative indirect models have been proposed (Shafie, 1980; Moore, 1981; Darbne et al., 1983; Page et al., 1983) in which either the growth would be regulated by inhibitors controlled by oestrogens, or oestrogens act indirectly by causing changes in sensitivity of cells to other agents.

The cyclic variations in blood levels of oestradiol and progesterone are considered to be responsible for the morphologic and physiologic changes observed in the endometrium, Growth in response to oestrogenic influence is a basic prerequisite for the regulation of

physiologic processes brought about later by progesterone stimulation. These include the synthesis and secretion of glycoprotein-rich material (Ferenczy et al., 1979).

In the past decade, the study of DNA synthesis, by means of historadio-autography (Gerschenson et al., 1979; Kirk & Irwin, 1980) and steroid biochemistry (Clark & Peck, 1979), has proved useful in the comprehension of the regulatory action of oestrogens on endometrial growth. The stimulation of normal endometrial cell growth by oestrogens has been obtained in mixed cultures of epithelium and stroma (Pietras & Szego, 1975; Eckert & Katzenellenbogen, 1981). While such results are impossible to interpret with respect to cell type, reports on the effects of oestrogens in separated epithelial and stromal cultures are contradictory (see Section 1.3200). Oestrogen was ineffective in cultures of separated rat endometrial epithelial and stromal cells (Casimiry et al., 1980) but, induced an increase of the labelling index and a shortening of the generation time of epithelial but not stromal cells in mixed cultures from rabbit endometrium (Gerschenson et al., 1974).

Studies on the effects of progestins on cell growth have been less extensive. Gerschenson et al. (1974, 1977) reported that P increased the labelling indices of both epithelial and stromal cells in mixed cultures from rabbit endometrium. In these epithelial cells, P prolonged the generation time and antagonised oestrogen-stimulated cell proliferation. In cultures of separated epithelial and stromal cells from rat endometrium (Casimiry et al., 1980) P inhibited the growth of epithelial but had no effect on stromal cells. On the other hand, P stimulated the incorporation of radioactive thymidine by rat decidual cells in culture (Peleg & Lindner, 1980). Low concentrations of P ant agonised the oest rogenic stimulation of growth and labelling index in cultured human endometrial carcinoma cells (Ishiwata et al., 1977) but

enhanced the incorporation of radioactive uridine and amino acid into acid-insoluble material. However, the inhibitory action of high concentrations of P used in these experiments is likely to induce toxic effects (Kuramoto et al., 1972). Thus, reports of steroid effects on normal endometrial cultures are sometimes contradictory - and it is difficult to discriminate between differences due to experimental conditions or to species. The posibility of functional heterogeneity in cultures of pure epithelial and stromal cells further complicates the interpretation of these studies.

Steroids have been shown in vitro to induce numerous morphological changes in uterine epithelium (Berliner & Gerschenson, 1976; Liszczak et al., 1977; Eckhevers et al., 1980). These include changes in number and size of mitochondria, lysosomal material, nuclear and nucleolar contents and modifications in membrane structures. These changes have been suggested to be correlated with growth. Berliner and Gerschenson (1976), based on general morphological and biochemical similarity between cultured cells and cells in vivo, suggested that endometrial cells cultured in defined medium can be used as a reliable system to study the specific direct actions of these hormones.

The interpretation of abnormal endometrial morphology requires a precise understanding of the natural history of individual endometrial carcinoma and the relationship of each to hormonal influences. Endometrial carcinomata are among the most common gynaecological malignant neoplasms.

Available clinical and biochemical data suggests that a persistent unopposed oestrogen stimulus plays an important role in the genesis of endometrial hyperplasia and adenocarcinoma. This hyperoestrogenic stimulation is reflected by a proliferation of all the endometrial components including the glands surface epithelium, stromal fibroblasts, and smooth muscle cells (Fenoglio et al., 1982). This

leads to an increase in the total amount of endometrial tissue. The epithelial cells are columnar with nuclear pseudostratification and increased mitotic activity and an increase in DNA-dependent RNA synthesis. At the ultrastructural level, this is reflected by an increase in number of free ribosomes, rough endoplasmic reticulum (RER), golgi, mitochondria, glycogen complexes, lysosomes, lipid bodies, microfilaments, surface microvilli and cilia (Wessel, 1965; Fasske et al., 1965; Ferenczy, 1975, 1979). That the nuclei are engaged in active DNA synthesis is confirmed by the presence of the diffuse distribution of the nuclear chromatin in a euchromatin pattern. In stromal fibroblasts, the RER, golgi and mitochondria are prominent, as are lipid and lysosomal bodies (Ferenczy, 1977).

5.010 THE ROLE OF RECEPTORS

In accordance with the basic paradigm of steroid hormone action, present evidence indicates that the steroid stimulated cell growth requires the presence of specific cellular receptors (Leake, 1976). However, it now appears clear that the mere presence of receptor proteins is not sufficient to predict the responsiveness of cells to steroid hormones (Leake, 1981a). Apart from the trivial explanation than a particular steroid may not be involved in the regulation of growth of a given cell type, it should be borne in mind that the binding of steroid to receptors is only the first step in a chain of events which ultimately results in a biological effect. This point is illustrated by the classical studies from Gordon Tomkins' group using lymphoma cell lines, which are killed by glucocorticoids (Sibley & Tomkins, 1974).

5.100 EFFECTS OF STEROID HORMONES ON DNA SYNTHESIS

Due to the limitations in experimental procedures and availability of cells, the rate of DNA synthesis was assessed in rat endometrial epithelial cell populations over a period of only two days. FCS contains multiple factors which affect cell proliferation and

considerable variation may be found in the hormone content (Hayashi & Sato, 1976) and mitogenic potential of different batches (Honn et al., 1975). Therefore, charcoal stripped serum from a single batch was used in these experiments. Since such stripping may remove both beneficial (Hayashi & Sato, 1976; Bradshaw et al., 1983) and inhibitory (Pigott et al., 1982) factors, effects of steroids on endometrial cells were investigated, with various concentrations of FCS, FCS-DCC(4), FCS-DCC(56), and serum-free medium.

5.110 OESTROGENS

Interactions of various concentrations of oestradiol-17β with different concentrations of sera were studied in relation to DNA synthesis (Table 5.1). In presence of a 10% concentration of serum, E_2 neither stimulated nor inhibited. In FCS (5%) E_2 was inhibitory, whereas in the same concentration of FCS-DCC(4) or FCS-DCC(56) E_2 stimulated DNA synthesis significantly. Oestradiol (10^{-9}M) increased the rate of DNA synthesis in serum-free medium (SFM).

Higher concentrations of tamoxifen (10^{-8}M) stimulated DNA synthesis with FCS and FCS-DCC(4), irrespective of serum concentration (Table 5.2). Tamoxifen (10^{-10}M) showed an inhibitory effect in presence of FCS-DCC(56). An overall increase was observed by tamoxifen in SFM.

5.120 PROGESTINS

FCS did not induce any effect with any concentration of progesterone (Table 5.3), whereas progesterone (10^{-7}M) inhibited DNA synthesis with FCS-DCC(4). Lower concentrations of P enhanced the growth with added 10% FCS-DCC(4). No significant effect was observed with FCS-DCC(56). A decrease in rate of DNA synthesis was noticed with progesterone in SFM.

As can be seen from Table 5.4, MPA caused an increase in DNA synthesis with FCS except at high concentration (10^{-7}M) with 10%

Table 5.1: Oestradiol-17 β modulation of ^{3}H-thymidine incorporation into immature rat endometrial epithelial cells in the presence of serum, stripped serum and serum-free medium

This table shows the percentage of cells labelled in response to the various concentrations of oestrogen. Cells were prepared (see Section 2.512) and cultured for 12h. Medium was then changed to the appropriate one, as indicated and cells were further grown for 48h. Cells were pulsed with ^{3}H-thymidine for the last 3h and autoradiographs were processed as described in Section 2.731. Each value is the mean $\pm$ S.D. of triplicate experiments.

* $P < 0.05$

** $P < 0.005$

*** $P < 0.001$

Medium supplemented with		Oestradiol-17β Concentration			
		Control	10^{-8}M	10^{-9}M	10^{-10}M
FCS	10%	36.3±5.2	22.9±6.7***	32.4±4.2*	31.0±6.1
	5%	21.2±4.0		7.3±1.7***	5.7±1.8***
	1%				
FCS-DCC-4°C	10%	17.2±5.7	26.1±6.6	20.0±8.4	
	5%	11.6±3.2	19.8±2.6**	20.7±4.4**	
	1%	5.2±2.1	1.5 ±3.0		
FCS-DCC 56°C	10%	5.2±1.5		5.9±2.9	6.0±1.4
	5%	2.5±0.8	1.9±1.0	10.0±2.0**	7.2±1.3*
	1%				
Serum-free EGF,INS,C		4.2±1.2	7.7±2.9 *	14.3±2.2**	5.9 ±0.3
		6.1±1.5	4.7±3.0	14.8 ±1.8**	

PERCENTAGE OF LABELLED NUCLEI

Table 5.2: Tamoxifen modulation of ^{3}H-thymidine incorporation into immature rat endometrial epithelial cells in the presence of serum, stripped serum and serum-free medium

This table shows the percentage of cells labelled in response to the various concentrations of tamoxifen. Cells were prepared, cultured and processed as for Table 5.1. Each value is the mean $\pm$ S.D. of triplicate cultures.

* $P < 0.05$

** $P < 0.005$

*** $P < 0.001$

Medium supplemented with		Tamoxifen Concentration			
		Control	10^{-8}M	10^{-9}M	10^{-10}M
FCS	10%	22.0±4.2	33.3±4.8***	24.7±4.0	14.0±9.1
	5%	18.2±5.5	35.8±6.2***	22.5±3.5	24.2±6.9
	1%	3.6±1.7	7.9±1.4*	8.4±2.3*	4.1±2.6
FCS-DCC-4°C	10%	23.6±9.1	61.3±7.9***	31.7±7.7	30.3±6.5
	5%	13.6±5.4	32.2±6.6***	16.9±3.6	25.8±4.7***
	1%	5.5±1.8	9.1±1.8*	3.0±0.9	4.1±1.0
FCS-DCC-56°C	10%	16.8±4.3	9.5±5.8	7.5±6.0	7.4±2.0**
	5%	6.9±1.0	10.6±3.5	6.6±2.7	2.9±1.2*
	1%	2.3±1.1	1.7±0.3	2.0±1.0	1.0±0.7
Serum-free		1.7±0.6		1.1±0.8	4.6±0.9*
EGF,INS,C		6.0±1.2	15.1±3.0**	22.7±3.9***	8.9±1.4*

PERCENTAGE OF LABELLED NUCLEI

Table 5.3: Progesterone modulation of ^{3}H-thymidine incorporation into immature rat endometrial epithelial cells in the presence of serum, stripped serum and serum-free medium

This table shows the percentage of cells labelled in response to the various concentrations of progesterone. Cells were cultured and processed as for Table 5.1. Each value is the mean $\pm$ S.D. of triplicate cultures.

* $P < 0.05$

** $P < 0.005$

*** $P < 0.001$

NORMAL ENDOMETRIAL CELLS

Progesterone Concentration

Medium supplemented with		Control	10^{-7}M	10^{-8}M	10^{-9}M
FCS	10%	21.2±4.2	15.9±4.9	25.0±3.0	28.2±9.4
	5%	16.7±4.6	24.7±6.0**	29.9±9.3***	25.6±4.7**
	1%	6.0±1.8	8.6±1.8	9.1±2.9	8.2±2.3
FCS-DCC-4°C	10%	35.0±7.1	16.9±2.3***	51.6±8.8***	48.2±10.3***
	5%	2.9±5.5		17.0±3.1	
	1%	7.8±1.6	3.2±0.7*	4.7±1.5*	9.0±1.5
FCS-DCC-56°C	10%	8.8±3.0	12.1±2.6	11.0±1.8	9.8±2.9
	5%	6.9±1.2	13.6±10.1	10.9±4.7	10.2±4.9
	1%	1.9±0.5	1.3±0.2	2.0±0.5	1.7±0.6
Serum-free		2.6±0.6	0.5±0.3	0.5±0.1	1.9±0.2
EGF,INS,C		6.8±1.1	6.4±2.0	5.2±0.9	4.9±0.6

Each point is mean value of 3 experiments.

Table 5.4: Medroxyprogesterone Acetate (MPA) modulation of ^{3}H-thymidine incorporation into immature rat endometrial epithelial cells in the presence of serum, stripped serum and serum-free medium

This table shows the percentage of cells labelled in response to the various concentrations of MPA. Cells were cultured and processed as for Table 5.1. Each value is the mean $\pm$ S.D. of triplicate cultures.

* $P < 0.05$

** $P < 0.005$

*** $P < 0.001$

NORMAL ENDOMETRIAL EPITHELIAL CELLS

Medium supplemented with		Concentration of Medroxyprogesterone Acetate (MPA)			
		Control	10^{-7}M	10^{-8}M	10^{-9}M
FCS	10%	26.6±4.9	12.9±3.4***	39.7±10.2***	34.1±6.7**
	5%	17.0±9.1	39.6±5.1***	42.1±5.1***	54.3±7.9***
	1%		2.2±0.5	5.8±2.1	5.3±1.4
FCS-DCC-4°C	10%	10.1±1.8	10.0±2.3	16.9±4.0**	2.9±0.4**
	5%	7.3±0.5	3.6±0.3	6.6±1.2	12.1±2.3
	1%	2.6±0.4	3.1±1.0	5.9±1.7*	1.4±0.1
FCS-DCC-56°C	10%	38.8±5.0	17.9±4.1***	31.0±6.3**	25.0±4.9***
	5%	16.1±3.1	10.6±1.3*	28.6±7.4***	42.3±16.3***
	1%	3.3±1.2	3.4±1.0	7.1±3.0	6.4±5.9*
Serum-free		2.9±0.8		1.0±0.2	1.4±0.2
EGF,INS,C		8.8±5.1		5.0±2.7	13.2±3.9*

Each point is mean value of 3 experiments.

serum. Lower concentrations of MPA were inhibitory in a medium supported by FCS-DCC(4). 10^{-7}M MPA at 5% concentration of the same serum also depressed DNA synthesis. MPA (10^{-7}M) reduced with 5% or 10% FCS-DCC(56), whereas, lower concentrations of MPA were stimulatory with lower concentrations of FCS-DCC(56) MPA also inhibited growth in SFM.

5.130 GLUCOCORTICOIDS

Table 5.5 shows that cortisol, at a concentration of 10^{-7}M, was stimulatory both in presence and absence of serum. The greatest response was seen with FCS-DCC(56). Dexamethasone at 10^{-9}M had little effect, but higher concentrations (10^{-7}M, 10^{-8}M) were observed to be stimulatory in all sera preparations (Table 5.6).

5.140 INTERACTIONS BETWEEN STEROID HORMONES

In a series of experiments the combined effects of the different steroid hormones on cell proliferation were investigated using maximally effective concentrations with 10% FCS and FCS-DCC(56) (See Fig.5.1). Oestradiol (10^{-9}M) plus progesterone (10^{-8}M) significantly enahnced thymidine incorporation in presence to FCS. In a medium containing FCS-DCC(56), oestradiol (10^{-9}M) alone and in combination with cortisol (10^{-7}M), dexamethasone (10^{-7}M) and tamoxifen (10^{-9}M) increased the rate of DNA synthesis.

5.200 STEROID RECEPTOR MODULATION

Histochemical receptor assays for oestrogen were performed on mixed cell populations by Dr.Louis Pertschuk (New York) (see Section 2.570). Although, qualitatively very nice fluorescent staining was observed no difference was found between control and hormone stimulated cells (Fig. 5.2 a,b).

Another method, using antibodies to receptor, to locate oestrogen receptor in rat endometrial epithelial cells, established by Dr.Shanthi Raam (Boston), was also assessed (see Section 2,570). In

Table 5.5: Cortisol modulation of ^{3}H-thymidine incorporation into immature rat endometrial epithelial cells in the presence of serum, stripped serum and serum-free medium

This table shows the percentage of cells labelled in response to the various concentrations of cortisol. Cells were prepared and processed as for Table 5.1. Each value is the mean $\pm$ S.D. of triplicate cultures.

* $P < 0.05$

** $P < 0.005$

*** $P < 0.001$

Medium supplemented with		Concentration of Cortisol: Control	10^{-7}	10^{-8}	10^{-9}
FCS	10%	20.0±3.4	32.6±5.0***	31.2±9.9***	21.7±4.9
	5%	14.4±6.8		24.8±5.2**	25.1±6.5***
	1%	2.4±1.2	1.8±1.1	1.0±1.0	6.3±2.7*
FCS-DCC-4°C	10%	30.1±6.1	51.7±8.8***	22.8±7.4**	40.5±5.9**
	5%	11.3±2.0	18.6±3.00**	12.1±1.1	30.4±16.3***
	1%	2.9±1.0	2.9±0.6	7.2±1.3*	1.7±1.0
FCS-DCC-56°C	10%	2.6±1.8	9.7±2.0**	13.1±1.9**	14.3±12.6***
	5%	2.0±0.8	2.9±1.0	3.9±0.3	6.4±1.2*
	1%	1.5±0.6	4.5±1.3*	2.9±0.5	3.3±0.9
Serum-free		0.8±0.2	3.2±1.1	1.0±0.2	1.6±0.5
EGC, INS		1.9±1.4	3.7±0.9	2.2±1.0	2.0±1.3

PERCENTAGE OF LABELLED NUCLEI

Table 5.6: Dexamethasone modulation of ^{3}H-thymidine incorporation into immature rat endometrial epithelial cells in the presence of serum, stripped serum and serum-free medium

This table shows the percentage of cells labelled in response to the various concentrations of dexamethasone. Cells were prepared and processed as for Table 5.1. Each value is the mean $\pm$ S.D. of triplicate cultures.

* $P < 0.05$

** $P < 0.005$

*** $P < 0.001$

Medium Supplemented with		Concentration of Dexamethsone			
		Control	10^{-7}M	10^{-8}M	10^{-9}M
FCS	10%	21.6±3.8	36.6±5.4***	41.3±4.9***	30.0±7.9**
	5%	7.9±4.0	19.1±1.7***	26.4±3.9***	12.5±2.8*
	1%	4.3±2.1		1.8±0.6	3.1±1.2
FCS-DCC-4^{o}C	10%	30.5±6.6	57.3±7.1***	50.5±7.6***	27.1±8.9*
	5%	19.1±4.2	32.8±5.0***	49.8±10.1***	29.3±4.9**
	1%	4.7±2.2	6.9±3.0	13.1±4.0**	3.3±1.6
FCS-DCC-56^{o}C	10%	8.6±2.3	19.1±2.4**	17.4±3.9**	9.5±3.0
	5%	4.5±1.7	16.9±2.7***	9.3±2.0*	8.5±2.4*
	1%	1.4±0.2	4.7±0.9	7.0±1.1	1.6±0.5
Serum-free EGF,INS		2.3±1.1	6.5±1.4*	3.9±2.0	2.9±1.7

PERCENTAGE OF LABELLED NUCLEI

Figure 5.1: Steroid modulation of ^{3}H-thymidine incorporation into immature rat endometrial epithelial cells

This figure represents the percentage of nuclei labelled in response to different steroids, alone and in combinations, in the presence of 10% whole serum (FCS) or 10% charcoal stripped serum (FCS-DCC(56)). Cells were prepared and processed as for Table 5.1. Each point is the mean $\pm$ S.D. for triplicate cultures.

* $P < 0.05$

** $P < 0.005$

*** $P < 0.001$

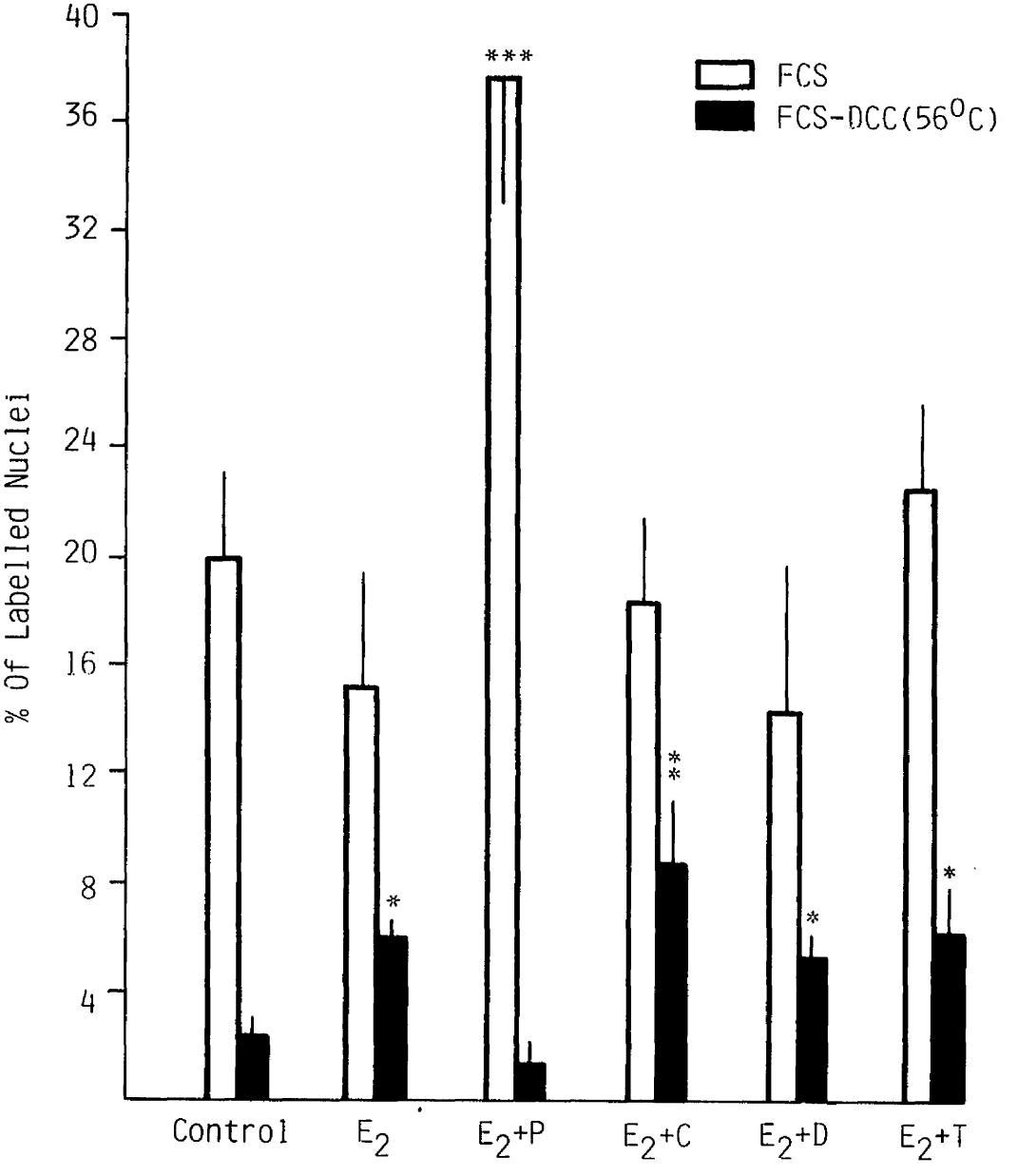
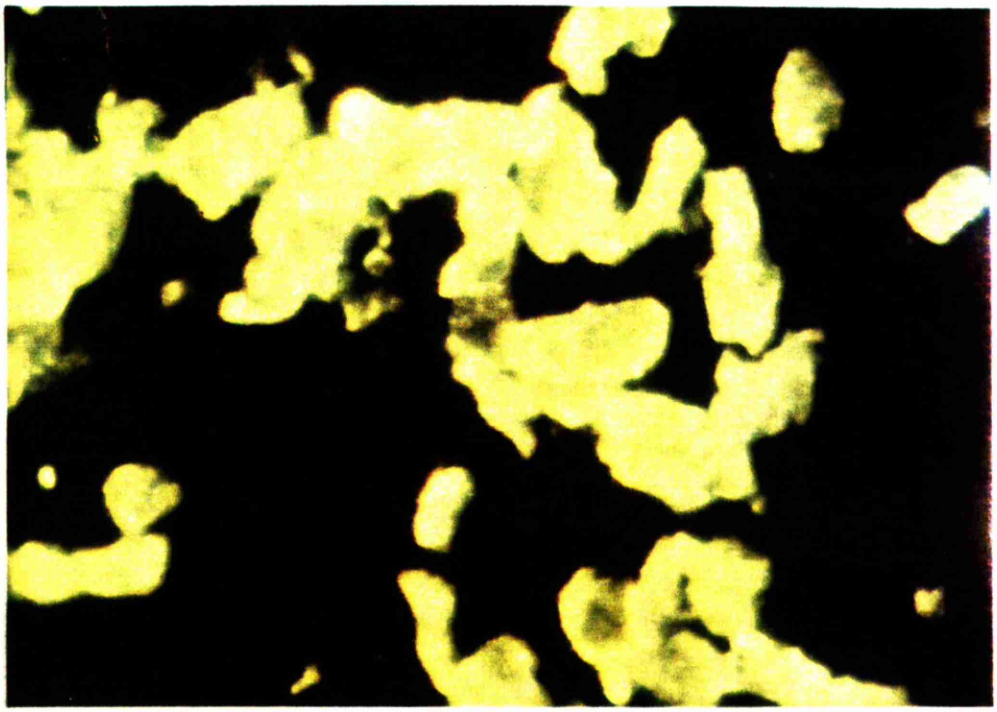

40
36
32
28
24
20
16
12
8
4
% Of Labelled Nuclei
FCS
FCS-DCC(56°C)
***
**
*
*
*
*
Control
E2
E2+P
E2+C
E2+D
E2+T

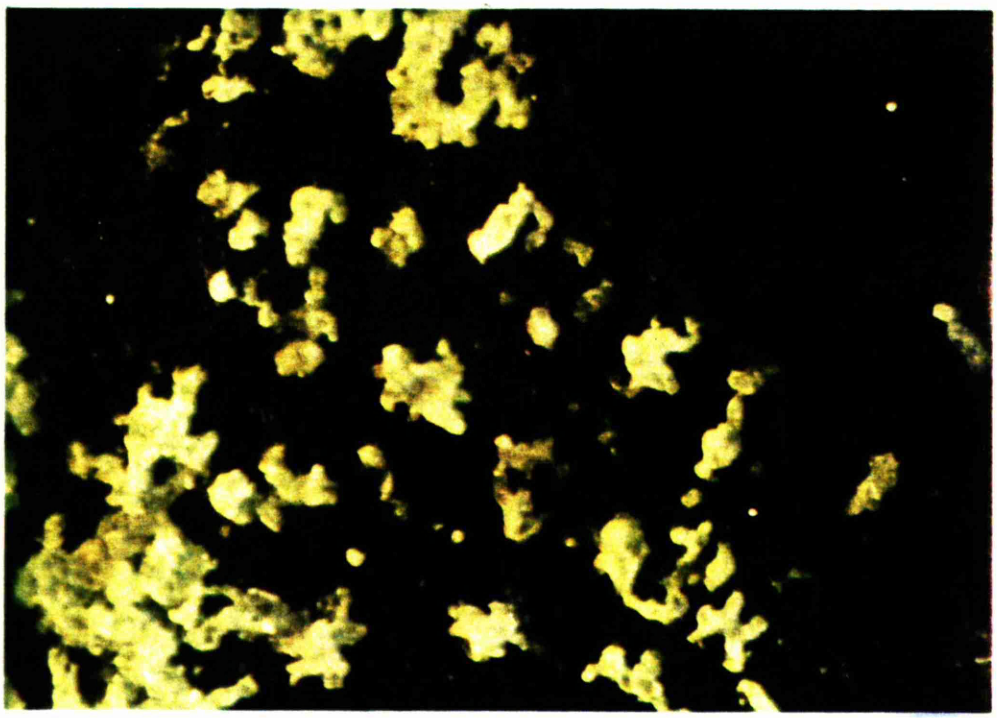
Figure 5.2: Histochemical staining of oestrogen receptor (ER)

Immature rat uterine mixed cells were processed foroestrogen receptor by Dr.L.P.Pertschuk using E_2-BSA-FITC as described in Section 2.570.

a) control culture x 440

b) cells stimulated with oestradiol (10^{-9}M) for 6 days prior to assay x100





control cells, fluorescence was observed in the cytoplasm, though a little staining was also located in the nucleus (Fig. 5.3a). Cells stimulated by E_2 for two days, exhibited staining mainly in the nucleus (Fig. 5.3b).

5.300 MORPHOLOGICAL CHANGES

No significant effects of steroids on endometrial cells were observed at the light microscopic level. The ultrastructural studies of cells cultured with various steroids revealed that these steroids induced diverse morphologies.

5.310 SURFACE FEATURES

Human endometrial epithelium showed an interesting change in surface structure in response to steroids. The frequency of junctions between adjacent membranes of cells cultured with oestradiol (10^{-9}M), when compared to control cells, appeared to be substantially increased (Fig. 5.5b). Combined treatment of these cells with oestradiol (10^{-9}M) and progesterone (10^{-8}M) induced changes in their microvillous structures. Simultaneous stimulation with both these hormones enhanced the size and number of microvilli on free surfaces of the cells (compare Figs. 5.4 & 5.6).

5.311 Cytoskeleton

Topographical fluorescence microscopic images of the features of the cellular cytoskeleton showed a difference in staining of rat endometrial epithelial cells. Oestradiol (10^{-9}M) increased staining for actin in these cells (Figs. 5.7 a,b), whereas progesterone, alone and in combination with oestradiol, showed no significant difference (Figs. 5.7 a,c).

5.320 CYTOPLASMIC CHANGES

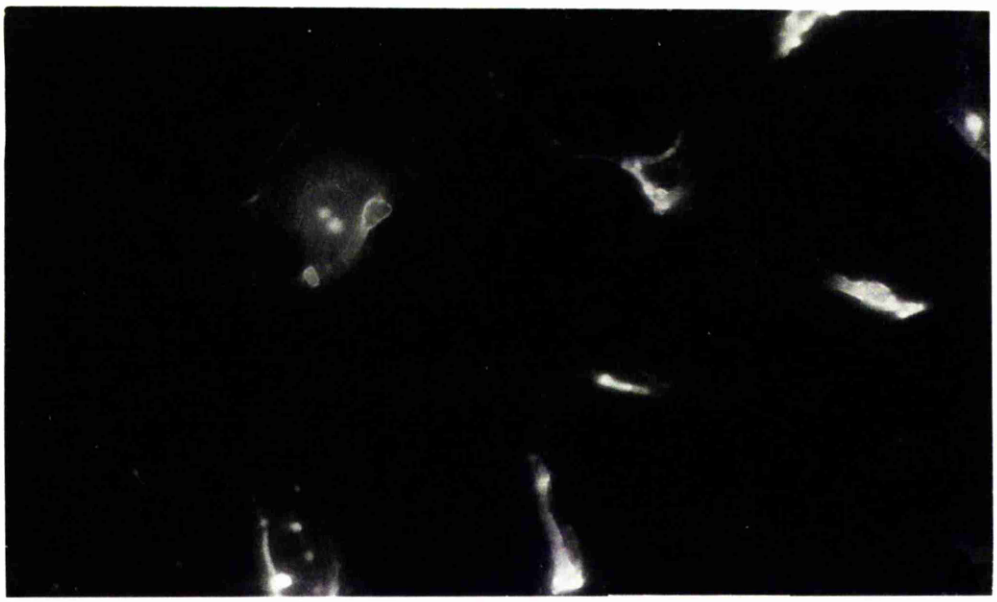
Steroid hormones, overall, brought about a considerable increase in cytoplasmic volume. In human endometrial epithelial cells

Figure 5.3: Immunocytochemical staining for oestrogen receptor

Immature rat endometrial epithelial cells were processed for oestrogen receptor by Dr.S.Raam, using a rabbit antibody to ER as described in Section 2.570.

a) control cells x 440

b) cells stimulated by oestradiol (10^{-9}M) for 2 days x 440



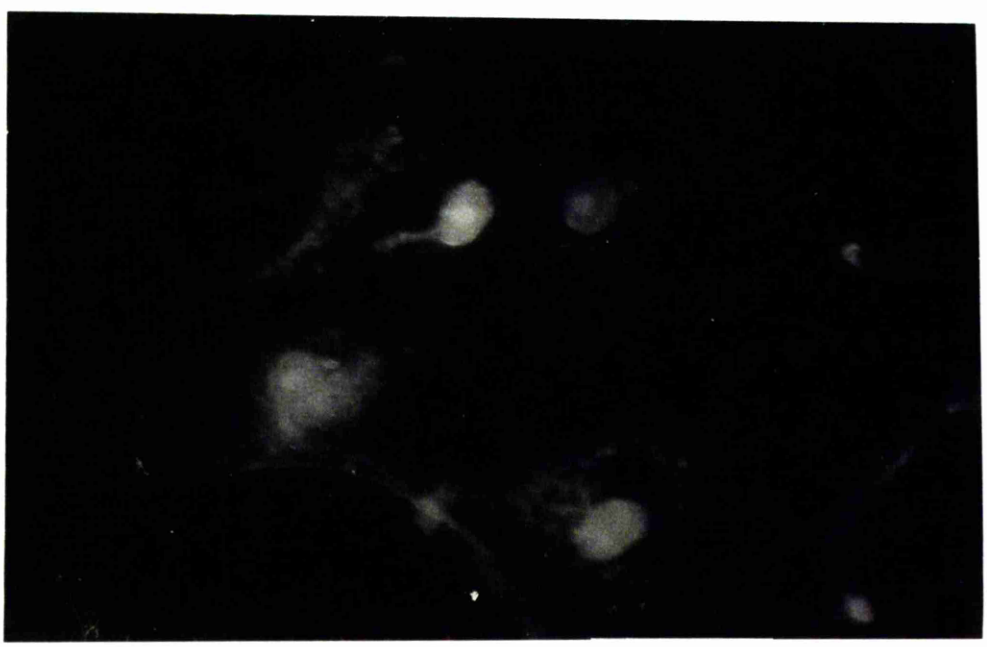
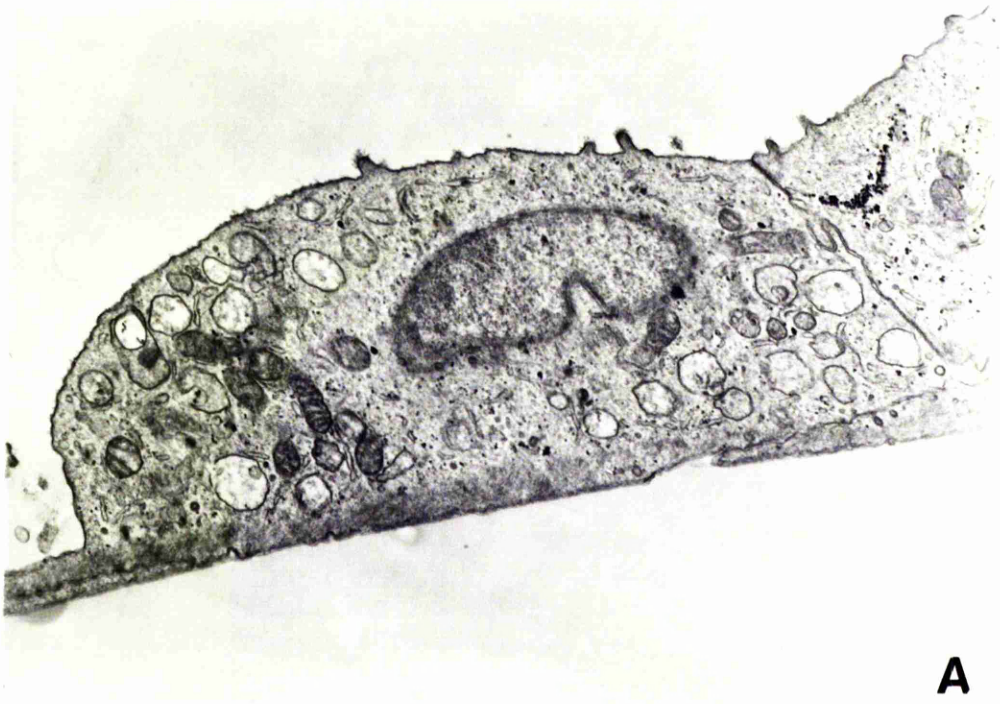


Figure 5.4: TEM of human normal endometrial epithelial cells

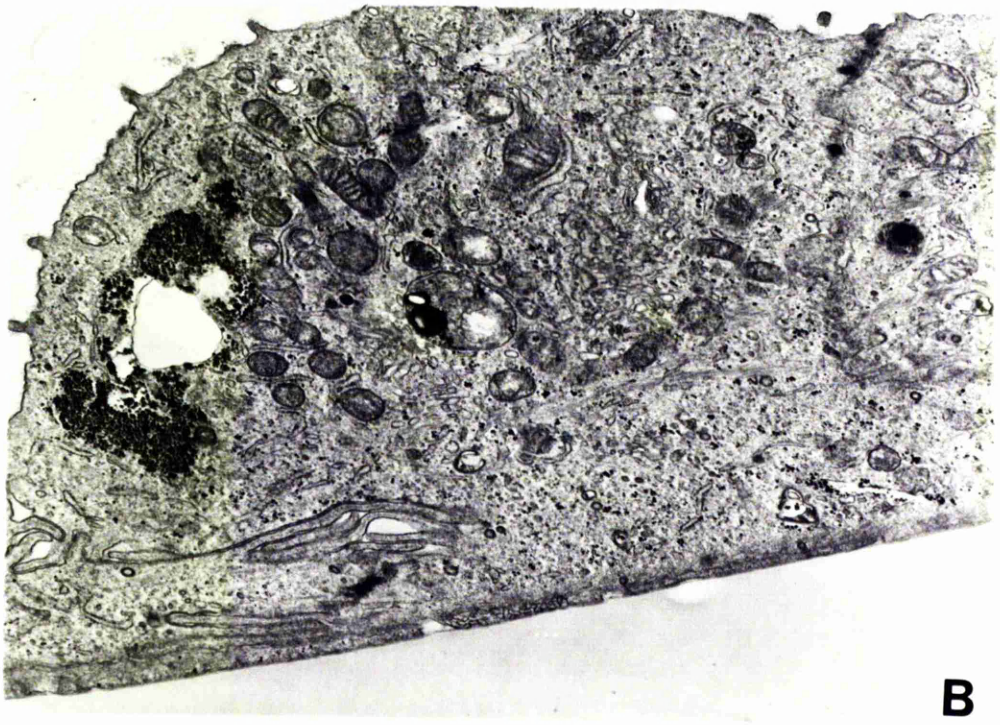
Cells were isolated (see Section 2.513) and cultured in 10% FCS for 12h. Medium was, then, replaced with 10% FCS-DCC(56). After 6 days, cells were processed as explained in Section 2.621.

a) x 8520

b) x 14155



A



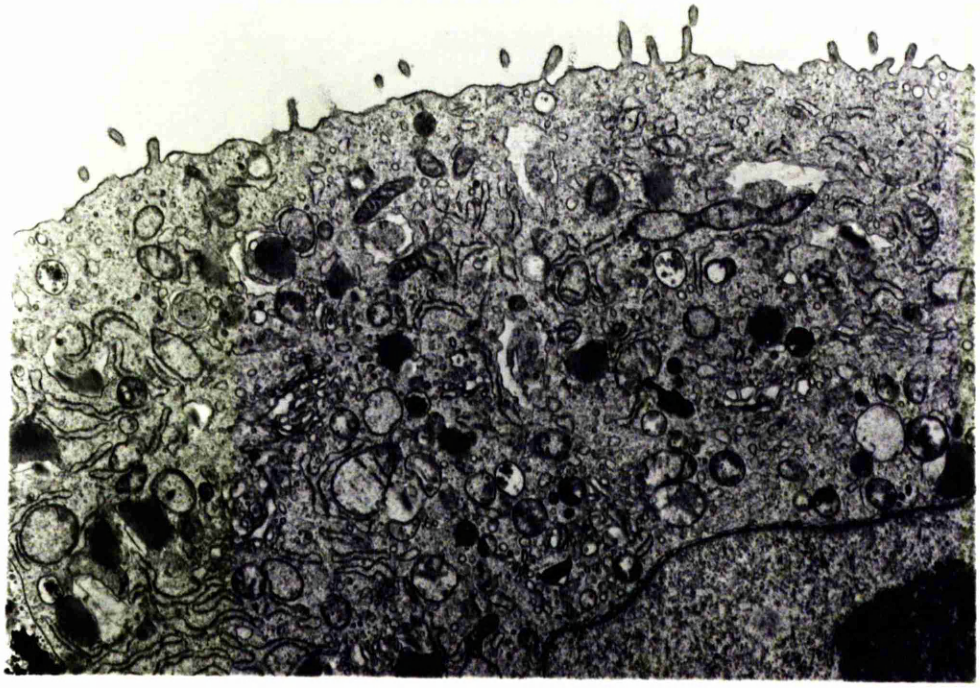
B

Figure 5.5: Effect of steroids on the morphology of human normal emdometrial epithelial cells

Cells were prepared and processed as for Figure 5.4. After 12h of culture, medium was replaced with a fresh one containing

a) progesterone (10^{-8}M) x 14155

b) oestradiol (10^{-9}M) x 40440



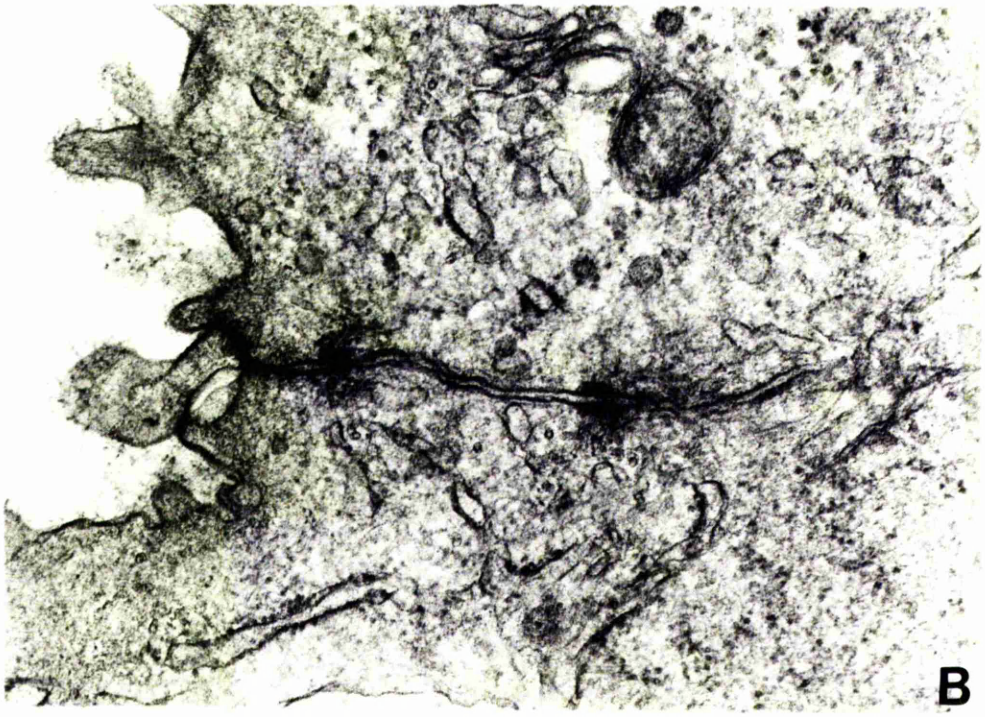
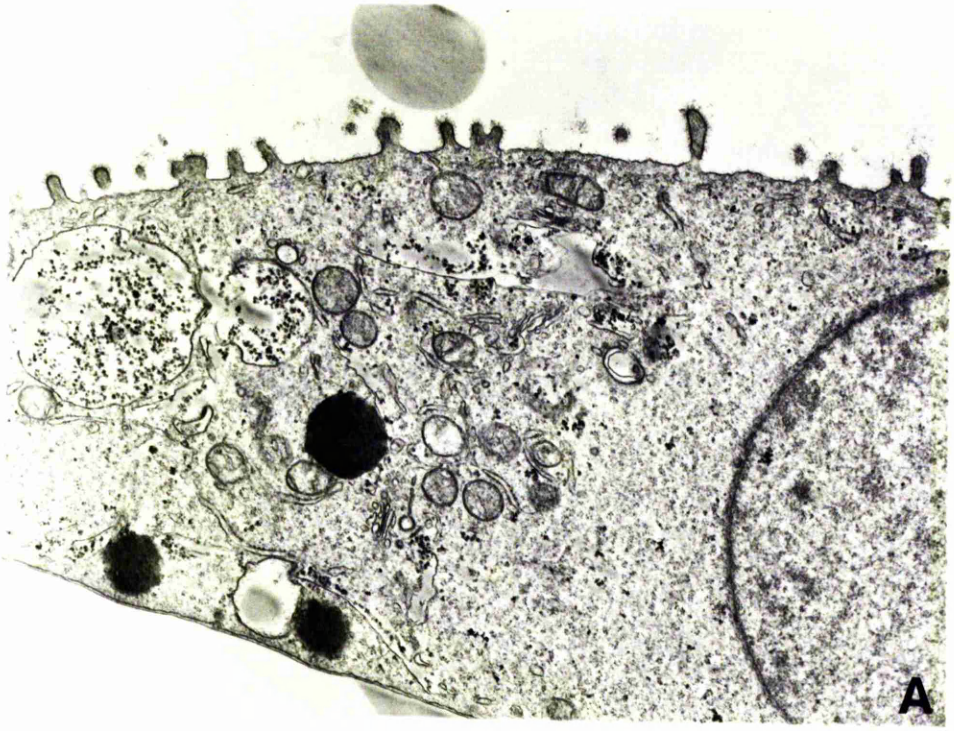

B

Figure 5.6: Effect of steroids on the morphology of normal human endometrial epithelial cells

Cells were prepared and processed as for Figure 5.4. Fresh medium replaced after 12h contained oestradiol (10^{-9}M) plus progesterone (10^{-8}M)

a) x 12855

b) x 8522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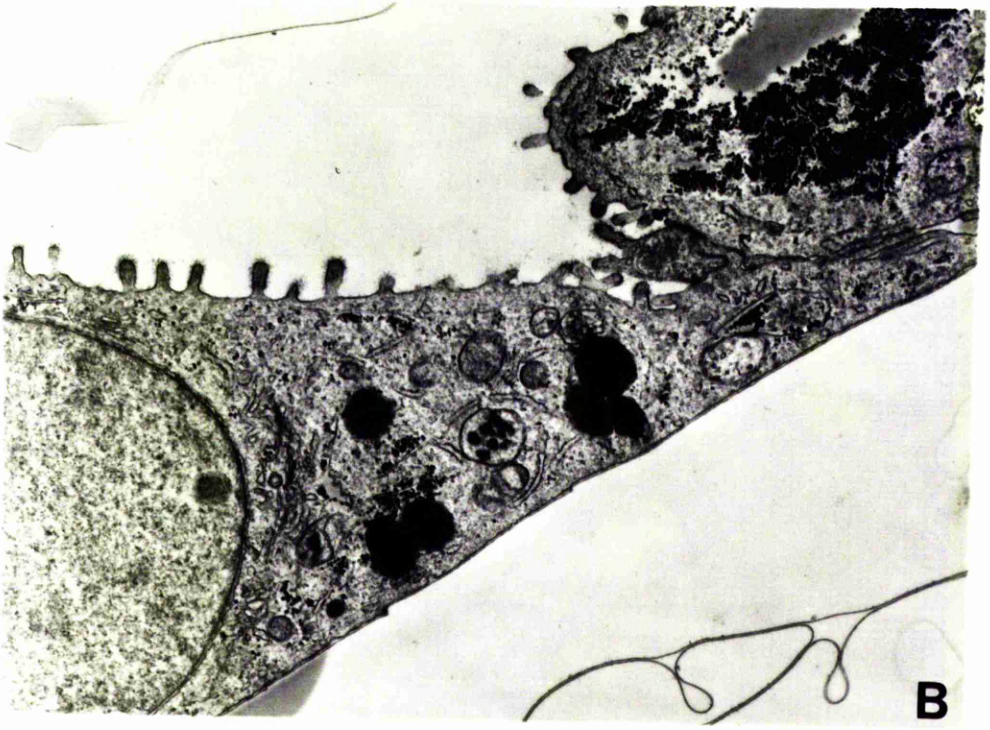

B

Figure 5.7: Actin staining of rat endometrial epithelial cells

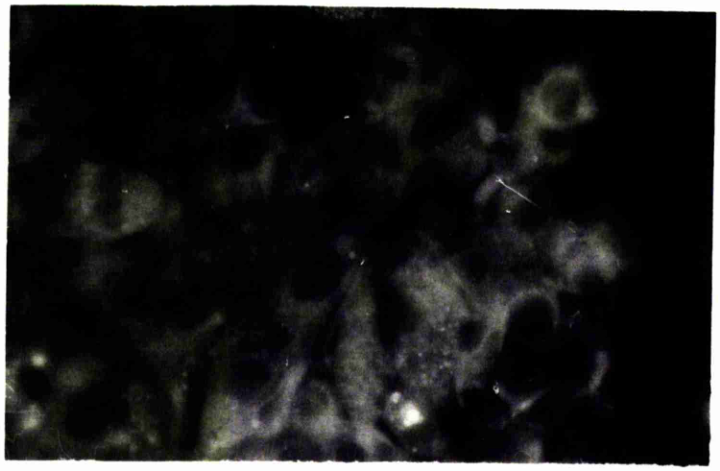
Cells were prepared and processed as for Figure 5.4. After 12h plating, cultures were stimulated for 48h with steroids as follows:

a) control x 250

b) oestradiol (10^{-9}M) x 360

c) progesterone (10^{-8}M) x 250

Cells were stained with NBD Phallacidin as described in Section 2.580



progesterone (10^{-9}M) induced abundant Rough Endoplasmic Reticulum (RER) Fig. 5.5b). Progesterone combined with oestradiol (10^{-9}M) promoted both lysosomal material and the frequency of Golgi apparatus in the cytoplasm of these epithelial cells (Figs. 5.4; 5.6). In rat endometrial epithelial cells, RER was not only increased quantitatively, but it was dilated in response to oestradiol (10^{-9}M) and/or cortisol (10^{-7}M), (see Figs. 5.8 a,b; 5.9 a,b). RER was more rounded in the cells cultured with cortisol (Fig. 5.9a). Overall mitochondria were also observed to be increased in these cells in response to oestradiol and cortisol (Figs. 5.8 & 5.9).

In rat uterine mixed cell populations, oestradiol increased the number of mitochondria (Figs. 5.10 a,b). RER appeared to increase quantitatively in response to oestradiol or progesterone in these fibroblasts (Figs. 5.10b, 5.11a). RER, in progesterone stimulated cells, was also very much dilated (Fig. 5.11a). Cortisol (10^{-7}M) increased production of glycogen.

5.400 HUMAN ENDOMETRIAL CANCER CELLS

5.410 STEROID REGULATION OF DNA SYNTHESIS

The endometrial cancer cells from a postmenopausal patient showed differential responses to the steroids in 5% FCS-DCC(56), in terms of thymidine incorporation (Fig. 5.6). The receptor status of the tumour was ER_c+, ER_n+, PR_c+, PR_n-. Oestradiol stimulated DNA synthesis maximally at 10^{-9}M, whereas the anti-oestrogen tamoxifen gave maximum stimulation at 10^{-8}M. Progesterone and MPA both depressed the growth at the levels of 10^{-8}M and 10^{-9}M respectively.

The rate of DNA synthesis was also assessed with steroids in FCS and charcoal stripped serum in the same tumour cells (see Table 5.7). Oestradiol enhanced the growth significantly with FCS-DCC(56).

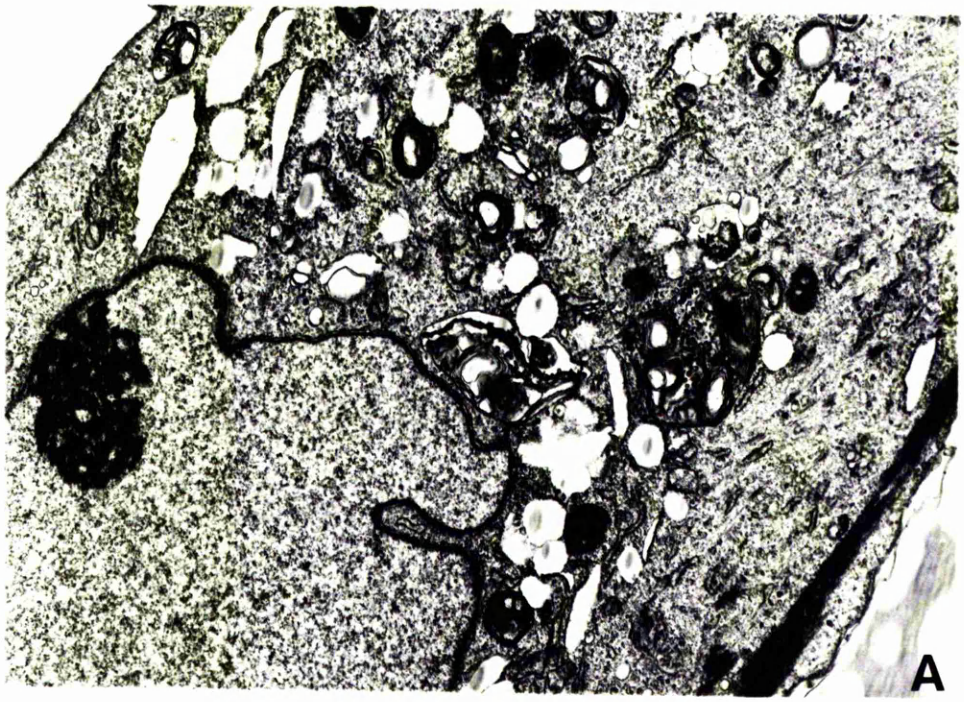
Figure 5.8: TEM of epithelial cells from rat endometrium

Cells were prepared and cultured as for Figure 5.4. After 12h, medium was changed to the following:

a) control x 12855

b) oestradiol (10^{-9}M) x 8522

After 48h of stimulation, cells were processed for TEM as described in Section 2.621


A

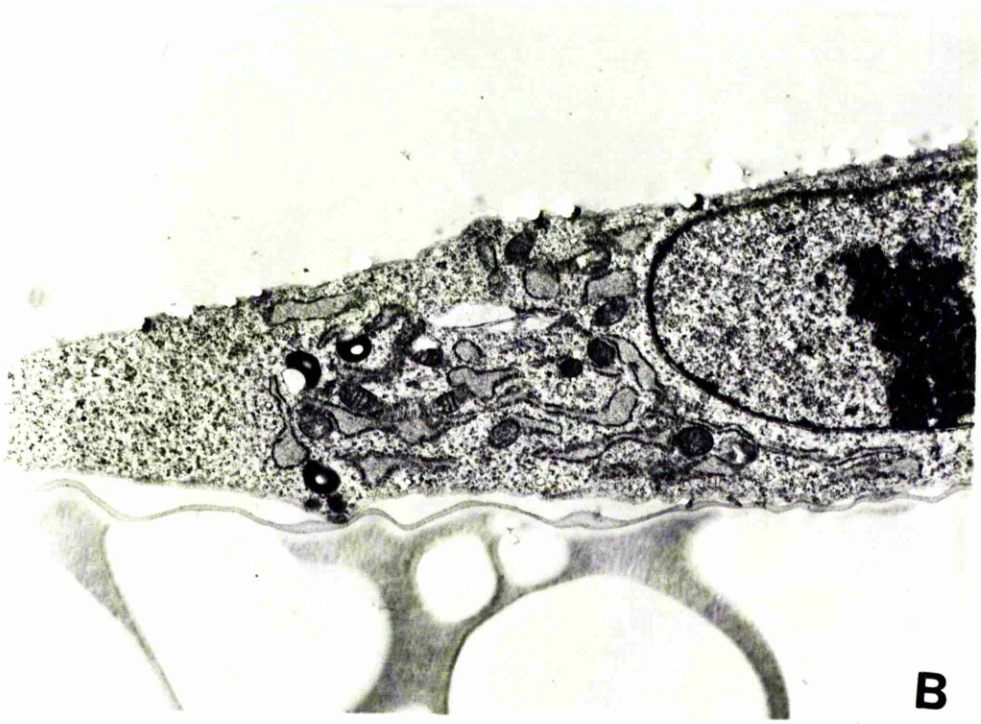
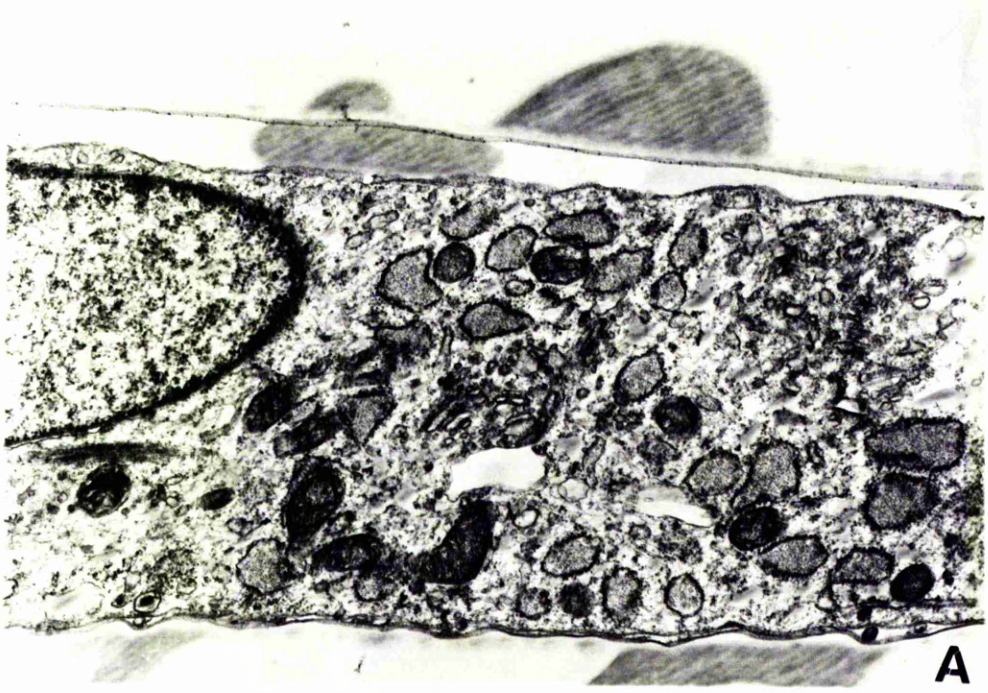

B

Figure 5.9: TEM of epithelial cells from rat endometrium

Cells were prepared and processed as for Figure 5.8. Cultures were stimulated for 48h as follows:

a) cortisol (10^{-7}M) x 14155

b) cortisol (10^{-7}M) plus oestradiol (10^{-9}M) x 14155


A

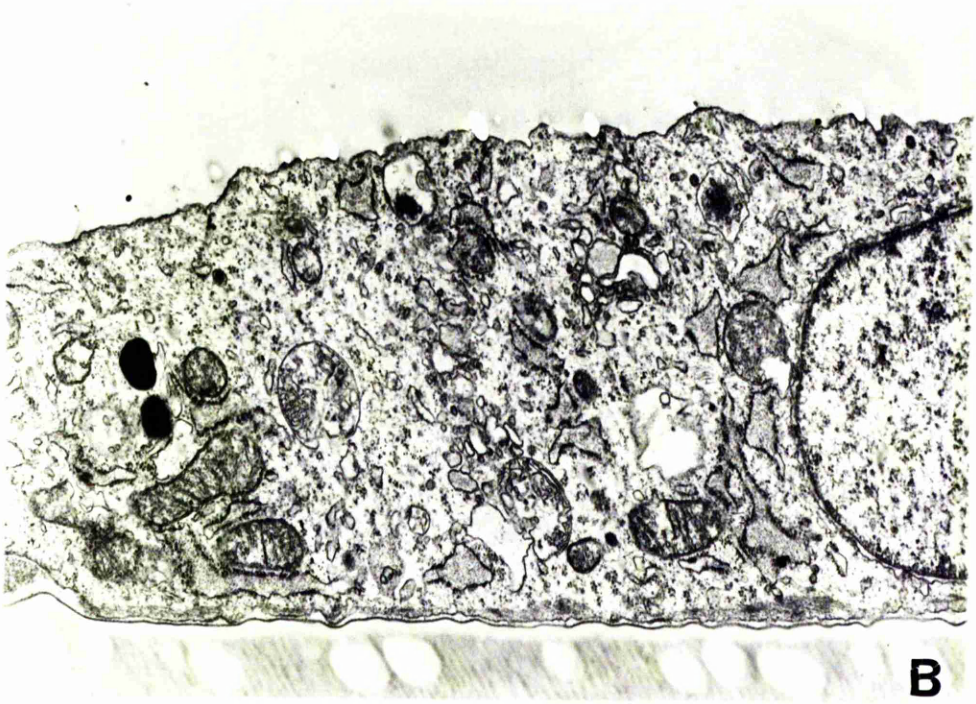
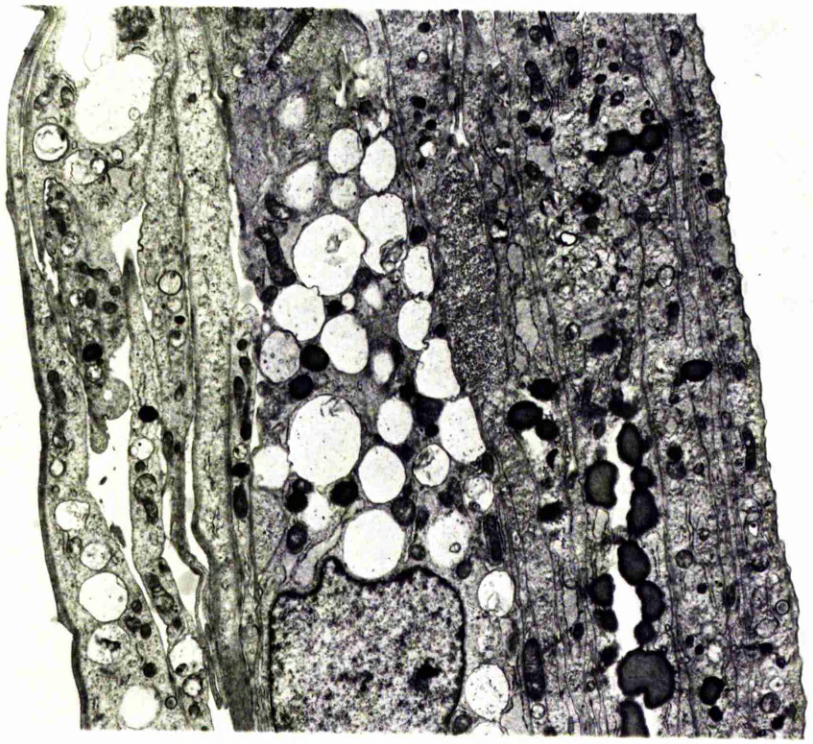

B

Figure 5.10: TEM of a rat uterine mixed cell population

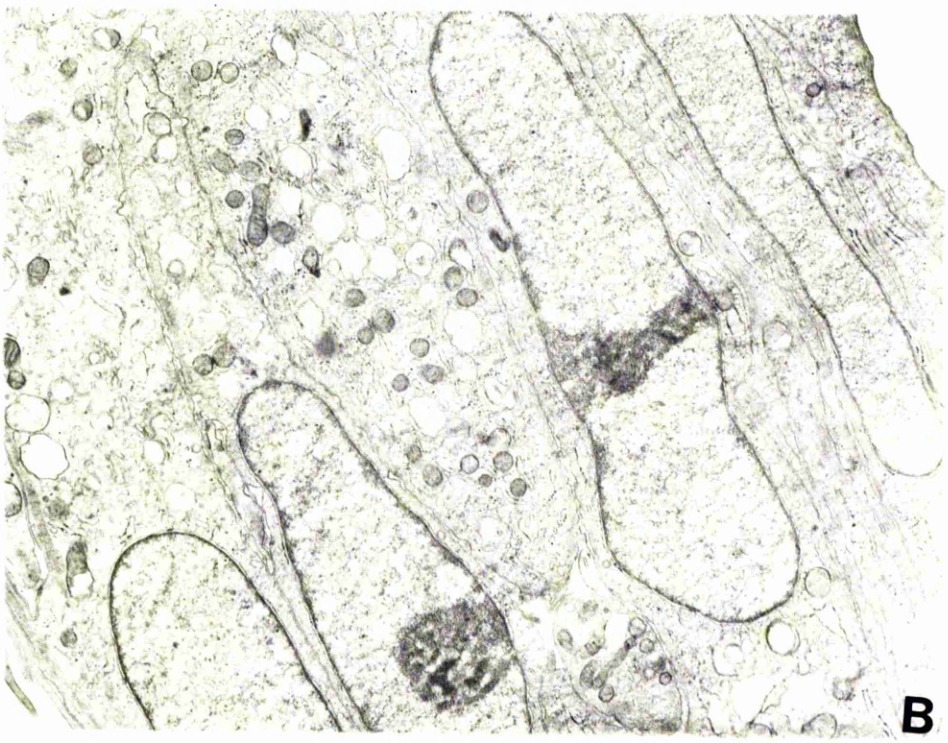
Cells were isolated (see Section 2.511) and cultured for 12h in 10% FCS. Medium was then changed and oestradiol (10^{-9}M) was added and left for a further 48h. Cells were then processed for TEM as described in Section 2.621.

a) x 8522

b) x 13611



A



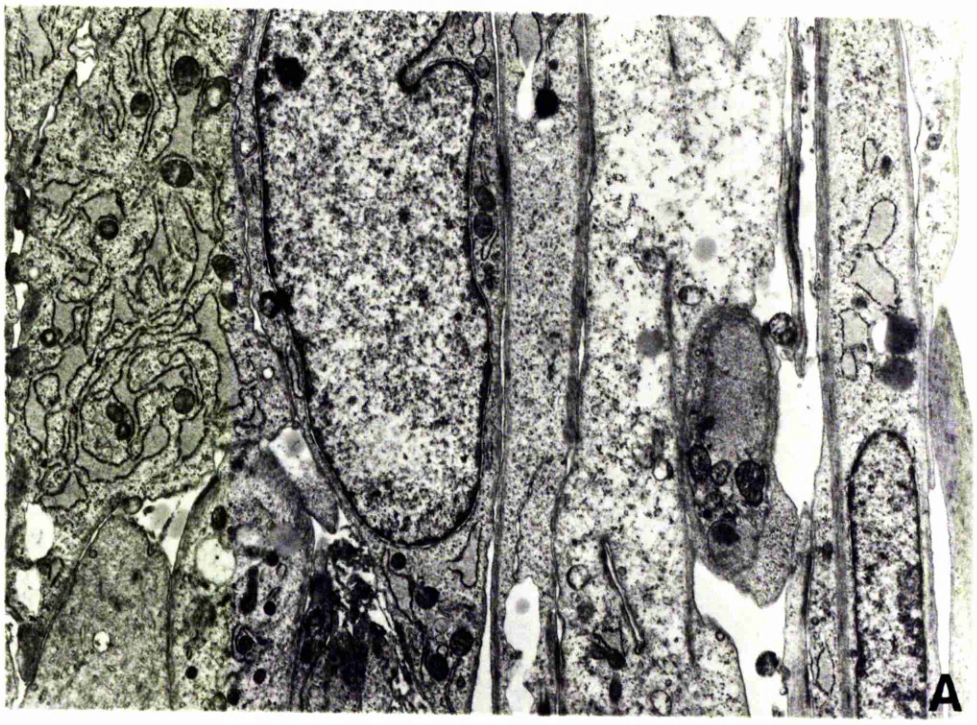
B

Figure 5.11 TEM of a rat uterine mixed cell population

Cells were prepared and processed as for Figure 5.10 but stimulated with:

a) progesterone (10^{-8}M) x 13611

b) cortisol (10^{-7}M) x 13611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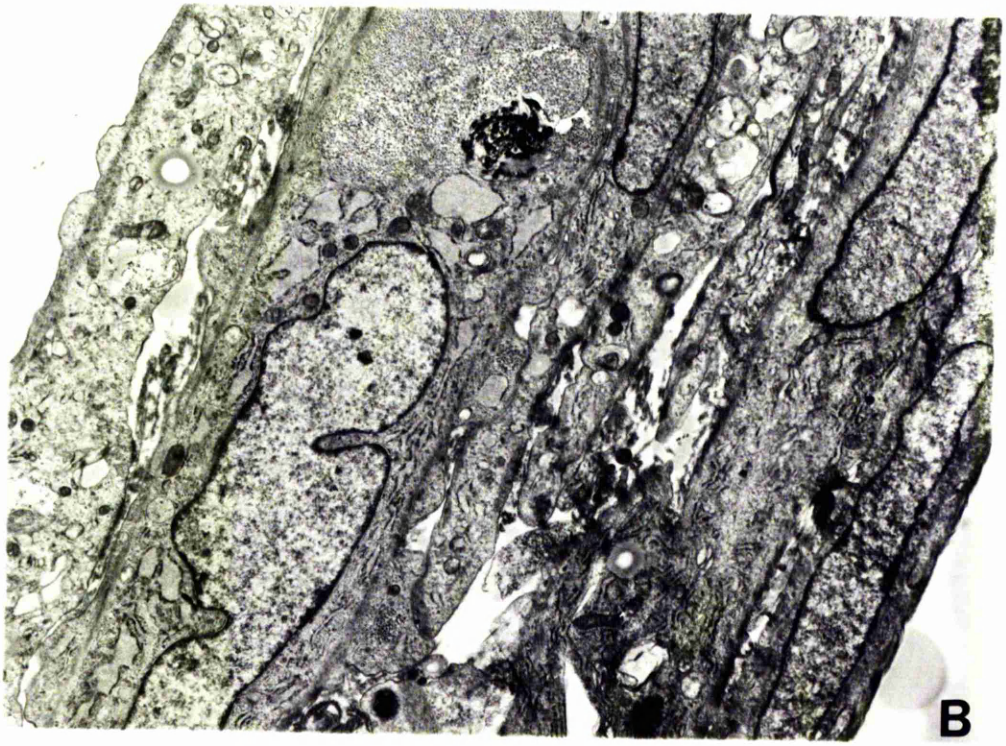

B

Table 5.7: Hormonal modulation of ^{3}H-thymidine incorporation into endometrial cancer cells in the presence of FCS, FCS-DCC(4) and FCS-DCC(56)

Cancer cells were prepared and processed as for Figure 5.12. Cells were stimulated with the following steroids over the 48h.

oestradiol (10^{-9}M)

progesterone (10^{-8}M)

tamoxifen (10^{-9}M)

dexamethasone (10^{-7}M)

MPA (10^{-8}M)

Receptor status of the parent tumour was as follows:

ER_c+, ER_n+, PR_c+, PR_n-

Patient was post-menopausal. Each value is the mean of two experiments.

* $P < 0.05$

** $P < 0.005$

*** $P < 0.001$

Steroid Hormones	MEDIUM SUPPLEMENTED WITH		
	FCS	FCS-DCC(4)	FCS-DCC(56)
Control	10.2 ± 3.1	18.0 ± 6.5	13.9 ± 4.6
Oestradiol-17β (10^{-9}M)	20.7 ± 10.8	8.7 ± 7.4*	32.0 ± 10.1*
Progesterone (10^{-8}M)		9.8 ± 5.8*	9.3 ± 5.6
Tamoxifen (10^{-9}M)	4.2 ± 1.6**	4.6 ± 1.9***	
Dexamethasone (10^{-7}M)	13.7 ± 4.2		19.9 ± 7.4
MPA (10^{-8}M)	3.4 ± 2.0**	4.5 ± 2.1***	

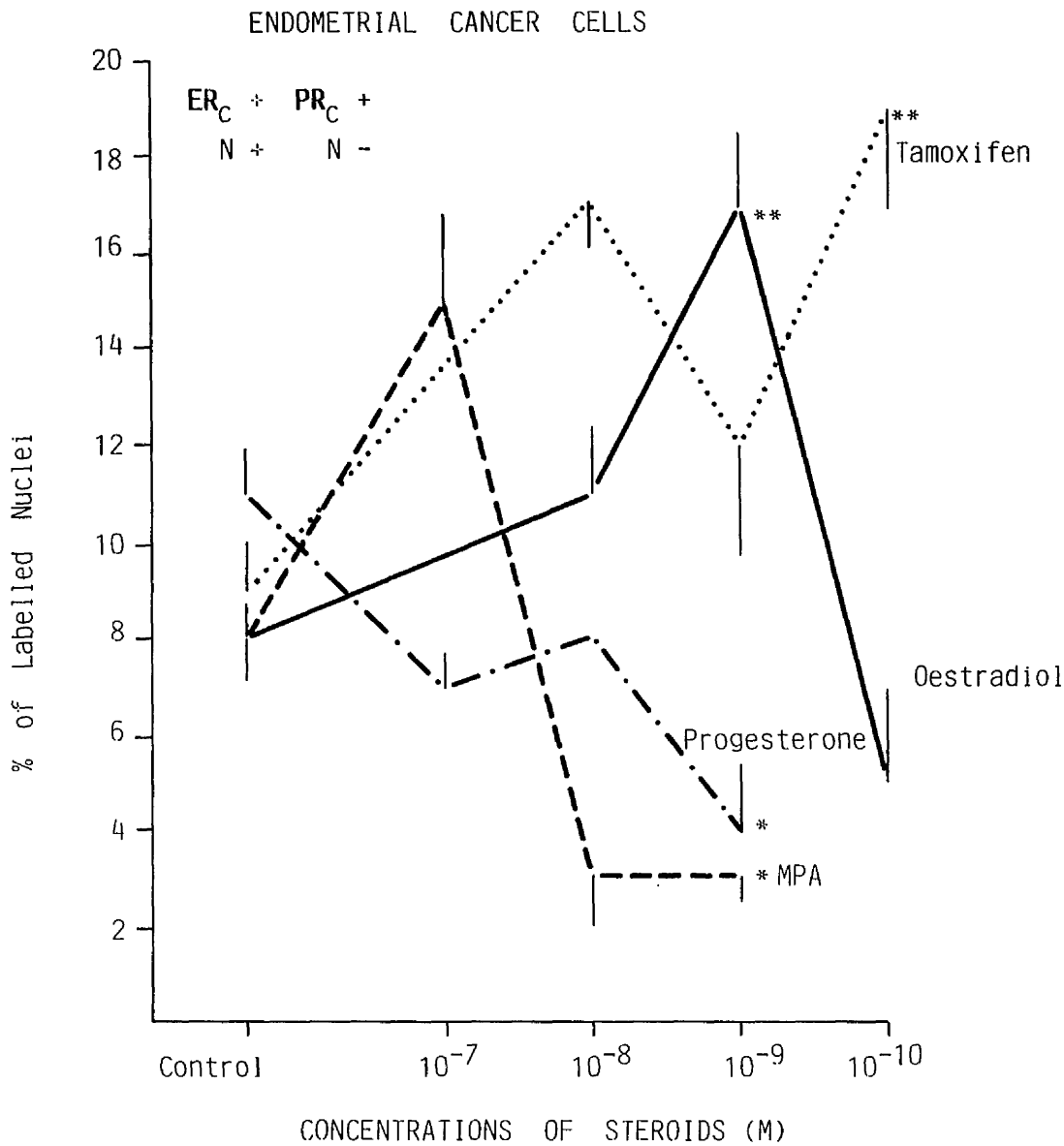
Figure 5.12 Hormonal modulation of DNA synthesis in endometrial cancer cells

Cancer cells were isolated (see Section 2.514) and grown as described in Section 2.520. Effects of various concentrations of different steroids on ^{3}H-thymidine incorporation were assessed in the presence of 5% FCS-DCC(56), over 48h. The receptor status of the parent tumour (post-menopausal) was ER_c+, ER_n+, PR_c+, PR_n+.

Each value is the mean of two parallel experiments.

* $P < 0.05$

** $P < 0.005$



Each value is mean of duplicate experiments. Effects of steroids were assessed in presence of 5% FCS-DCC ($56^{O}C$) over two days.

The cells were consistently responsive to tamoxifen and MPA. Both of these compounds showed inhibitory effects of DNA synthesis in both FCS and FCS-DCC(4). No significant change was observed with dexamethasone.

In a tumour cell population, lacking both oestrogen and progesterone receptors, oestradiol and dexamethasone inhibited thymidine labelling, where progesterone and MPA were stimulatory. Oestrogen plus progesterone induced the maximum response (Fig. 5.13).

In a study with a tumour cell population lacking oestrogen receptor but containing progesterone receptor, steroid-induced changes were observed in the presence of FCS and in serum-free medium (see Fig. 5.14). A decline in the rate of DNA synthesis was observed in the presence of progesterone, MPA and dexamethasone in both serum-free and FCS-DCC(56) medium, whereas cortisol only depressed DNA synthesis in FCS-DCC(56). Oestrogen and tamoxifen stimulated DNA synthesis in SFM but failed to show any significant change in the presence of FCS. In another series of experiments with the same cell population, the combined effects of different steroids on DNA synthesis were investigated using maximally effective concentrations in the presence of FCS (5%) and SFM (see Fig. 5.15). The cells grown with oestradiol for 24h were further stimulated for another 48h with various other steroids. Progesterone decreased DNA synthesis after oestrogen stimulation in the presence of FCS while being unresponsive in SFM, whereas oestradiol plus progesterone enhanced DNA synthesis further after stimulation with oestradiol (24h) in SFM and FCS. Dexamethasone failed to modify DNA synthesis in E_2-stimulated cells in any way. The continued presence of oestradiol together with either progesterone or tamoxifen over the 72h period increased the rate of DNA synthesis in the presence of serum or SFM.

Figure 5.13: Hormonal modulation of DNA synthesis in endometrial cancer cells

Cells were prepared and processed as for Figure 5.12. Influences of various steroids on ^{3}H-thymidine incorporation into endometrial cancer cells was assessed in the presence of 5% FCS-DCC(56). Concentrations of steroids used were as follows:

Oestradiol (E_2, 10^{-9}M)

Progesterone (P, 10^{-8}M)

Dexamethasone (D, 10^{-7}M)

Medroxyprogesterone Acetate (MPA, 10^{-8}M)

Each bar is the mean $\pm$ S.D. of triplicate culture.

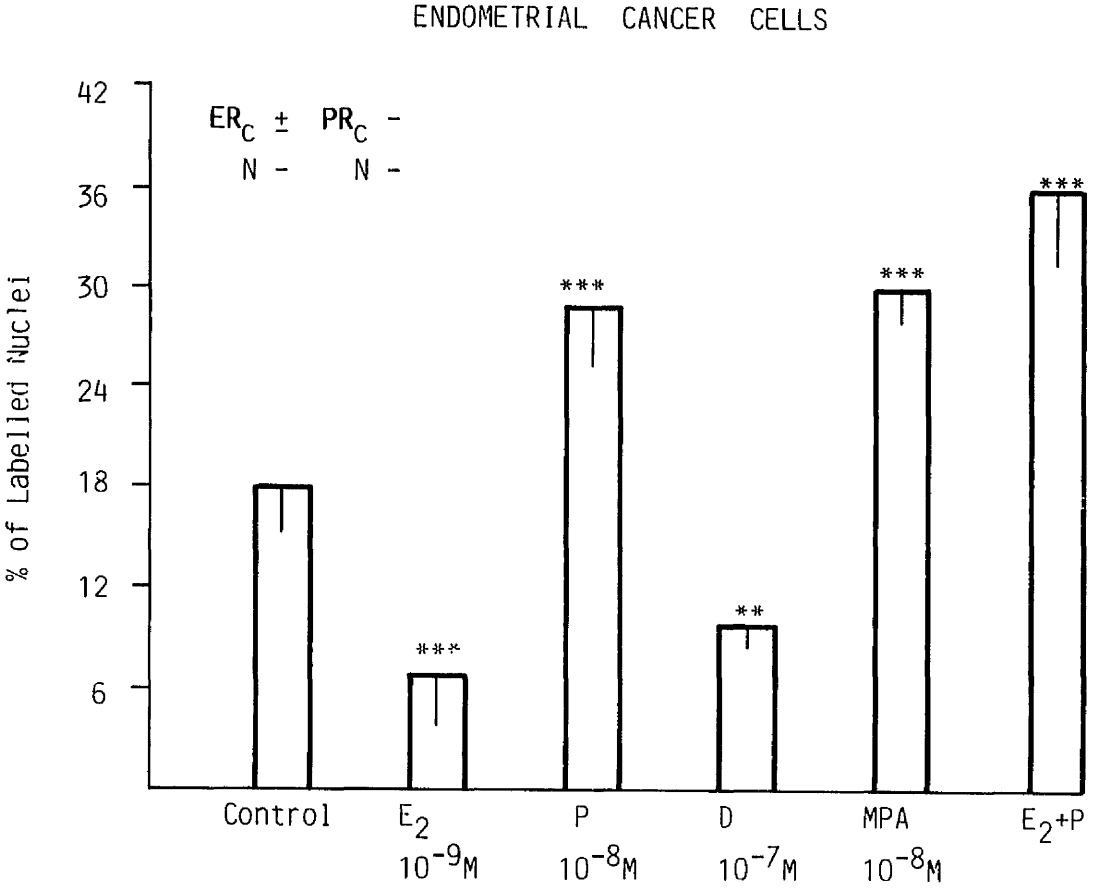
Receptor status of parent tissue (post-menopausal) was:

ER_c $\pm$	PR_c -
ER_n -	PR_n -

* $P < 0.05$

** $P < 0.005$

*** $P < 0.001$



Each point is mean of 3 experiments. Cells were grown in 5% FCS-DCC (56^{o}C). Influence of steroids on DNA synthesis was assessed after two days.

Figure 5.14: Hormonal modulation of DNA synthesis in endometrial cancer cells over two days

Cells were prepared and processed as for Figure 5.12. Effects of steroids on incorporation of ^{3}H-thymidine were assessed in endometrial cancer cells in the presence of 5% FCS-DCC(56) and SFM, over two days. Receptor status of the parent tissue was:

ER_c-	PR_c+
ER_n-	PR_n+

Concentrations of steroids used were:

oestradiol (E_2, 10^{-9}M)

progesterone (P , 10^{-8}M)

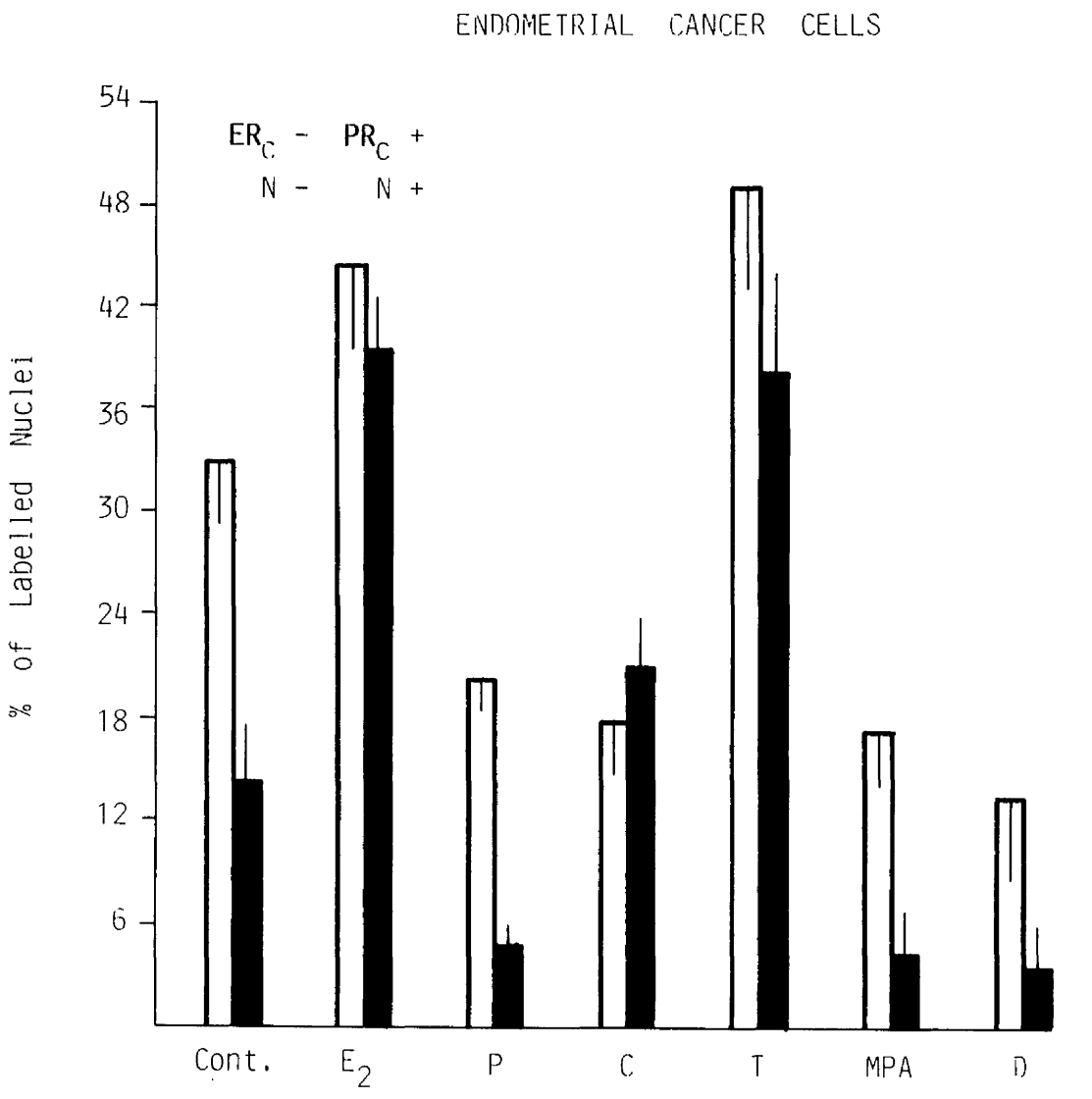
cortisol (C, 10^{-7}M)

tamoxifen (T, 10^{-9}M)

Medroxyprogesterone Acetate (MPA, 10^{-8}M)

Dexamethasone (D, 10^{-7}M)

Each point is the average of two independent readings.



Cells were cultured in presence of 5% FCS-DCC (56°C) □ or SFM ■. Effects over two days of steroids on DNA synthesis was assessed.

Figure 5.15: Hormonal modulation of DNA synthesis in endometrial cancer cells

Cells were prepared and processed as for Figure 5.12. Cancer cells in primary culture were stimulated by either individual steroids or by combinations for the first 24h. The medium was then replaced with new medium containing the same or different hormones and left for another 48h. ^{3}H-thymidine incorporation was determined after 72h (total), in the presence of 5% FCS-DCC(56) □ or SFM ■

Receptor status of the parent tissue (post-menopausal) was:

ER_c-	PR_c+
ER_n-	PR_n+

Concentrations of the steroids used were:

oestradiol (E_2, 10^{-9}M)

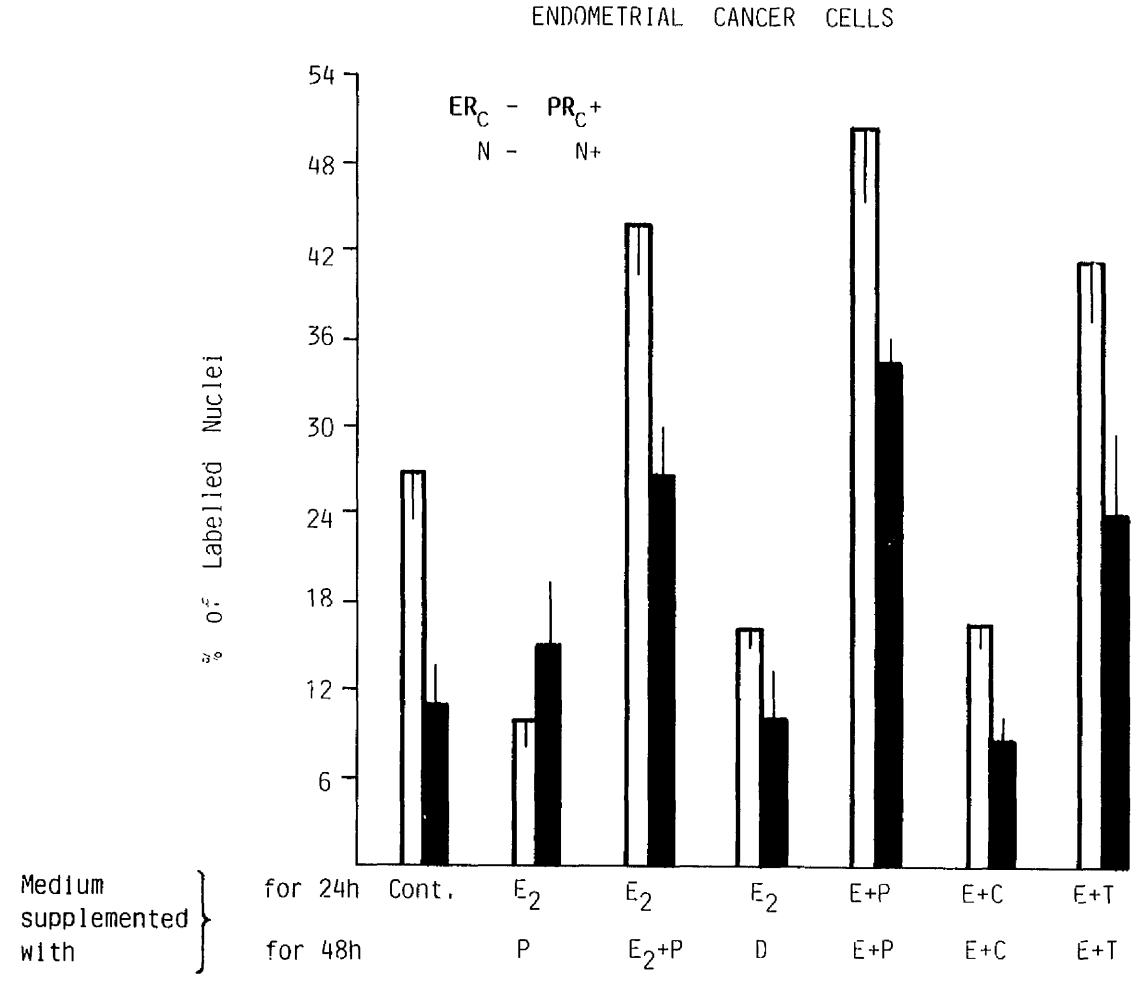
progesterone (P, 10^{-8}M)

cortisol (C, 10^{-7}M)

tamoxifen (T, 10^{-9}M)

dexamethasone (D, 10^{-7}M)

Each point is average of two experiments.



Effects of steroids over the 24 & 48 hours were assessed in presence of 5% FCS-DCC (56^{0}C) as well as in SFM

5.500 DISCUSSION

5.510 STEROIDS AND CELL PROLIFERATION

DNA labelling by ^{3}H-thymidine is considered to be one of the most suitable methods for the analysis of tissue responses to hormones *in vitro* (Ferenczy, 1979; Adams, 1980). Autoradiography was used in this study to estimate the rate of DNA synthesis. This method tends to minimise those effects of hormones which simply reflect changes in precursor uptake or pool sizes (Richman *et al.*, 1976). Although, a correlation between labelling index and thymidine incorporation into isolated DNA can be shown (Richman *et al.*, 1976), autoradiography is the method of choice in cultures that may contain different cell types with varying rates of DNA synthesis.

Three types of responses (stimulation, inhibition, no effect) could be demonstrated over a wide range of hormone concentrations but there was an optimum concentration for each hormone at which maximal DNA synthesis was induced under defined culture conditions. This suggests that the same population of cells which is stimulated by a lower concentration of a steroid, may be inhibited at higher concentrations of the same hormone.

Cultured "normal" untransformed cells show density-dependent regulation of growth (Holley, 1975), a complex phenomenon that results from a combination of causes which can vary for different cell types and for the same cell type under different conditions. The following factors have been shown to be involved in restriction of endometrial cell growth (Gerschenson *et al.*, 1979, 1980; Irwin, 1982).

a) Depletion of growth factors and nutrients from the medium

b) Production of inhibitors by the cells

c) Interactions between various components and factors present in the medium.

The results presented in this chapter did not provide any clear evidence to distinguish between these possibilities. However, they did show differences between the effects of individual steroids in different conditions.

Our initial finding that cultures did not respond to oestradiol (data not shown), led us to explore three possibilities.

a) Oestradiol would stimulate proliferation only in high density cultures and in such cultures the cells resulting from increased cell division do not stay attached (Gerschenson et al., 1981). Such floating cells were not detected when the attached cells were scored for ^{3}H-thymidine labelled nuclei.

b) The cells would metabolise oestrogen at such a high rate that the hormone could not act (Gerschenson et al., 1979).

c) A factor inhibiting response to oestradiol could be produced by the cells (Gerschenson et al., 1979, 1981).

Gerschenson et al. (1979) showed an oestrogen inhibitory factor existed that was dependent on density of rabbit endometrial epithelial cells in culture. It is also possible that the production of a steroid hormone inhibitory factor may be related to the density of, or critical ratio of, a certain cell type within the culture. It is interesting to point out that a report on the responsiveness of a cell line (L-929) to steroid hormones, mentioned that androsterone, a potent growth factor, exerts this effect in low, but not in high density cultures (Jung-Testas, I. & Baulieu, E.E., 1979).

The effects of steroids on DNA synthesis were of lesser magnitude in vitro than the response to the steroids shown by some cellular components of the uterus in vivo (Kaye et al., 1972; Tachi et al., 1972; Katzenellenbogen & Leake, 1974; Cowan & Leake, 1979; also see Sections 1.3100, 1.3200). The possible reasons for this divergence

in behaviour between the cultured cells and the intact uterus *in vivo* include the following:

a) In dispersed cell cultures normal tissue organisation is lost

b) The cultured cells may be deprived of the permissive action of other hormones or growth factors

c) In heterogenous cell populations only some of the component cell types may have been responsive to the hormones

d) Loss of receptors for certain steroids during initial tissue dissociation.

The experiments described in this chapter are consistent with the findings of Chen *et al.* (1973) and Berliner and Gerschenson (1976) who could show response to steroids in terms of cell division and morphology. But according to Liszczak *et al.* (1977) and Kirk and Irwin (1980), steroid hormones failed to modify response in endometrial epithelial cells. A possible explanation for both cases is that not all cells are cycling. A specific stimulus may activate one of the two (cycling and quiescent) cell populations (Gerschenson *et al.*, 1979). Another explanation could be offered on the basis of a recent finding in this laboratory. Larger epithelial cells seem to be more active in taking up ^{3}H-thymidine and cell size is, in turn, inversely related to the size of colony or density of culture (Field, 1983). It is not yet clear whether increased epithelial cell size is related to polyploidy or other factors. However, human endometrial epithelial polyploid cells have been shown to preferentially incorporate ^{3}H-thymidine over diploid cells (Kirk & Irwin, 1980). Recently, Borjesson & Sarpty (1981) also suggested the possibility of larger breast cancer cells being oestrogen-responsive.

Some authors have proposed that oestrogens might stimulate proliferation indirectly causing changes in sensitivity to other agents

(Sonnenschein & Sato, 1980; Gerschenson et al., 1981) or via other organs (Ikeda et al., 1982). Several authors have also described the existence of a small molecule which inhibits nuclear translocation or binding to DNA of active glucocorticoid receptors (Litwack, 1979) Chen et al. (1973) suggested a direct effect of oestradiol on target cells. While oestradiol is clearly the major stimulator of cell growth and proliferation in the uterus, there is increasing evidence that uterine sensitivity to oestrdiol is modulated by everal other hormones (Hsueh et al., 1976; Rochefort & Garcia, 1976).

All these ideas indicate that both positive and negative effectors may play important regulatory roles in the effect of ovarian hormones on endometrial cell proliferation. The influence of the same effectors on other parameters of metabolism should prove of interest.

5.511 DNA Synthesis and Serum Dependence

Physiological concentrations of steroid hormones present in whole serum may obliterate responses to additional hormones. Hence, serum was stripped of endogenous steroids by charcoal (see Section 2.351). To assess the interactions of steroid hormones with other components of serum and /or medium, endometrial epithelial cells were cultured in medium with normal whole serum (FCS), charcoal stripped serum (FCS-DCC(4), FCS-DCC(56)) or no serum at all.

Oestradiol-17β slightly depressed (Table 5.1) DNA synthesis in epithelial cells cultured with whole serum (FCS). Similar, almost unresponsive behaviour of breast tumour cells has been demonstrated in the presence of FCS (Darbre et al., 1983). Charcoal stripped serum at $4^{o}C$ or $56^{o}C$ showed increased responses to oestradiol, even at lower concentrations. An important observation was made concerning concentrations of different sera. The results indicate 5% serum to be appropriate to observe response to oestradiol. In contrast, Page et al.

(1983) demonstrated that MCF-7 cells were only responsive in the presence of 15% serum. These differences may be explained by considering the different characteristics of primary culture and established lines (Paul, 1970). The results from oestradiol stimulated cells in serum-free medium (SFM) agree with those of Lippman et al. (1975, 1976).

A stimulatory response was observed to tamoxifen over two days which was more consistent in the presence of whole serum (FCS) and FCS-DCC(4) (Table 5.2). An inhibitory response to tamoxifen in terms of growth of MCF-7 cells has recently been reported (Osborne, 1983; Sutherland, 1983). They showed this inhibition in the presence of FCS but over a longer period than studied here.

Relatively higher concentrations of progesterone depressed DNA synthesis significantly but only in FCS-DCC(4) (Table 5.3). MPA showed inhibition at a concentration of 10^{-7}M in FCS. A lower concentration (10^{-9}M) of MPA inhibited DNA synthesis in charcoal stripped serum. This decreased rate of DNA synthesis with MPA was only observed at a 10% concentration of serum (Table 5.4).

It appears from the above observation that some serum component(s) was required for steroid-induced alterations of growth. However, the cells did respond to oestradiol in the presence of charcoal stripped serum or in serum-free medium. A similar finding has been reported by Irwin (1982). According to him, high concentrations of whole serum (FCS) suppressed the stimulatory effects of ovarian steroids. However, the presence of serum seems to be more appropriate to look at stimulatory effects of glucocorticoids on endometrial cells. These observations are also in agreement with those of Irwin (1982). This difference in serum dependence between ovarian steroids and dexamethasone could be partial explained by their different binding to serum proteins since protein-bound steroids are biologically inactive

(Westphal, 1971). Pavlic and Katzenellenbogen (1978) have shown that under ordinary culture conditions the ratio of protein-bound to free E_2 in the medium increases linearly with the concentration of serum. As a result, the amount of E_2 available to the cells decreases dramatically with increases in the serum concentration in the culture.

An alternative explanation for the difference in serum dependence between dexamethasone and ovarian steroids would be that of a lower metabolic inactivation of glucocorticoids and MPA compared to the natural steroids E_2 and P. Thus, at optimal serum concentrations the higher cell densities achieved would result in higher activities of steroid metabolising enzymes. Pavlik and Katzenellenbogen (1978) have shown that confluent cultures of mixed epithelial and stromal cells from human endometrium rapidly metabolise E_2 to E_1.

5.520 MORPHOLOGICAL CHANGES IN RELATION TO GROWTH

The morphological changes reported here, indicate that steroids have specific effects on endometrial cells in primary culture. Oestradiol, in combination with progesterone, brought about a considerable increase in nuclear and cytoplasmic volume. Similar *in vivo* changes were noted by Tachi *et al.* (1974). Oestradiol stimulated cells have increased numbers of organelles and appear to be more metabolically active than cells in control cultures as evidenced, for example, by increased amounts of rough endoplasmic reticulum. The simultaneous presence of oestradiol and progesterone in the medium promotes increased surface activity in terms of the number and length of microvilli. However, it does not appear that either E_2 or P alone is capable of exerting any comparable effects on surface microvilli in these cells. Observations made on several cell types have led to the proposal that the formation of microvilli requires the movement of filamentous material from beneath the plasma membrane into the microinjections

(Madison et al., 1979). Oestradiol alone managed to modify the membrane characteristics, like increased number of junctions between cell membranes and altered cytoskeleton. Similarly, the amplification of cellular junctions in a number of tissues can be correlated with altered and elevated biochemical changes which result from the administration of exogenous hormones or other stimulatory agents (Burghardt and Anderson, 1979). The results in this study clearly establish that the onset of increased microvilli on the surface in response to steroids is coupled with an increase in lysosomal material. Mattson and Kowal (1980) observed increased steroidogenesis in response to ACTH in adrenal cells, which is associated with an increase in microvilli structures and lysosomes.

Qualitative and quantitative changes were observed in rough endoplasmic reticulum in response to progesterone. Berliner and Gerschenson (1976) reported a 2-fold increase in the amount of RER in response to P or P + DES. Oestrogen (E_2) in ovariectomised rats, previously treated with P, has been reported to bring about distension of RER (Tachi et al., 1974).

The modification in the structure of a nucleus of endometrial cells in response to E_2 has been described (Vic et al., 1980). This supports the idea that transcription is stimulated when chromatin has been decondensed by E_2. Biochemical and ultrastructural results in this study support this idea and provide evidence that a specific effect of oestrogen can be observed on replication in a rapidly growing cell population, which is engaged in little secretory activity, while cells treated with P appear to be engaged in the production of proteins for secretion. This holds exciting possibilities for the induction of differentiation in hormone dependent tumours using progestins.

5.530 STEROID RECEPTORS AND GROWTH PATTERNS OF HUMAN ENDOMETRIAL CANCER CELLS

This study provokes further speculation about the function of serum in the growth ofendometrial cancer cells. Serum factors significantly influence the response of cancer cells to a variety of steroids. In general, whole serum supports certain steroids to depress growth, whereas stimulatory behaviour can be observed more often in FCS-DCC(56) or SFM. Whole serum is normally required to grow cells in culture at a faster rate (Page et al., 1983), and this situation is ideal to observe inhibition in growth (Sutherland, 1983). Serum factors decrease the quantity of hormone available to cells (Darbre et al., 1983). The stimulation in growth by physiological concentrations of steroids can best be observed with a medium containing minimum quantities of influencing factors e.g. SHBG etc. (Lippman, 1983). The results reported in this study clearly demonstrate that the rate of DNA synthesis in endometrial cancer cells was increased in response to steroids, particularly in SFM or in a medium containing FCS-DCC(56).

Another observation made was that the presence of progesterone receptor (PR) in the cells seemed to be necessary to observe progesterone-induced reduction of DNA synthesis. Satyaswaroop et al. (1978) reported the lack of responsiveness of a human endometrial cell line to progestins. They presented data on oestradiol receptor (ER) in these cancer cells but did not measure PR. A number of endometrial cancers have been found to contain ER but not PR. These tend to be insensitive to endocrine therapy (Leake, 1981b). Several other reasons may be proposed for these negative results. For example, Satyaswaroop et al. (1983) demonstrated that the lack of progesterone sensitivity in cultured endometrial carcinoma explants in vitro is due to progesterone receptor instability under culture conditions. This may explain the

similar negative results reported by Shapiro et al. (1975), who failed to show any difference in protein synthesis in human endometrial cancer cells either with oestrogens or progestins. In contrast, Satyaswaroop et al. (1983) showed distinct E_2-induced growth only in receptor-containing endometrial carcinoma.

Soutter and Leake (1978) also demonstrated increased DNA synthesis in response to oestradiol in explants of endometrial carcinoma containing ER. The results in the present study indicate that the rate of DNA synthesis can be stimulated by oestradiol and tamoxifen in cultured cells from both tumours, containing or lacking ER, depending on serum conditions (see Figs. 5.2-5.5 and Table 5.7). Surprisingly, in a cell population derived from a tumour lacking both ER and PR, oestradiol decreased and progesterone increased DNA synthesis. This situation can be explained by suggesting an alternate mechanism of action. Shafie (1980) reported that oestrogen had an indirect action on tumour growth since the growth of MCF-7 cells was entirely dependent on E_2 in nude mice but was independent of oestrogen in culture. Several other investigators (Ikeda et al., 1982; Sirbasku, 1983) have proposed similar indirect mechanisms in tumour growth in other oestrogen target tissues and have attributed this to the involvement of other factors. It seems reasonable to propose that certain steroids can affect the growth of endometrial tumour cells through both receptor-mediated and receptor-independent mechanisms.

5.540 INTERACTION BETWEEN STEROID HORMONES

The stimulation of normal endometrial epithelial cells by maximally effective concentrations of E_2 was synergised by P. A similar response was also observed by simultaneous stimulation with E_2 and glucocorticoids but only in the presence of FCS-DCC(56) (Fig. 5.1). In endometrial cancer cells E_2 plus P also increased the labelling

index significantly (Fig. 5.5). In the same tumour cell population, E_2 plus glucocorticoids depressed DNA synthesis, but only did so in the presence of FCS-DCC(56). The finding of a biphasic response of the E_2 + glucocorticoids complex may be explained on the basis of the presence or absence of ER. Two criteria used to quantify different growth factors as acting through different mechanisms are their ability to synergise when used together at maximally effective concentrations and to interact differently with a third growth fractor (Irwin, 1982). Applying these criteria to the present data, the synergism between E_2 and P and opposite effects of E_2 with glucocorticoids on cancer cells, suggests that these three types of steroids affected growth by acting through different mechanisms presumably involving their receptor machineries.

Oestradiol produced a significant increase in DNA synthesis in normal endometrial epithelial cells in medium supplemented by FCS-DCC(56). The combination of E_2 with cortisol further increased the response, implying that glucocorticoids may be important for E_2 to be effective in the cells containing ER (Freshney et al., 1983). In a tumour cell population, lacking ER but containg PR, E_2 in combination with glucocorticoids depressed the growth. The mechanism of this is not clear unless glucocorticoids use and act through PR as well as their own receptors. Consistent with the latter interpretation, the above mentioned effects were obtained using physiological concentrations of steroid hormones. Progesterone and cortisol have been shown to interact with both PR and glucocorticoid receptors (King and Mainwaring, 1974). However, dexamethasone binds to the PR only to a limited extent (Walters & Clark, 1977). Therefore, the effect of dexamethasone is probably a specific event mediated through the glucocorticoid receptor. On the other hand, the present studies showed that cortisol and P had opposite

effects on E_2-stimulated growth suggesting that the effects of these two steroids on cancer cell growth were not solely mediated through interaction with the same receptor.

CHAPTER 6 - GENERAL DISCUSSION

6.000 CULTURE CONDITIONS AND RESPONSIVENESS

An understanding of how hormones affect cell proliferation, necessarily involves knowledge of the regulatory processes of this cellular function. The regulation of the frequency of replication in animal cells is a complex phenomenon for which much of the molecular basis is currently unknown. A review of the literature published during the last decade on the characteristics of cells derived from endometrial tissue reveals great variations in culture survival, DNA synthesis and growth. Cells obtained from malignant tissue are the most difficult to study because of low cell numbers and lack of *in vitro* growth capacity. These have hindered attempts to quantitate the effects of any factors which may alter survival time.

Attempts to elicit steroid responses in culture have met with varying degrees of success. This may, in part, be due to some inherent limitations in culture techniques, the skill of the operator in maintaining the tissue in culture or the nature of the experimental model. The results presented in this thesis showed that endometrial cells in primary culture retained the potential to express differentiated functions in response to steroids.

The results in Chapter 5 showed that individual hormones prefer separate sets of conditions to induce an optimal response in endometrial epithelial cells. A series of experiments were carried out to investigate the contribution of serum in growth responses to steroids. They revealed differences in the ways in which the different steroids affected the rate of DNA synthesis. Steroid hormone binding proteins (SHBG etc) present in serum, are the main hind rance to the availability of free hormone to cells in culture (Westphal, 1971). Page *et al*. (1983) confirmed the presence of factors in serum, which could positively influence the expression of a growth response at least to

oestradiol. Thus, it appears that some serum component(s) was required for steroid stimulation of growth. In contrast, Lippman and Bolan (1975) and Lippman et al. (1976) showed steroid responses in MCF-7 cells only in serum-free conditions. Where no response is seen in serum-free medium, two separate reasons may contribute:

a) not enough growth to observe any stimulation

b) lack of stromal cells to produce some components to induce or support the response.

Another important factor may be associated with cell density in culture. Hormonal requirements may vary according to the density of cells (Holley, 1975). Very high density endometrial epithelial cultures contain a higher number of diploid cells than do sub-confluent cultures (Kirk et al., 1978). It is also quite possible that steroid action on epithelial cells is modifed by other hormones or growth factors. Experiments directed along these lines should prove interesting, together with hormonal effects on differentiation as opposed to proliferative responses. The timing of observation could be an important factor regarding the responsiveness. Fleming et al. (1980) demonstrated fluctuations in ER levels but could not conclude whether steroids induced the changes in receptor content or only affected the time at which the peak of ER concentration appeared.

Analysis of medium components is another effective approach to the assessment of steroid responsiveness in cultured cells. Another interesting finding raised the possibility that cells could metabolise weak hormones to produce active compounds or that a hormone could induce dividing cells to synthesize and/or secrete a factor which could modulate the subsequent response to hormones (Gerschenson et al., 1979).

It can be concluded, therefore, that the hormone responsiveness of endometrial cells is compatible with the results

reported by Gerschenson's and Lippman's groups although valid comparisons are often difficult because of different hormone concentrations, differences in periods of culture, different types and concentrations of serum and a variety of different culture conditions.

6.100 PHYSIOLOGICAL RELEVANCE OF THE IN VITRO STUDIES

6.110 THE ORIGIN OF CULTURED CELLS

The results presented in Chapter 3 give strong support for the conclusion that endometrial epithelial cultures are indeed derived from epithelium of the endometrium. This conclusion is further strengthened by several lines of evidence presented in Chapter 5, which indicate that the epithelial cells had the potential to express differentiated functions *in vitro* in response to steroids. The features of epithelial cells *in vitro* were consistent with those of the epithelial fraction of the endometrium *in vivo*.

In vitro findings presented in the previous chapter also support the concept of glandular epithelium *in vivo* as a population which has the potential to proliferate and differentiate in response to the appropriate stimuli. Furthermore, the present studies have shown that ovarian steroids play a direct role in the control of proliferation and in the expression of differentiated functions in the epithelial cells of the rat and human endometrial cells.

6.120 HORMONE INDUCED GROWTH

Primary cultures of epithelial cells were consistently responsive to steroids and growth factors, as evidenced by morphological changes, growth and DNA synthesis. However, the stimulatory or inhibitory effects of various hormones on growth *in vivo* were poor as compared to that observed *in vivo*. This difference may be explained by considering the culture conditions.

Mechanistic studies of steroid hormone action are frustrated by the inherent variations of the hormonal responses observed in uncloned

epithelial cells. A uniformity of response might be obtained from studies on cloned cells. However, epithelial cultures do promise to be a useful tool for studying growth factor interactions in vitro.

The fact that there is general morphological and biochemical similarity between cultured cells and the cells in vivo, suggests that endometrial cells cultured in defined media can be used as a reliable system to study specific, direct actions of hormones. The data confirm previous experimental information and suggest the usefulness of this method as an indicator of overall changes in cell characteristics. The evidence presented that cultured endometrial cells respond to physiological concentrations of ovarian steroids suggests that this system will be a useful in vitro model for the study of uterine function and for evaluating the oestrogenic potency of new compounds being tested for contraceptive or therapeutic activities. In addition, the methodological advantages of cell culture which enable hormone responsiveness to be studied for rather prolonged periods should provide a valuable complement to clinical studies thus obviating some of the difficulties inherent in obtaining information about human reproduction and uterine function from in vivo studies alone. It should also eliminate the problems of premature cell death associated with organ or "explant" culture.

6.130 CLINICAL SIGNIFICANCE

As described in Sections 1.4300 and 1.4400, disturbance in one or several of the intrinsic mechanisms involved in the growth of the uterine epithelium could result in hyperplasia or/and carcinoma. The cells that have undergone malignant transformations are often less subject to normal growth regulation. Thus, one might expect malignant endometrial cells to respond aberrantly to normal signals. Since the events of endometrial transformation involve intrinsic changes at the cellular level, cell culture, which is exempt from tissue and systemic

influences is ideal for investigating whether an alteration of endometrial cell response to hormone actually occurs at the cellular level. The onset of endometrial cancer may reflect, in part, a breakdown in the normal regulation of DNA synthesis. Much of the early work on steroid-dependent DNA synthesis was carried out *in vivo* (Kaye *et al.*, 1972; Martin *et al.*, 1973; Leake *et al.*, 1975). To avoid the complexity of the *in vivo* situation, and to assess specific responses of individual cell types, the value of primary cultures of both normal and malignant endometrial epithelial cells have been investigated. This approach can help in selecting optimum therapy for individual patients. Potentially this system also permits *in vitro* comparison of the functional properties of epithelial and stromal components in response to various hormones or carcinogens. It could act as a test system for screening new anti-cancer drugs. This concept entails the use of hormones to accelerate tumour cell replication and to enhance the susceptibility of these cells to the lethal effects of cytotoxic agents. Since endometrial cancer consisted of both hormone-dependent and independent cell populations (see Section 1.4500), such a mixed cell population could best be eradicated by a combination of hormonal and cytotoxic therapy.

6.200 FUTURE PROSPECTS

The research described in this thesis indicates that the future holds great promise for increased understanding of hormones and their cellular response in culture. In turn, this will provide an insight into how cell-cell interactions occur *in vivo* and the relative importance of each type of modulation can be assessed. Endometrial epithelial cell cultures should provide a useful system for future studies in the following areas of endocrine research:

6.210 GROWTH REQUIREMENTS

Overall progress in the understanding of cellular growth requirements and the development of new media may be rather slow. Nevertheless a complete understanding of the growth requirements of endometrial cells may soon be reached. Serum, in addition to providing classical hormones and growth factors, compensates for the deficiencies of the defined media by supplying additional nutrients and trace elements. It provides proteins which bind vitamins, lipids, metals and hormones and may stabilise and modulate the action of these ligands. Serum further provides factors for attachment and spreading, buffering capacity and protection against proteases. When serum is omitted from the culture medium, substitutes must be found for all its functions.

6.220 CELLULAR AND MOLECULAR ASPECTS

In the present studies some factors which affect the responsiveness of endometrial epithelial cells to ovarian steroids have been identified. This offers an opportunity to study in vitro the process leading to the unresponsive state and the mechanism involved therein. The present work suggests that both altered surface activities and DNA content may be the results of action of an ovarian steroid regulated gene product in endometrial epithelial cells. It is not unreasonable to assume that new, uncharacterised dimensions of cytoplasm exist and that even more complicated levels of molecular organisation will be found in cells.

6.230 CELL-CELL INTERACTIONS

Little is known about the role of each cell type in cell-cell interactions which results in induction of functional differentiation. The extracellular matrix produced by stromal cells in vitro may serve as the ideal substrate on which to examine epithelial cells as they express changes in their differentiated functions in response to the stromal

signals. By mixing separately cultured cell types, the role of cell contact on epithelio-mesenchymal interactions could also be studied. Using such recombinations of epithelium and stroma from normal, hyperplastic or neoplastic endometria, might permit study of interactions involved in the normal-to-neoplastic transition *in vitro*.

CHAPTER 7 - REFERENCES

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