GROWTH OF HUMAN BREAST CELLS IN PRIMARY CULTURE

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A Thesis submitted for the Degree of Doctor of Philosophy in the Faculty of Science.

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This thesis is dedicated to my parents.

ACKNOWLEDGMENTS.

I am indebted to my supervisor, Dr. R.E. Leake, for his help, encouragement and friendly support during the course of this project and in the writing of this thesis. I should also like to thank Dr. R.I. Freshney for his help, encouragement and advice on the practical problems of tissue culture.

I am grateful to all the members of Lab. C26, in particular Sheila Cowan for her friendship and proofreading of this thesis.

Thanks are also due to the members of the Dept. of Medical Oncology, especially Elaine Hart for her friendship and advice on tissue culture.

I am grateful to Laurence Tetley of the EM unit at the Dept. of Zoology for his skillful help and advice on the techniques of scanning electron microscopy. I am indebted to the surgeons at various hospitals in the Glasgow area for provision of human breast tumour tissue without which these studies could not have been performed.

I should like to thank Professor Smellie for provision of facilities within the Dept. of Biochemistry and Professors Calman and Kaye for provision of facilities within the Dept. of Medical Oncology. I acknowledge receipt of a Medical Research Council studentship.

I am grateful to the Medical Illustration Unit for the preparation of a poster demonstration, and particularly 'Wullie'for the black and white prints on P84 paper. I should also like to thank the Photographic Unit for helping to brighten up this thesis with colour prints.

I am also very grateful to Mrs. M. Nimmo for the typing of this thesis.

ABBREVIATIONS

The standard abbreviations, as recommended in the Biochemical Journal "Policy of the Journal and Instructions to Authors", (1981), are employed throughout this thesis with the following additions:-

AFP	α- foetoprotein
ATP	Adenosine triphosphate
BMAMF	A human breast stromal fibroblast cell strain
BPE	Bovine pituitary extract
BSA	Bovine serum albumin
^{o}C	Degrees Centigrade
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CK-BB	Brain-type isozyme of creatine kinase
CM_	Conditioned medium from BMAMF breast stromal fibroblast cell line grown in the absence of exogenous oestradiol
CM_/E	CM_ supplemented with 10^{-9}M oestradiol
CME	Conditioned medium from BMAMF breast stromal fibroblast cell line grown in the presence of exogenous oestradiol(10^{-9}M)
CME/C	CME supplemented with 10^{-7}M cortisol
CP-3	Chromosomal protein fraction 3
CPD	Critical point dehydration
DCC	Dextran coated charcoal
DES	Diethyl stilboestrol
DMBA	Dimethyl benz(a) anthracene
DMEM	Dulbecco's modification of Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
E_2	Oestradiol
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra-acetic acid
EGF	Epidermal Growth Factor
EMA	Epithelial membrane antigen
ER	Oestrogen receptor
ER_c	Soluble oestrogen receptor
ER_n	Nuclear oestrogen receptor
ER o/o	Oestrogen receptor negative
ER +/+	Oestrogen receptor positive

FITC	Fluorescein isothiocyanate
FCS	Foetal calf serum
FD	Freeze drying
G_{50}	Half of the growth achieved with serum supplemented culture medium
GAG	Glycosaminoglycan
GH_3/C14-AGF	Autocrine growth factor from GH_3/C14 cells/tumours
GR	Glucocorticoid receptor
(^3H)	Tritium labelled
HEPES	4-(2-hydroxyethyl-1-piperazine-ethanesulphonic acid)
HIDCCFCS	Heat-inactivated charcoal stripped foetal calf serum
HIDCCFHS	Heat-inactivated charcoal stripped female human serum
HMFG-1	Antiserum to human mammary fat globule
HMFG-2	Antiserum to human mammary fat globule
hnRNA	Heterogeneous ribonucleic acid
hPL	Human placental lactogen
HPLC	High performance liquid chromatography
IF	Intermediate filament
Kd	Dissociation constant
KDGF	Kidney-derived growth factor
LE61	Antiserum to keratin 18
MCF-7	A human breast cancer cell line
M8	Antiserum to human milk fat globule membrane
Mr	Relative molecular mass
mRNA	Messenger ribonucleic acid
MSA	Melanocyte stimulating activity
MTCA	Mean total colony area
MTCN	Mean total colony number
MTGF-Pit	Pituitary gland derived mammary tumour growth factor
MTV	Mammary tumour virus
NAP	Nucleoacidic protein
NIH-3T3	Mouse embryo fibroblast cell line
NMU	N-nitrosomethylurea
PBS-A	Dulbecco's phosphate buffered saline A(without calcium and magnesium)
PGE1	Prostaglandin E1
PGE2	Prostaglandin E2
PGF2α	Prostaglandin F2α
PR	Progesterone receptor
RNA	Ribonucleic acid

SDS-PAGE	Polyacrylamide gel electrophoresis performed in the presence of sodium dodecyl sulphate
SEM	Scanning electron microscopy
SHBG	Sex hormone binding globulin
STO	Mouse embryo fibroblast cell line
Swiss 3T3	Mouse embryo fibroblast cell line
T_3	Tri-iodothyronine
TEM	Transmission electron microscopy
Tfm	Testicular feminisation
TGF- α	Alpha-type transforming growth factor
TGF- β	Beta-type transforming growth factor
UDGF	Uterine derived growth factor

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SUMMARY

Human breast tumours were enzymatically disaggregated with collagenase and established in primary culture on a variety of feeder layers of mitomycin-C treated mouse embryo fibroblasts. The NIH - 3T3 and STO cell lines proved the most successful in promoting the growth of primary breast cultures (successful cultures from approximately 80% of breast tumours).

Primary cultures were selected from different categories of breast tumours on the NIH - 3T3 and STO feeder layers. For the oestrogen receptor (ER) positive category, the premenopausal group was selected on both feeder layers. In the ER negative group, the NIH - 3T3 feeder layer selected for the postmenopausal group, while the premenopausal group was selected by the STO cells. A mitogenic response to cortisol or oestradiol alone was observed for the well differentiated ER positive groups on the NIH - 3T3 feeder layer, whereas the postmenopausal ER negative and the premenopausal abnormal ER groups responded well on the STO feeder layer. Differences were also observed in the responses to the cortisol/oestradiol combination on both feeder layers. On the NIH - 3T3 feeder layer, the ER positive group showed twice the response of the ER negative group and the premenopausal group showed over 2.5 times the response of the postmenopausal group. In contrast, on the STO feeder layer, the ER negative group showed over 50% greater response to the hormone combination than the ER positive group, while the postmenopausal and premenopausal groups did not differ in their degree of response. These results have suggested the existence of an indirect autocrine- or paracrine-type regulatory mechanism for breast epithelial cell growth which may be independent of the ER. The response of the different feeder layers to steroids was briefly studied but no direct effects were observed.

Epidermal growth factor (EGF) and prolactin were mitogenic

for primary breast cultures although the former also increased plating efficiency while the latter reduced it. The effect of EGF was potentiated by cortisol with regard to colony area. The steroids, cortisol and oestradiol, alone or in combination antagonised the mitogenic action of prolactin and also reduced plating efficiency. Experiments with conditioned medium from human breast stromal fibroblasts suggested the existence of an additional indirect paracrine-type mechanism for the growth regulation of mammary epithelium.

Hormones and growth factors have been found to exert differential effects on both the plating efficiency and growth of the primary breast cultures. The difference in the response of primary cultures to these external signals, when grown on different feeder layers, emphasises the importance of the stromal-epithelial interaction in growth regulation in vivo. The magnitude of the mitogenic responses to external signals is dramatically less than the in vivo effects. The existence of autocrine and paracrine growth regulatory mechanisms is proposed in addition to the well recognised endocrine mechanism. The results presented have confirmed that a complex interplay of hormones and growth factors is involved in the modulation of mammary epithelial cell growth.

Characterisation of the primary cultures has been performed by both immunocytochemical and scanning electron microscopy (SEM) studies. The mammary origin of the primary cultures was indicated by the studies with the M8 and HMFG-2 antibodies. The epithelial nature was confirmed by staining with anti-desmoplakin and anti-keratin antibodies. Unequivocal proof of the purely epithelial nature of the cultures came from the specific staining with the LE61 antiserum to keratin 18 which is present only in the glandular epithelium and not the basal, myoepithelial cells. Localised regions of breast colonies and, less commonly, single cells were intensely stained. This intense staining and the local concentration of heavily labelled cells in the autoradiographic studies may

both reflect the heterogeneity within the cultures and local synchronisation or improved communication between specific cells.

The expression of keratin 18, a marker of simple epithelia, has been investigated under various conditions with regard to possible changes in epithelial differentiation. The effects of various steroids and retinoic acid were assessed by immunofluorescence studies. The steroids elicited little change in the expression of keratin 18 although cells grown in the presence of cortisol/tamoxifen exhibited an irregular morphology. Retinoic acid, however, appeared to induce a phenotypic alteration in the pattern of keratin 18 expression. The more linear and diffuse arrangement suggested either a reduced expression of keratin 18 or alternatively masking of keratin 18 antigenic determinants. These observations were particularly interesting as vitamin A is recognised as having an important role in epithelial growth and differentiation. Vimentin, the characteristic intermediate filament type of mesenchyme derived cells, was co-expressed with keratin in primary breast cultures, as has been shown for other epithelial cells in primary culture.

The SEM studies demonstrated that, although primary cultures did not respond in a mitogenic manner to oestradiol, they maintained a functional state of differentiation. The epithelial nature of the primary cultures was once again verified. Changes in the surface morphology of some cultures in response to oestradiol were observed. Cultures derived from ER positive biopsies showed a greater degree of roundedness and an increase in the number and length of microvilli in response to physiological levels of oestradiol. Primary cultures established from ER negative tumour biopsies, however, showed no change in surface morphology in the presence of oestradiol.

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1. Introduction

The mammary gland is a target organ for several trophic hormones. These hormones play a major role during the different phases of mammary growth, in particular during puberty and lactation. Growth regulation of the mammary gland involves not only oestrogens and progestins, but also several other mammogenic hormones and possibly other growth factors as described in section 1.4.

1.1 Control Mechanisms

Co-ordination of the diverse activities of different cells in the human body is achieved by the integration of the nervous and endocrine systems. The nervous system is responsible for detecting changes in the internal and external environments and responds rapidly by generating electrical impulses. These impulses are generated from the brain and rapidly conducted along nerve processes to the effector tissues. In contrast, the effects of the endocrine system are mediated through hormones which are chemical messenger substances synthesised in specific ductless glands. These hormones are released into the bloodstream and transported to their target organs where they elicit their biological effects.

Hormones may be subdivided into two groups with respect to their mode of action. The first group includes the catecholamine and polypeptide hormones which generally induce rapid and transient responses. These hormones interact with specific cell surface receptors and mediate their effects through a second messenger. Binding of these hormones to their receptors generally results in activation of the membrane-bound enzyme adenyl cyclase which catalyses the formation of the second messenger, cyclic AMP from ATP. Increased intracellular cyclic AMP levels result in the activation of protein kinase(s) which phosphorylates one or more proteins in the cell to invoke the action of the hormone.

The other group of hormones includes the thyroid and steroid hormones which enter the target cells and interact with intracellular receptors. The mechanism of action of steroid hormones will be discussed in section 1.2 with particular reference to oestrogen action.

1.11 Steroid Hormones

Steroid hormones are lipophilic molecules, which retain a common cyclophenanthrene structure since they are all derived from the parent C_{27} compound cholesterol. In terms of their biological effects, steroid hormones may be classified into the following categories: i) oestrogens; ii) progestins; iii)androgens; iv) corticosteroids; v) vitamin D related steroids and vi) ecdysteroids.

Steroid hormones exhibit a continuous spectrum of biological effects and some hormones antagonise or synergise the effects of others. For example, the antagonism between oestrogens and androgens is well established (Huggins & Clark, 1940) as is the synergism between oestrogens and progestins (King & Mainwaring, 1974).

1.12 Plasma Proteins

In the bloodstream, an equilibrium exists between free steroid hormone and hormone bound to plasma proteins (albumin, specific steroid hormone binding globulins), in favour of the bound form. The concentration of free steroid hormone is therefore very low, for example, the concentration of oestradiol is about 10^{-10} M, with the majority bound to sex hormone binding globulin (SHBG) (Sandberg et al, 1957). The affinities of the binding proteins range from weak (Kd $\sim 10^{-3}$ M) to very strong (Kd $\sim 10^{-10}$ M) (Clark & Peck, 1981). The level of free hormone available for entry into the cells is determined by these binding proteins which play an important role in hormone-receptor dynamics.

1.2 Molecular Mechanism of Steroid Hormone Action

The mode of action of steroid hormones is described in this section with reference to oestrogen action. The role of steroid hormones in the regulation of gene expression is well established. However, there is controversy regarding the localisation of the oestrogen receptor (ER) in the intact cell, the binding of the hormone receptor complex to acceptor sites and the nature of these sites.

1.21 Hormone entry into cells

It is regarded that steroid hormones penetrate the phospholipid bilayer of the cell membrane by passive diffusion down a concentration gradient (Gorski & Gannon, 1976; Giorgi & Stein, 1981) although specific mechanisms for cellular uptake of steroids have been reported (Milgrom et al, 1973; Rao et al, 1976). Steroid hormones enter both target and non-target cells as shown by the presence of radiolabelled hormone in all tissues shortly after injection of labelled hormone (Jensen & Jacobson, 1960,1962). In target cells, the hormone is retained by high affinity binding to a specific macromolecule (receptor) in a non-covalent manner while in non-target cells the hormone diffuses out.

1.22 The Classical "Two Step" Model

Until recent reports on the subcellular localisation of ER (King & Greene, 1984; Welshons et al, 1984), the Classical "two step" model for steroid hormone action was widely accepted. It was independently proposed for oestrogen action by Jensen et al, (1968) and Gorski et al, (1968). Binding of oestradiol to its 'soluble' cytoplasmic receptor was shown to result in the formation of a hormone - receptor complex (Step 1) which was subsequently translocated to the nucleus (Step 2). Prior to translocation, the hormone - receptor complex undergoes an 'activation' step which endows it with a greater affinity for DNA and chromatin. An alteration in the sedimentation coefficient for the receptor on sucrose density gradients from 4S to 5S is also observed.

This is said to be due to the addition of a 50K polypeptide (Notides & Nielsen, 1974, 1975) and is referred to as transformation which is an event unique to ER.

The 'Classical' model was based on results from fractionation studies from immature and ovariectomised rats with low endogenous oestrogen levels. Unoccupied receptor was regarded as being present in the cytosol fraction (Toft & Gorski, 1966; Toft et al, 1967) with the majority of the labelled hormone-receptor complex being associated with the nuclear fraction (Shyamala and Gorski, 1969).

1.23 The Equilibrium Model

The validity of the 'Classical' model has been questioned by Sheridan's group (Martin & Sheridan, 1980, 1982; Sheridan et al, 1979) who have proposed that unbound receptor is in equilibrium, partitioned between the cytoplasm and nucleus according to the free water content of these intracellular compartments. Their autoradiographic data, obtained using thaw-mount techniques (Sheridan et al, 1979) conflicts with that of Jensen et al, (1968). Within five minutes of incubation of $0^{o}C$, specifically bound oestrogen was found to have an extensive nuclear localisation at the periphery of uterine sections. The improved methodology for autoradiography, which is least susceptible to redistribution artefacts, has been suggested to account for the difference in receptor localisation. Further experiments have shown that receptor appears artefactually in the soluble fraction due to the homogenisation of the tissue in large volumes of buffer with respect to the tissue mass (Martin & Sheridan, 1980).

1.24 Reinvestigation of oestrogen receptor localisation

1.241 Enucleation studies to localise receptor

Welshons et al, (1984), have employed enucleation techniques to reinvestigate the subcellular distribution of unoccupied ER in the GH_3 cell line, derived from rat pituitary cells. Cytochalasin-B induced enucleation has yielded cytoplast and nucleoplast

fractions. The cytoplasts contain cytoplasm within an intact plasma membrane while the nucleoplasts comprise nuclei with a small amount of cytoplasm and intact membrane. Unoccupied ER were predominantly found in the nucleoplast enriched fraction. The cytoplast fraction, however, contained only 5-10% of the concentration of ER present in the whole cells.

1.242 Immunocytochemical studies on the localisation of ER

A panel of monoclonal antibodies to ER protein (oestrophilin), each of which recognises different epitopes, have been employed to localise ER. In studies on frozen, fixed sections of human breast tumours, human and rabbit reproductive tract and fixed MCF - 7 cells, specific staining was obtained exclusively in the nuclei of stained cells. Furthermore, after short-term exposure to physiological levels of E_2, no increase in intensity of nuclear staining in MCF - 7 or rabbit uterine cells has been observed (King & Greene, 1984). The applications of ER immunocytochemistry in breast cancer are further discussed in section 1.551.

It is unlikely that the exclusive nuclear localisation of occupied and unoccupied receptor is due to fixation artefacts. Interference in the interaction between oestrophilin and the monoclonal antibodies (which have antigenic determinants ranging from the DNA binding region to the steroid binding region), by cytoplasmic macromolecules is also unlikely (Press et al, 1984).

These results however, do not discount the possibility that some unoccupied receptor may have a diffuse cytoplasmic localisation which is beyond the limit of detection for immunohistochemical techniques. Nevertheless, they provide very strong evidence that the majority of ER is associated with the nucleus. It remains to be seen whether there is a role for extranuclear receptors in oestrogen action within target cells.

1.25 Parallelism between steroid and peptide hormone action

It has been proposed that steroid hormones, like the peptide hormones, have primary recognition sites on the outer surface of the plasma membrane (Pietras & Szego, 1984; Szego, 1984). After treatment with oestradiol, recognition of oestradiol has been reported to occur at the plasmalemma within 10-40 seconds. This is subsequently followed by internalisation of the hormone into the 50-200nm organelle fraction (Pietras & Szego, 1984) and transfer into the Golgi and lysosome fractions. The interaction at the cell surface elicits alterations in Na^+, K^+-ATPase activity, availability of cAMP, calcium fluxes (Szego & Pietras, 1981) and are exemplified by the morphological changes observed in endometrial cells such as increases in the number and length of microvilli (Rambo & Szego, 1983).

The accumulation of lysosomes after hormone treatment in the peri-and supra-nuclear areas has been demonstrated (Szego & Seeler, 1973). This has led to the proposal that a lysosomal link exists in the information transfer system from the cell surface to the nucleus, since immunochemical studies indicated that lysosomal proteins could enter the nuclear compartment (Nazareno *et al*, 1981). Indeed, Pietras & Szego (1979,1980, 1984) have demonstrated that receptors are mobilised from the plasma membrane to the nuclear compartment, in particular the nuclear matrix.

1.26 Nuclear Binding of Activated Hormone-Receptor Complex

1.261 Acceptor sites

The binding of the activated hormone-receptor complex to specific high affinity sites on the genome, referred to as "acceptor sites" (Spelsberg *et al*, 1971a), results in changes in gene expression associated with the physiological responses of oestrogens. These intranuclear binding sites on the chromatin have been characterised as various components:

i) solely DNA sequences (Higgins et al, 1973; Yamamoto & Alberts, 1974; Compton et al, 1982), ii) ribonucleoproteins (Liao et al, 1973), iii) non-histone proteins (Puca et al, 1974, 1975; Mainwaring, 1976), iv) nuclear matrix (Barrack et al, 1977; Barrack & Coffey, 1982), v) nuclear membranes (Jackson & Chalkley, 1974) and vi) protein - DNA complexes (Alberga et al, 1971; Spelsberg et al, 1971 ab; Ruh & Spelsberg, 1983)

Although the nature of the acceptor sites has yet to be unequivocally characterised, it is currently regarded that acceptor sites for steroid hormones (Klyzsejko-Stefanowicz et al, 1976; Hamana & Iwai, 1978; Ruh et al, 1981; Ruh & Spelsberg, 1983) comprise tightly bound protein-DNA complexes, termed nucleoacidic proteins (NAP) (Webster et al, 1976; Thrall et al, 1978). The chromosomal non-histone protein fraction CP-3, extracted with 5-7M guanidine hydrochloride, which binds activated ER and PR has been extensively studied (Spelsberg et al,1971 ab, 1972, 1979). Other chromatin protein fractions, however, do not contain acceptor activity. Although denaturing conditions have been used to extract chromatin fractions, the non-histone protein fractions have been reannealed to pure hen DNA yielding reconstituted NAP with native-like acceptor sites for ER and PR (Ruh & Spelsberg, 1983; Spelsberg et al, 1984). The highest acceptor activity for ER was present in reconstitutions using 5 and 7M guanidine hydrochloride extracts (Ruh & Spelsberg, 1983) while the PR acceptor activity was associated with the 7M extract. Furthermore, the existence of distinct nuclear acceptor sites for ER and PR in avian oviduct has been reported (Kon & Spelsberg, 1982).

The problems and potential artefacts encountered in the identification of acceptor sites for PR have recently been reviewed (Littlefield & Spelsberg, 1985). The major criticism of these reconstitution experiments involves degradation of DNA or protein, or even both, but the dissociation constants determined in cell-free binding assays are of the correct order of magnitude, indicating that the results are biologically relevant.

1.262 Role for DNA in acceptor sites

There has been increasing evidence that DNA has an important role in the acceptor sites, with steroid hormone receptors themselves possessing distinct polynucleotide and steroid binding domains (Andre & Rochefort, 1973; Wrange & Gustafsson, 1978). Studies employing recombinant DNA techniques have shown that specific DNA sequences, flanking and within the mammary tumour virus (MTV) (Payvar et al, 1983), egg white protein (Mulvihill et al, 1982; Compton et al, 1982) and rabbit uteroglobin DNA (Bailly et al, 1983) preferentially bind activated PR or glucocorticoid receptor (GR) in the case of MTV. Deletion studies indicate that regulatory sequences are controlled by steroid hormones (Ringold, 1983; Gustafsson et al, 1984; Renkawitz et al, 1984; Rousseau, 1984).

In the avian lysozyme gene, two distinct GR binding sites have been identified, with the upper site required for regulated expression of the promoter (Chandler et al, 1983; Renkawitz et al, 1984). Indeed, in all GR binding sites studied, a hexanucleotide sequence has been identified which may play an important role in the interaction of receptor with the regulatory element. Deletion studies, which have shown that sequences for progesterone and dexamethasone induction map onto the same region of the lysozyme gene, have led to the hypothesis that hormone regulated induction is controlled by the interaction with the same binding site (Renkawitz et al, 1984). The mechanisms by which regulatory regions affect transcription have yet to be resolved.

Multiple binding domains have been identified for activated GR in MTV DNA, although not all these domains are essential for hormonal responsiveness of MTV or heterologous promoters fused to the binding regions. Each binding domain contains at least two binding sites at which a tetrameric receptor complex appears to interact (Payvar et al, 1983; Gustafsson et al,1984).

The preferential binding of steroid receptors to the 5' consensus sequence of various regulated genes *in vitro* represents only a 40-fold greater affinity than non-specific binding to DNA (Bailly *et al*, 1983). The affinity determined for *in vivo* binding of receptor to chromatin is, however, many orders of magnitude greater (Kd $\sim 10^{-9}$M). This suggests that the full three-dimensional conformation of DNA alone cannot account for the biological affinity and it seems that non-histone proteins are involved, perhaps by delineating the consensus sequence. The exact nature of the steroid receptor-chromatin interaction, however, remains to be elucidated.

1.27 Modulation of Cellular Oestrogen Receptor Levels

The regulation of cellular ER levels by receptor replenishment and processing has been investigated using the rat uterus and MCF-7 cells as model systems. Studies in the rat uterus have shown that after an injection of oestradiol, there is both a dose-dependent loss of soluble receptor and a simultaneous rise in the level of nuclear receptor. Subsequently an increase in the level of unoccupied receptor occurs, with control values attained by 11-16 hours and exceeded by about 50% at 32 hours (Sarff & Gorski, 1971). Receptor replenishment, i.e. the restoration of 'soluble' receptor after nuclear translocation of hormone-receptor complexes, appears to be essential in maintaining the responsiveness of target tissues to further hormone treatment (Anderson *et al*, 1974). In contrast to glucocorticoid receptor replenishment which occurs through recycling alone (Rousseau *et al*, 1973), ER replenishment is thought to involve both resynthesis and recycling (Kassis and Gorski, 1983). In MCF-7 cells and in the rat uterus after oestrogen treatment, nuclear translocation is followed by an irreversible time-dependent decrease in the level of nuclear receptor termed processing (Horwitz & McGuire, 1978c,1980; Pavlik *et al*, 1979). A simultaneous restoration of soluble receptor levels, however, does not accompany processing. The mechanism of processing has been investigated by measuring the loss of specific oestrogen binding in 0.6M KCl extracts. Such studies have shown that

within five hours post-oestrogen exposure, only 30% of cellular receptors are detectable in nuclear extracts (Horwitz & McGuire, 1978a).

Inhibitor studies have demonstrated that processing is inhibited by exposure of the cells to actinomycin D and chromomycin A_3 (specific G-C base pair intercalators) but not by other inhibitors of DNA, RNA or protein synthesis (Horwitz & McGuire, 1978b, 1980). Partial or total inhibition of processing has been demonstrated using antioestrogens (Horwitz & McGuire, 1978c) giving support to the concept that processing is necessary for oestrogenic stimulation (Horwitz & McGuire, 1978a). In the R_3 cell line (a subline of MCF -7) which is oestrogen-independent, processing does not occur and induction of PR by oestrogen is undetectable (Horwitz & McGuire, 1978c, 1980; Nawata et al, 1981).

Recent studies suggest that processing is a multistep process involving kinetic changes in the properties of the receptor, in addition to a decrease in hormone binding (Strobl et al, 1984). Two different populations of rapidly and slowly dissociating ER's have been detected in MCF-7 cells (Strobl et al, 1984). The rapidly dissociating population of high affinity ER's is detectable within one hour of oestrogen treatment. It is only this class of receptor which is lost during processing (Strobl et al, 1984). The other slowly dissociating class of receptors is detected if the cells are treated with oestradiol and actinomycin D and fails to bind hormone again. After continued hormonal exposure, molecular alterations occur to the receptor rendering nuclear ER into a more tightly bound, less exchangeable form (Jakesz et al, 1983 a b; Kasid et al, 1984). Immunological studies indicate that there is a distinct modification to the receptor (Jakesz et al, 1983b).

In contrast to Horwitz & McGuire (1978a), it has been suggested that the modifications to the receptor and oestrogenic responses (as assessed by PR induction) are independent of processing which may occur simultaneously (Eckert & Katzenellenbogen,1982; Kasid et al, 1984). It is moreover surprising that processing is important in oestrogen action in MCF-7 cells but not in the rat uterus.

An equilibrium model for ER replenishment has been proposed by Kassis & Gorski (1983), similar to that for glucocorticoid receptors (Munck & Foley, 1976) with ER existing in three different states. The three different forms of receptor include active and inactive forms of receptor with high and low affinity for ligand respectively as well as a transformed receptor with high affinity for nuclear components.

1.28 Binding characteristics of the oestrogen receptor

Experimentally, the ER has been found to occur as both a soluble form that predominates in the absence of steroid (Jensen *et al*, 1968; Shyamala & Gorski, 1969) and a nuclear form after oestrogen treatment of target tissues. Increased ionic strength or oestrogen treatment result in transformation of the soluble form to the nuclear form. At high salt concentrations, un-transformed receptor sediments as a 4S monomer while as an 8S aggregate at low salt concentrations.

Positive cooperativity has been observed for oestrogen binding at sub-physiological levels of soluble receptor (Notides *et al*, 1981). However, other studies have shown non-cooperative steroid binding characteristics for intact cells, tissues and 4S receptor immobilised onto hydroxy-apatite (Sakai & Gorski, 1984; Muller *et al*, 1985). Monomeric 4S oestradiol-receptor complexes were found to coelute with transformed receptor during hydroxy-apatite chromatography only after heating. This led Sakai & Gorski, (1984), to conclude that the 4S↔8S change does not occur *in vivo* and that transformation, a membrane associated event, does not require the aggregation of ER binding units. They have suggested that receptor monomers may exist in equilibrium between high and low affinity conformations and that dimerisation of the high affinity receptor may force the equilibrium towards the transformed (high affinity) state. Furthermore, in conjunction with receptor localisation studies, it has been postulated that unoccupied and occupied receptor are immobilised by binding to an undefined nuclear component (Gorski *et al*, 1984).

1.29 Oestrogen induced responses

The responses to oestrogen in target tissues are discussed with regard to the rat uterus (Section 1.291) and breast cancer (Section 1.551).

1.291 Uterotropic responses

After an injection of oestradiol to an immature rat, the responses which occur have been classified into 'early' and 'late' uterotropic responses. In the uterus, oestradiol induces generalised growth with hypertrophy followed by hyperplasia. The biosynthetic and metabolic processes involved in the uterotropic responses have been comprehensively reviewed (Katzenellenbogen & Gorski, 1975; Segal et al, 1977; Clark & Peck, 1979).

1.2911 Early responses

Uterine hypertrophy begins with increased RNA synthesis which is maximal after 2-4 hours (Knowler & Smellie, 1973) and is followed by a generalised increase in protein synthesis. In the immature rat injected with oestradiol, the earliest response after 15 minutes is a rise in RNA Polymerase II activity which peaks at 30 minutes with control values attained by two hours (Glasser et al, 1972; Borthwick & Smellie, 1975). The hnRNA synthesised matures into mRNA and after one hour results in the aggregation of ribosomes into polysomes.

Early responses relate to the ability of oestrogen to optimise biosynthetic activity through the mobilisation of various physiological functions. These include histamine release, eosinophil migration, increased nucleotide uptake, amino acid uptake, glucose metabolism, vascular permeability, etc. Some responses, such as increased vascular permeability and glycogen levels are not regarded as receptor-mediated events. Receptor-mediated responses such as oestrogen-induced protein synthesis, include the induction of brain type creatine kinase, CK-BB,(Reiss & Kaye, 1981) and glucose-6-phosphate dehydrogenase. In the case of CK-BB, mRNA's have been detected within 15 minutes of oestradiol injection (De Angelo & Gorski, 1970).

1.2912 Late responses

The metabolic and biosynthetic events are followed by the 'late' uterotropic responses characteristic of cellular hypertrophy and hyperplasia. These include the sustained high polymerase I activity (Hardin et al, 1976), increased protein synthesis (Mueller et al, 1958), DNA synthesis, cell division (Kaye et al, 1972; Stormshak et al, 1976) and increased uterine wet weight (Hishaw, 1959).

1.292 Induction of progesterone receptor

PR synthesis has also been shown to be regulated by oestrogen. Treatment of uterine tissue and MCF-7 cells with oestradiol results in a rise in the level of PR (Corvol et al, 1972; Hsueh et al, 1975; Horwitz & McGuire, 1978a). In castrated animals, PR is present primarily in the 4S form but after oestrogen treatment, the 7-8S form predominates (Toft & O'Malley, 1972). In vitro studies on strips of hamster uteri treated with oestradiol have shown a significant rise in the level of soluble PR over 24 hours. The induction of PR has been blocked by cycloheximide and actinomycin D, suggesting that it is dependent on RNA and protein synthesis (Leavitt et al, 1978). These qualitative and quantitative changes in soluble PR have been attributed to de novo synthesis (Clark & Peck, 1979).

Conversely, progesterone has been shown to decrease the level of ER's in immature rat uterus by interfering with ER_c replenishment (Hsueh et al, 1976). The subsequently reduced sensitivity of the uterus to oestrogen (Hsueh et al, 1975) is therefore due to the progesterone induced reduction in ER_c levels.

1.3 Regulation of Mammary Epithelial Cell Growth

1.31 Mechanism of oestrogen action on cellular proliferation

The role of oestrogens on specific protein synthesis and cellular proliferation in target tissues is well established (O'Malley & Means, 1974; Gorski & Gannon, 1976). Although these two

effects may overlap, they need not be mutually dependent events. The exact mechanism of oestrogen action on cellular proliferation is, however, unclear (Sonnenschein & Soto, 1980) as the effects of oestrogens, on established cell lines from oestrogen-responsive tumours, have varied significantly. The MCF-7 cell line was initially reported to respond to physiological concentrations of oestradiol by increases in cell number and macromolecular synthesis (Lippman & Bolan, 1975; Lippman *et al*, 1976). Later reports, however, have indicated that these cells are not growth responsive to oestradiol (Zava *et al*, 1977).

The major paradox regarding the mechanism of oestrogen action concerns the oestrogen requirement of various cell lines to grow as tumours *in vivo*, while *in vitro* oestrogens have no apparent effect on the growth of these cells (Shafie, 1980; Sonnenschein & Soto, 1980). The various hypotheses for oestrogen action are:-

i) Direct positive hypothesis in which oestrogens directly induce the proliferation of target cells.

ii) Indirect positive hypothesis according to which oestrogens trigger the synthesis and/or release of growth factor(s) mitogenic for oestrogen-responsive cells.

iii) Indirect negative hypothesis which is based on oestrogen inhibition of the action of a chalone for oestrogen-responsive cells.

iv) A combined direct/indirect hypothesis based on the inter-relationships between prolactin, oestradiol, progesterone and adrenal corticoids, both *in vivo* and *in vitro*.

1.311 The Direct Positive Hypothesis

Proliferative changes have been observed in the uterus *in vivo* and are associated with the *in vivo* variations in serum oestrogen levels (Epifanova, 1958; Kimura *et al*, 1978). Administration of oestrogen has also been shown to increase DNA synthesis and cell proliferation in the rat uterus (Kaye *et al*, 1972; Stormshak *et al*, 1976). These *in vivo* observations, however, have not shown that oestrogens play a direct role in cell proliferation.

In vitro studies have shown that the responses to physiological levels of oestrogen, such as DNA synthesis and increased cell number are of a lesser magnitude than the *in vivo* situation (Chen *et al*, 1973; Pavlik & Katzenellenbogen, 1978). Recent studies on 21-23 day old rats have shown that injection of oestradiol into one uterine horn directly stimulated DNA synthesis two-fold in the isolated nuclei of that horn rather than the 15-fold stimulation found *in vivo*. No effect on DNA synthesis was observed in the other uterine horn injected with control vehicle (Stack & Gorski, 1984). Although indirect oestrogen action through an intermediary organ has been eliminated, the possibility still exists that an autocrine or paracrine oestromedin may be involved (see section 1.312).

Observations on clonal cell lines derived from oestrogen-dependent tumours have shown that there is no growth response to oestradiol in culture (Sorrentino *et al*, 1976). A direct - positive mechanism for oestrogen action on target cells is therefore difficult to reconcile with the results from cell culture studies and has led to the proposal of different types of indirect mechanisms.

1.312 The Indirect Positive Hypothesis

A model for the indirect positive mechanism of oestrogen-responsive growth *in vivo* was proposed by Sirbasku (1978). It centred on the concept of oestrogen-induction of growth factors (oestromedins) which, at least in part, act as primary mitogens for oestrogen-responsive uterine, kidney and mammary tumour growth. The initial proposal has undergone modification to cover three different types of oestromedin regulation (Sirbasku 1981; Ikeda *et al*, 1982) as shown in Fig. 1.1.

An endocrine mechanism may operate for oestromedins, with biosynthesis and/or release from oestrogen target tissues, followed by the concentration of oestromedin within the tumours. For example, mammary oestromedins may be synthesised and released by the uterus, kidney and pituitary. Non-endocrine mechanisms may also operate, in which oestrogen action results in the release of

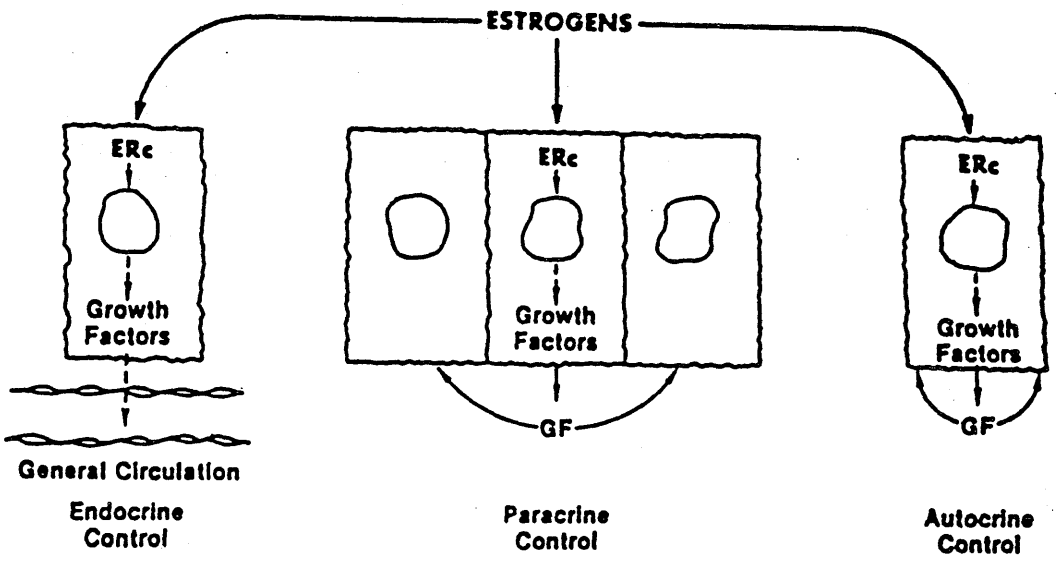


Fig. 1.1 Proposed mechanisms for indirect models of oestrogen-promoted growth.

(Reproduced from Ikeda et al, 1982)

locally acting autostimulatory oestromedins. Tumour epithelial cells may produce their own growth factor which acts on the same epithelial cell population by an autocrine mechanism. Alternatively, adjacent cell types (stromal or myoepithelial) may produce growth factor for the epithelial cells by a paracrine mechanism.

The properties of oestromedins so far discovered, are discussed below. These growth factors are acid-extractable, heat-stable polypeptides which have been purified from various target organs (e.g. sheep uterus, kidney and pituitary), tumours (GH3/C14, MTW9/PL) and cell lines derived from these tumours as well as the MCF-7 and T47-D lines.

1.3121 Uterine derived growth factor

The presence of an oestrogen inducible growth factor activity in rat uterine extracts was first reported by Sirbasku (1978). This activity, obtained at neutral pH, was found to be a specific mitogen for rodent cell lines derived from oestrogen-responsive or dependent pituitary, kidney and mammary tumours. Extraction of this activity at neutral pH revealed that it was not related to steroid hormones or lipid components, but was heat and trypsin labile with an apparent molecular weight of 70000 (Sirbasku et al, 1981). Other studies on growth factors such as epidermal growth factor (EGF), have identified them as proteins with molecular weights greater than 70000 in neutral extracts, but as low molecular weight species (4-6000) when purified under acidic or dissociating conditions. Using a similar approach, uterine derived growth factor (UDGF) has subsequently been purified and found to be a heat-stable protein of molecular weight 2-6000 (Ikeda & Sirbasku, 1984).

UDGF has also been purified from pregnant sheep uteri using acetic acid extraction followed by heat treatment and subsequent chromatography. The apparent molecular weights determined by SDS-PAGE and HPLC for UDGF are 4200 and 6200 respectively,(Ikeda & Sirbasku, 1984). UDGF has been shown to be mitogenic for mammary, uterine and pituitary tumour cells derived from

oestrogen responsive tumours but not normal diploid rat fibroblasts. Other growth factors such as EGF, melanocyte stimulating activity (MSA) and somatomedin C (insulin-like growth factor I) have been unable to substitute for this activity (Ikeda & Sirbasku, 1984). UDGF, under serum-free conditions, has been shown to promote half of the 10% serum stimulated growth (G_{50}) for MTW9/PL cells at 8ng/ml without supplementation by other hormones, attachment and growth factors. In these growth assays, UDGF has stimulated the incorporation of labelled precursor into DNA in addition to a logarithmic increase in cell number (Ikeda & Sirbasku, 1984). After a single addition of UDGF to MTW9/PL cells, growth of the cells in culture was found to cease after 48 hours unless more factor was added. These results suggest that UDGF both promotes and sustains the growth of MTW9/PL cells in culture. A basal level of uterine extract is a prerequisite for a growth response, implying that undefined factor or essential nutrients are present in the extract.

Differential mitogenic responses to UDGF have been observed in endometrial tumour cells (G_{50} = 36ng/ml) and uterine smooth muscle cells (G_{50} = 176ng/ml) (Ikeda & Sirbasku, 1984). These observations are of particular interest since they suggest that target cells are more sensitive to the actions of these growth factors. UDGF has also been identified in the oestrogen-induced accumulation of rat uterine luminal fluid (Sirbasku & Leland,1982; Leland et al, 1983), indicating that it may be an endogenous autocrine oestromedin which has a role in the oestrogen-responsive or pregnancy related uterine growth or tumourigenesis (Ikeda & Sirbasku, 1984).

1.3122 Mammary tumour growth factor

Ikeda et al (1984a), have purified a 3900 dalton polypeptide growth factor from sheep pituitaries (MTGF-Pit) which is a distinct species from UDGF previously identified in uterine extracts purified under similar denaturing conditions at acid pH (Ikeda & Sirbasku, 1984). MTGF-Pit is also a different species from the 22000 dalton form of growth hormone, native prolactin (24000 daltons) and cleaved prolactin (16000 daltons).

However, until MTGF-Pit is characterised, it is not possible to discount the possibility that it is a degradation product of cleaved prolactin, although this seems unlikely since the latter has not been identified in any of the experiments.

In assays under serum-free conditions, 20 - 75ng/ml MTGF-Pit accounted for half the growth (G_{50}) of T47-D, MTW9/PL and MCF-7 cells in 2 - 10% serum containing medium. The mitogenic response of mammary tumour cells to MTGF-Pit was not replaced by growth hormone, prolactin or other growth factors such as EGF, platelet-derived growth factor or fibroblast growth factor. The effect of MTGF-Pit on normal mammary epithelial cells has yet to be reported, but it was shown to be mitogenic for normal human diploid fibroblasts at levels greater than 1.0µg/ml, while normal rat fibroblasts were unresponsive (Ikeda et al, 1984a).

1.3123 Other oestromedins

Kidney derived growth factor (KDGF), with an apparent molecular weight of 4200 ± 400 as assessed by SDS-PAGE, has been purified using a modified procedure as it is negatively charged at neutral pH (pI ~ 4.2 - 4.7). KDGF is a distinct species from UDGF(pI ~ 7.3) and has been used in growth assays on MTW9/PL cells. Under the standard serum-free conditions, the G_{50} for KDGF was found to be 19ng/ml (Ikeda et al, 1984b). The effect of KDGF on other oestrogen-responsive cell types has yet to be reported.

A low molecular weight (2500-6200) autostimulatory growth factor GH3/C14-AGF has been partially purified from extracts of GH3/C14 rat pituitary tumours, cells in culture and their serum-free conditioned medium (Danielpour et al, 1984). GH3/C14-AGF has been shown to be mitogenic for MTW9/PL and GH3/C14 cells but not for 3T3 mouse embryo or diploid rat fibroblasts. Identification of the autostimulatory growth factor in rapidly growing, relatively autonomous GH3/C14 tumours has led to the proposal that it may have a role in the conversion of oestrogen-dependent to autonomous tumours (Danielpour et al, 1984).

The involvement of endocrine and autocrine growth factors is implicit in the indirect mechanism for oestrogen-responsive growth *in vivo*. Endocrine oestromedins, for example, mammary oestromedins, may be synthesised and/or released by oestrogen target organs such as the uterus, kidney and pituitary. A significant role has been postulated for endocrine oestromedins in the growth regulation of normal or early hormone-dependent tumour tissue (Ikeda *et al*, 1984b). Danielpour & Sirbasku (1984), have elegantly demonstrated the growth of oestrogen-dependent and responsive tumour cells in the absence of physiological levels of oestrogens, providing strong evidence for the concept of autocrine oestromedins.

Autocrine oestromedins, which are produced by autonomous cell types and are under ER control, support the growth of oestrogen-dependent and responsive cells and replace their requirement for oestrogens (Ikeda *et al*, 1984b). Autonomy appears to arise from unregulated production of locally acting oestromedins and not the loss of ER, with the result that autonomous tumours may be either receptor positive or negative. Unregulated autocrine oestromedin production offers the distinct selective advantage to autonomous cell types, allowing the conversion of the hormone-dependent to independent phenotype. This has led to the suggestion that autocrine oestromedins may have an important role in the conversion of hormone-dependent to autonomous growth (Ikeda *et al*, 1984b; Danielpour & Sirbasku, 1984).

1.313 The Indirect Negative Hypothesis

This hypothesis is based on the premise that oestrogens may reduce levels of serum-borne chalones for oestrogen-sensitive cells, oestrocolyones, which are synthesised in intermediary organs such as the liver (Sonnenschein & Soto, 1979, 1980; Soto & Sonnenschein, 1980). Studies in ovariectomised quails have shown that infusion of oestradiol into the portal vein, with its resultant metabolism does not bring about ER translocation, PR synthesis or any increase in protein per cell. However, an increase in the DNA content of the oviduct occurs,

with the magnitude of the response comparable to oestradiol administration via the jugular vein. In quails, the liver has been shown to be involved in mediating short-term responses to oestrogen, while in the rat, an indirect liver-mediated mechanism does not operate (Schatz et al, 1984), suggesting that species differences may exist for oestrogen-regulation of cell proliferation.

Combined 'in animal - in culture' studies suggest that proliferation of rat perinatal and pituitary tumour $C_2$9RAP oestrogen-sensitive cells is inhibited by foetal or newborn rat serum. The inhibitory effect, which is specific for oestrogen-sensitive cells, appears to be mediated by α-foetoprotein (AFP)(Sonnenschein & Soto, 1979, 1980; Soto & Sonnenschein, 1980). AFP has been shown to bind oestrogens with high affinity, Kd $\sim 10^{-9}$M in rats and mice (Nunez et al, 1971). A dose-dependent effect of AFP was observed on ER positive $C_2$9RAP cells which are oestrogen-responsive in vivo but not in vitro (Sonnenschein & Soto, 1979, 1980). These results suggested that AFP inhibited oestrogen-induced cell proliferation. The growth of the F_4C_1, ER positive, oestrogen insensitive rat pituitary tumour cells was however unaffected by similar AFP levels. Oestradiol was unable to antagonise the inhibitory effect on oestrogen-sensitive cells, indicating both that it is not a trophic hormone per se and that ER's are not involved in this inhibitory mechanism (Soto & Sonnenschein, 1980). Other studies in hamsters have shown that sera of newborn hamsters do not bind oestradiol with high affinity although high AFP titres are present (Schatz et al, 1983). The inhibition of oestrogen-sensitive proliferation in newborn hamsters is therefore not due to sequestration of oestrogens (Germain et al, 1978), suggesting the existence of other inhibitory mechanisms which are insensitive to oestrogens.

A dose-dependent inhibitory effect of heat-inactivated, charcoal stripped female human serum (HIDCCFHS) has been observed on cloned MCF-7 (C_7MCF7 - 173) oestrogen sensitive cells, while autonomous ER-negative, KLE human endometrial cells (Richardson et al, 1984) were not inhibited. The inhibition was abolished by addition of naturally occurring or synthetic oestrogens, or

Fig. 1.2 Proposed indirect negative model for oestrogen action on cellular proliferation. (Reproduced from Soto & Sonnenschein, 1985).

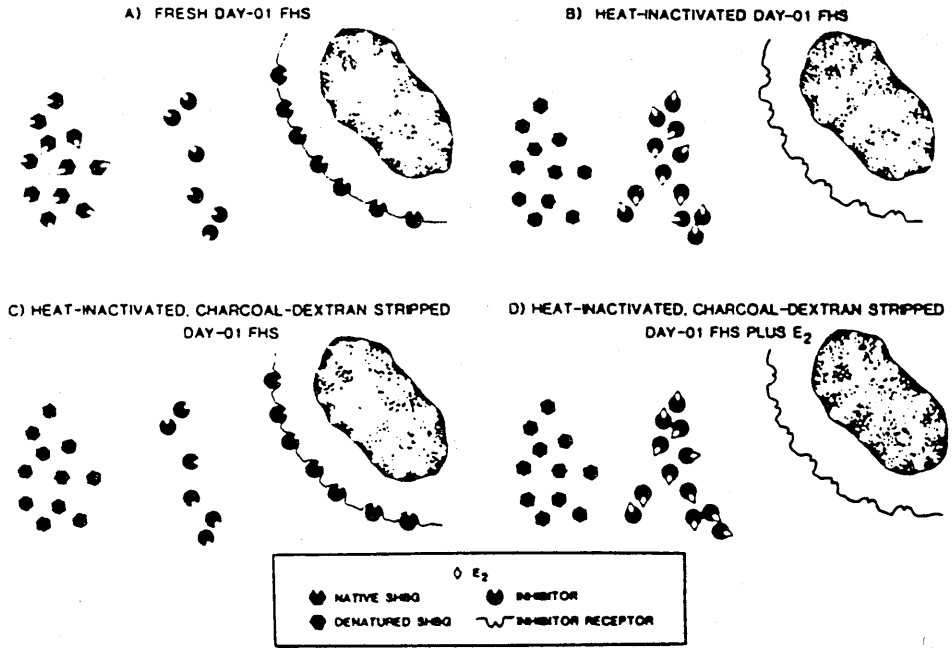
The presence of unbound oestradiol(E_2) regulates the interaction between the serum-borne chalone, oestrocolyone, and the oestrogen-sensitive cells.

a) E_2 is bound to plasma proteins(e.g.SHBG) and oestrocolyones. Unbound oestrocolyones bind to their cell surface receptors and prevent the proliferation of oestrogen-sensitive cells.

b) Heat inactivation denatures the plasma proteins and therefore releases E_2 which becomes bound by free oestrocolyones. Maximal cell proliferation proceeds.

c) Charcoal stripping removes unbound E_2 and the unbound oestrocolyones bind to their receptors thereby preventing proliferation of these cells.

d) When exogenous E_2 is added to heat inactivated, charcoal stripped serum, the oestrocolyones dissociate from their membrane receptors and bind to the free steroid permitting cell proliferation.


A) FRESH DAY-01 FHS
B) HEAT-INACTIVATED DAY-01 FHS
C) HEAT-INACTIVATED, CHARCOAL-DEXTRAN STRIPPED DAY-01 FHS
D) HEAT-INACTIVATED, CHARCOAL-DEXTRAN STRIPPED DAY-01 FHS PLUS E_2
◊ E_2
NATIVE SHBG
INHIBITOR
DENATURED SHBG
INHIBITOR RECEPTOR

by dilution of serum levels to below 5% (Soto & Sonnenschein, 1984 ab, 1985). These studies on C_7MCF - 173 cells have suggested two possible mechanisms for the inhibitory effect of HIDCCFHS (Soto & Sonnenschein, 1985). Firstly, oestradiol may act directly on target cells, conferring sensitivity to oestrocolyones. Alternatively, oestradiol binding to oestrocolyones may negate the inhibitory effects since the resulting conformational change would not allow recognition of the oestrocolyones by membrane receptors. A model based on the latter hypothesis is summarised in Fig. 1.2. The validation of such a model will require the characterisation of the oestrocolyones in addition to their membrane receptors.

Page et al, (1982) have demonstrated that serum contains a low molecular weight (400-1200) growth inhibitory factor that is required for oestrogen-responsiveness. Serum concentrations were found to regulate the oestrogen-responsiveness of MCF-7 cells with no response to oestrogen at low levels (0-1%) of serum. At 15% serum, however, these cells responded to oestradiol in terms of increased growth rate and macromolecular synthesis. Dialysis and charcoal stripping have been found to remove this factor which inhibits growth and hormone responsiveness.

1.314 Direct/Indirect Hypothesis

A new model for growth stimulation incorporating the relationships observed between oestradiol, prolactin, progesterone and cortical steroids both in vivo and in vitro has been proposed (Imagawa et al, 1985). Oestrogens have been suggested to act directly on growth through PR induction and indirectly through the stimulation of prolactin secretion. The indirect action has been suggested to be independent of oestromedin production by either mammary or intermediate tissues. Oestrogen action on the pituitary gland results in increased prolactin secretion which induces both PR and its own receptor (prolactin). Prolactin receptors may also be induced by adrenal corticoids resulting in a stimulation of growth through synergism with prolactin. According

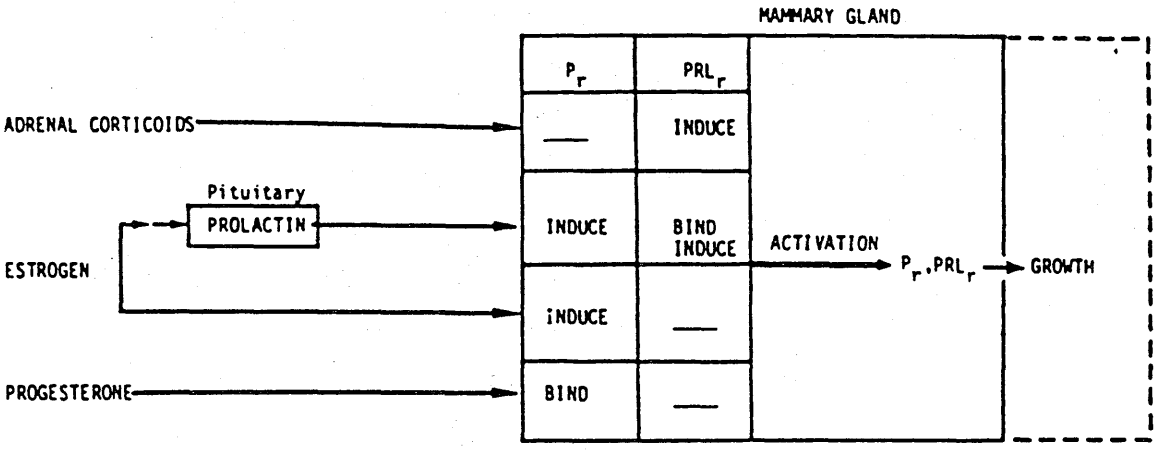


Fig 1.3 Proposed direct/indirect model for hormone action in the mammary gland.

It is proposed that cellular proliferation is dependent on the induction of progesterone receptor (Pr) and prolactin receptors (PRLr). Oestradiol may have both a direct effect on growth, mediated by induction of Pr and an indirect action by the stimulation of prolactin secretion. Prolactin induces both its own receptors in addition to Pr. Adrenal steroids induce prolactin receptor and synergise with prolactin in stimulating growth.

(Reproduced from Imagawa et al, 1985)

to this model which is summarised in Fig. 1.3 oestrogens need not be directly mitogenic for mammary epithelial cells.

1.315 Conclusions

Evidence for the different models of oestrogen action on epithelial cellular proliferation has been presented in this section. It appears that a combination of direct and indirect mechanisms may operate, with a direct effect on specific protein synthesis and an indirect effect, via non-epithelial cells, resulting in cellular proliferation. The sequence analysis of the oestromedins has yet to be performed and may perhaps show the existence of a family of growth factors for oestrogen-sensitive cells. The relationship (if any) between the oestromedins and the factors involved in mesenchymal-epithelial interactions (see Sect.1.32) may also prove to be very interesting.

1.32 Regulatory Mesenchymal-Epithelial Interactions

1.321 Role in developing target tissue

Autoradiographic localisation of(^{3}H) diethylstilboestrol (DES) in developing urogenital sinus and Mullerian ducts of foetal mice has shown that binding is limited to mesenchymal derived cells (Stumpf et al, 1980). In neonatal mice, nuclear ER are limited to vaginal, cervical and uterine mesenchymal cells (Cunha et al, 1982). Although ER activity does not normally begin until 10-20 days postpartum, DES treatment during days 1-4 postpartum induced dramatic epithelial growth and cornification of vaginal epithelium by day 5 despite the absence of epithelial ER's (Cunha et al, 1982). An indirect mechanism for hormone action has been proposed (see Fig. 1.4) in which ER containing mesenchymal cells mediate the hormonal effects on epithelial cells by production of growth factors.

Early studies on androgen-dependent morphogenesis demonstrated that the effects of androgens on epithelial development are elicited by the mesenchyme (Cunha 1970, 1972). Tissue recombinants of urogenital epithelium (from urogenital sinus or foetal seminal vesicle) were grown in conjunction with mesenchyme of

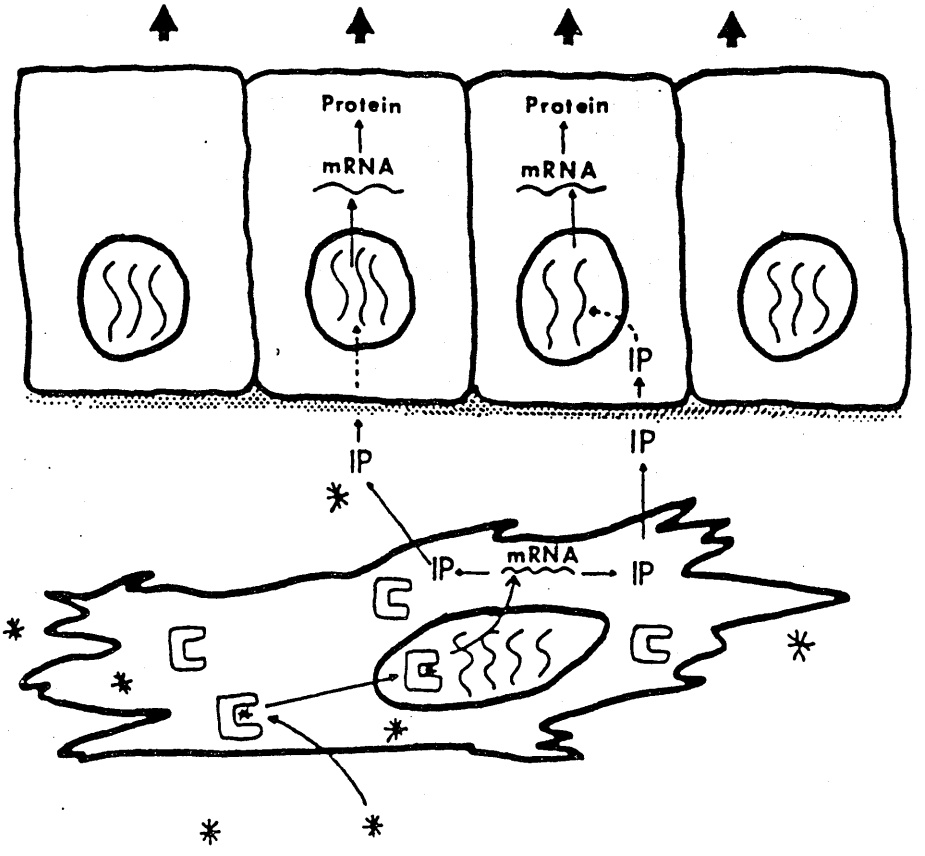


Fig. 1.4 Proposed model for a mesenchyme mediated mechanism of hormone action. A receptor-mediated mechanism is regarded as inducing the synthesis of protein inductor molecules which mediate the hormonal effects on the epithelial cells. The hormone is represented by asterisks and the receptor by an open C shape.

(Reproduced from Cunha et al, 1983)

either urogenital origin or non-target integument. Recombinants with urogenital mesenchyme were found to undergo androgen-dependent prostatic or seminal vesicle morphogenesis in male mice. The integumental mesenchyme recombinants, however, did not undergo further morphogenesis.

Further evidence for the putative role of the mesenchyme has come from recombinants of Tfm (testicular feminisation) and wild type tissues. A Tfm mutation renders the target organs insensitive to androgens either through defective or reduced androgen receptors (Ohno, 1977). Tfm mice are completely feminised with the urogenital sinus giving rise to a vagina instead of a prostate. The lack of androgen production by the foetal testes also results in mammary gland development (Ohno, 1977). Recombinants of wild type urogenital sinus mesenchyme and either Tfm or wild type epithelium resulted in testosterone-induced epithelial regression in male hosts. The use of Tfm mesenchyme with epithelium from either source, however, did not induce regression (Kratochwil & Schwartz, 1976; Drews & Drews, 1977). Similarly, androgen dependent prostatic induction has been observed in wild type recombinants, although it does not occur with Tfm mesenchyme (Cunha & Lung, 1978; Lasnitzki & Mizuno, 1980). Moreover, studies with wild type urogenital sinus mesenchyme and Tfm bladder epithelium have shown that morphogenesis and differentiation occur in androgen receptor deficient Tfm epithelium. Autoradiographic analysis of (^{3}H)-dihydrotestosterone binding in recombinants of wild type urogenital mesenchyme and adult bladder epithelium has shown specific binding in stromal cell nuclei (Shannon & Cunha, 1984).

In rodent prostate recombinants with varying proportions of epithelium and mesenchyme from 16 day old foetal urogenital sinus, a correlation has been found between the level of mesenchyme and the mass of prostatic acinar tissue (Chung & Cunha, 1983). Similarly, in the mammary gland regulation of epithelial growth and morphogenesis has also been reported to be under the regulation of mammary stroma (Daniel & De Ome, 1965). These studies highlight the regulatory role of mesenchymal or stromal cells in complex hormonal effects on epithelium during morphogenesis and cytodifferentiation.

1.322 Regulation of epithelial morphogenesis, differentiation and function

Further evidence for a mesenchymal role in epithelial differentiation comes from recombinants of uterine epithelium and vaginal or cervical mesenchyme which have been reported to differentiate into stratified squamous vaginal or cervical epithelium (Cunha, 1976). These mesenchyme-induced morphological changes are coupled to altered biochemical activity. For example, recombinants expressing uterine differentiated properties synthesise proteins characteristic of the uterus (Cunha et al, 1983). The normal expression of seminal vesicle and prostate development by recombinants of mesodermal seminal vesicle epithelium/urogenital mesenchyme and urogenital epithelium/seminal vesicle mesenchyme respectively has been observed (Cunha, 1972). Normal development in these heterotypic recombinants occurs as both mesenchymes contain androgen receptor and have the ability to act as gland inductors.

Recombinant studies on endoderm-derived bladder epithelium and urogenital or seminal vesicle mesenchyme, which have resulted in prostatic development, suggest that endodermal bladder epithelium does not have the capacity to express mesodermal specific differentiation (Cunha & Lung, 1978). These studies support the concept that the developmental response in recombinants is dependent on both mesenchymal induction and the capacity of the epithelium to respond.

1.323 Role in adult development

The dramatic morphological and functional changes occurring in the mammary gland and reproductive tissues in both sexes after puberty involve similar developmental processes to those occurring in primary development. For example, the characteristic ductal growth and differentiation of mammary epithelium associated with foetal mammary development, is induced by the mesenchyme (Kratochwil, 1969). Indeed both foetal and adult mammary epithelium and stroma are capable of promoting mammary morphogenesis (Daniel & De Ome, 1965; Sakakura et al, 1979).

1.324 Mesenchymal-epithelial interactions in carcinogenesis

The progression of atypical hyperplasias into endometrial and cervical carcinomas has been reported (Langley, 1976; Sherman & Brown, 1979). A role for mesenchymal-epithelial interactions (Cunha et al, 1977) has been shown in ovarian-independent vaginal hyperplasia and cornification (Takasugi, 1976). Mice with this condition retain the atypical vaginal epithelium through adulthood if they have been treated with oestrogen during perinatal life. A specific requirement exists for mesenchymal-epithelial interactions during oestrogen treatment for the induction of this condition and its subsequent maintenance in adult life by the interaction between abnormal epithelium and stroma (Cunha et al, 1977). Even ablative treatments such as adrenalectomy, ovariectomy and hypophysectomy have been unable to eliminate this condition.

A role for mesenchymal-epithelial interactions in carcinogenesis has been proposed, with neoplasia representing an abnormal state of differentiation (Pierce et al, 1978; Mintz, 1978). Embryonal carcinoma cells, for example, may be regulated when inserted into the blastocyst (Pierce et al, 1978; Mintz, 1978). Other studies on bladder and mammary tumours have supported the concept of a regulatory role for mesenchymal-epithelial interactions in carcinogenesis (Sakakura et al, 1981; Fujii et al, 1982). Recombinant studies in male hosts have shown that non-glandular epithelial transitional cell carcinomas are induced by urogenital mesenchyme to express a glandular, adenocarcinomatous phenotype (Fujii et al, 1982).

1.325 Conclusions

The regulatory nature of the mesenchymal-epithelial interactions in epithelial morphogenesis has been illustrated in this section. It is particularly interesting that the mesenchyme, which contains nuclear steroid receptors, mediates some hormonal responses of the epithelium. The regulatory role of the mesenchymal-epithelial interactions persists during foetal development, maturity and carcinogenesis.

1.33 Substrate interactions in vitro

1.331 Growth on plastic or glass substrates

The majority of studies have been performed by culturing cells on solid non-physiological surfaces such as plastic or glass which impose a two-dimensional morphology (Vic et al, 1982). In serum supplemented media, cells have been found to attach to serum proteins, such as fibronectin, which are adsorbed onto the substrate (Hynes, 1981). Proliferation of non-transformed mammalian cells is known to be regulated by cell shape (Folkman & Moscano, 1978) although the exact mechanism is unclear. Rounded cells, for example, are associated with reduced adhesion points and a disappearance in microfilament bundles (Bragina et al, 1976; Bissell et al, 1977). Transformed cells, however, are anchorage independent in terms of growth and are not subject to the same regulatory effects on growth control in vitro (Shin et al, 1975).

Studies on mammary differentiation on standard substrates, in response to lactogenic hormones have met with little success (Lasfargues, 1957; Feldman, 1971; Ceriani, 1976). This has led to the use of alternative substrates such as collagen gels, extracellular matrices and feeder layers.

1.332 Growth on and within collagen gel matrices

Preferential attachment and subsequent proliferation of rat mammary epithelial cells has been observed on basement membrane type IV collagen in comparison with stromal types I and III collagen (Wicha et al, 1979). The type IV matrix is required for normal growth both in vivo and in vitro (Wicha et al, 1979, 1980). There is a reduced requirement for cortisol and EGF (Salomon et al, 1981) although it is clear that the basement membrane collagen is partly responsible for normal mammary function.

Morphological and biochemical differentiation of mammary epithelial cells has been observed on floating collagen gels but not when the cells are grown on plastic or attached gels (Emerman & Pitelka, 1977; Yang et al, 1979). Limited differentiation occurred since lactose was not produced (Burwen & Pitelka,

1980). Functional differentiation on floating gels has collectively been attributed to i) the interaction with stromal collagen fibrils; ii) increased oxygen supply; iii) improved access of hormones and nutrients to basolateral cell surfaces and iv) a change in cell shape from squamous to cuboidal or columnar cells. Floating of the gels allows the cells to adopt a preferential shape for secretory activity (Emerman et al, 1979; Haeuptle et al, 1983) and secretion of basal lamina (Liotta et al, 1979). Different glycosaminoglycan (GAG) profiles have been observed for cells cultured on floating gels than on attached collagen or plastic substrates (Parry et al, 1985). Additionally, a difference in GAG distribution was found in cells grown on or within collagen gels than on plastic.

1.333 Growth on extracellular matrix

It is evident that extracellular matrix (ECM) constituents have a very important role in cellular growth, morphology and differentiation (Hay, 1981; Gospodarowicz et al, 1980). ECM components of epithelial tissues are arranged into a basal lamina comprising several components including collagen type IV, proteoglycans, GAG's, laminin and fibronectin (Hay, 1981). This basal lamina separates the epithelial cells from the mesenchyme and stromal elements. ECM's for in vitro studies are generally prepared by EDTA or Triton X-100 treatment of cell monolayers and have supported growth of normal and transformed cells (Gospodarowicz et al, 1981, 1982; Biran et al, 1983).

Long-term growth and differentiation of normal mammary epithelial cells has been observed on a complex biomatrix derived from rat mammary glands (Wicha et al, 1982). This biomatrix contains basal lamina components such as type IV collagen, laminin and fibronectin. It provides conditions more representative of the in vivo situation as shown by α - lactalbumin levels which are 5-fold those on floating collagen gels and 50-fold those on plastic (Wicha et al, 1982). In contrast, rabbit cells grown on a homologous rabbit biomatrix produced significantly less casein than on floating gels (Wilde et al, 1984). Proliferation and secretory activity were found to be mutually exclusive events on

floating gels while with the homologous rabbit biomatrix system, both DNA and casein synthesis were increased with insulin, cortisol and prolactin although oestradiol and progesterone had an inhibitory effect. Rates of lactose synthesis by rabbit cells on floating gels and homologous biomatrix dropped rapidly (Wilde et al, 1984) while rat cells on a rat biomatrix were able to secrete lactose for over 30 days in culture (Wicha et al, 1982). These contrasting results with the homologous biomatrices may reflect differences in culture techniques or matrix preparation. The use of organoids (Wicha et al, 1982) rather than single cells (Wilde et al, 1984), may improve cellular integrity and the inherent interdependent stabilising actions of membrane components with ECM constituents.

1.334 Growth on feeder layers

Mammary epithelial growth has been stimulated by the presence of feeder cells (Taylor-Papadimitriou et al, 1977 ab; Stampfer et al, 1980; Smith et al, 1981; Ehmann et al, 1984). Coculture of normal mouse mammary epithelial and stromal cells has shown that stromal cells have a permissive role in the response of the epithelium to oestradiol (McGrath, 1983). Confrontation of epithelium and stroma resulted in a significant homogeneous inhibition of epithelial growth as measured by labelling indices and addition of oestradiol was found to reinitiate epithelial growth. This response specifically required the presence of stroma since confrontation of mammary epithelium by mammary epithelium did not reinitiate epithelial growth. Although one BALB/C stromal cell line did not have a permissive effect in terms of augmentation of the response to oestrogen, these results suggest that the specificity lies within the stromal cells. Morphological studies indicated that oestradiol may alter the stromal-epithelial interaction by local stromal invasion, perhaps through plasminogen activator (Butler et al, 1979) or collagenase release (Shafie & Liotta, 1980). When the stromal and epithelial cell types were separated by a plastic barrier which permitted mixing of the medium, DNA synthesis was not reinitiated by oestradiol (McGrath, 1983). However, serum-free mouse mammary fibroblast conditioned medium was found to stimulate (^{3}H) thymidine incorporation into epithelial cell DNA in the absence of mammogenic

hormones (Enami et al, 1983). These conflicting results suggest that a species specificity may exist and direct contact of mammary epithelium and stroma may not necessarily be required for growth.

Epithelial growth stimulation by feeder cells appears to involve a combination of conditioning effects on the medium and substrate. Feeder cell conditioned medium has been shown to stimulate mammary epithelial growth (Taylor-Papadimitriou et al, 1977 ab; Armstrong & Rosenau, 1978; Enami et al, 1983; Gray et al, 1983). Furthermore, the cellular nature of the substrate may be a more appropriate surface for attachment and growth of epithelial cells (Stampfer et al, 1980; Gray et al, 1983). Morphological differences have been found in colonies of rat tracheal epithelial cells on 3T3 feeder layers and in the presence of feeder cell conditioned medium (Gray et al, 1983). The colonies on feeder layers were found to be smaller but more homogeneous with a larger cell number than colonies grown in the presence of conditioned medium.

Both fibroblastic (Taylor-Papadimitriou et al, 1977 ab) and epithelial cells (Freshney et al, 1984; Ehmann et al, 1984) have proved successful as feeder layers. Conditioned medium from certain epithelial cell lines has been shown to promote plating efficiency while other epithelial cell lines specifically stimulate mammary epithelial growth (Stampfer et al, 1980; Smith et al, 1981). The factors which determine the efficacy of feeder cells are unclear but there appears to be some specificity in terms of growth stimulation of mammary epithelial cells.

1.335 Does a change in cell shape induce differentiation?

The response of mammary epithelial cultures, on 3T3 adipocytes or fibroblasts, to lactogenic hormones has been shown to be dependent on the substrate interaction (Levine & Stockdale, 1985). Coculture of mammary epithelium with 3T3 - L1 adipocytes or 3T3-C2 fibroblasts had a permissive effect on hormone-dependent differentiation. The 3T3 - L1 system was more successful in supporting differentiation with casein synthesis in the 3T3-C2

system about 60% of that with the 3T3 - L1 system (Levine & Stockdale, 1985). Floating collagen studies have suggested that the change in cell shape and orientation relate to changes in milk protein synthesis (Emerman et al, 1977; Haeuptle et al, 1983). These results have been reinterpreted by Levine & Stockdale, (1985), who suggest that these features are not due to a causal relationship but are characteristics of differentiated mammary epithelium. Casein synthesis was not supported by co-culture with the parental Swiss 3T3 or newborn foreskin fibroblasts, indicating that the substrate does not play a passive or non-specific role. ECM, obtained after EDTA treatment, alone was unable to sustain hormone-dependent differentiation although it promoted mammary epithelial growth (Levine & Stockdale, 1984). This has led to the suggestion that stromal elements represent the common factor between differentiation on ECM, floating collagen gels and certain cell lines (Levine & Stockdale, 1985). It appears that some cellular substrates may enable more rapid basal lamina assembly than other substrates perhaps through production of extracellular matrix constituents and soluble factors which facilitate basal lamina assembly.

1.336 Conclusions

The importance of substrate interactions has been described in this section with particular emphasis on the cellular interaction with basal lamina components, whether they are present in the form of collagen gels, extracellular matrix or feeder layers. At present it remains unclear whether the changes in cell shape observed in primary culture are due to epithelial differentiation in culture or the selection of particular strains of differentiated mammary epithelial cells from the original suspension.

1.4 The Mammary Gland: Physiology and Endocrinology

1.41 Embryological development

The primitive mammary streak, a band of ectodermal cells, can be identified in humans during the fifth week of foetal development. This mammary (ectodermal) ridge widens to form a pit and the ectodermal anlage invades the underlying mesenchyme giving rise to the definitive mammary primordium. By the fifteenth week, secondary epithelial sprouts have developed, dividing the parenchymal tissue into 15-25 epithelial strips.

At six months gestation, the secondary mammary branches have begun to canalise, with formation of glandular lumina complete at eight months. In the last two months of pregnancy, the mass of breast tissue increases four-fold and parenchymal tissue differentiates into lobuloalveolar structures, although the alveoli regress postnatally to ductal structures. Highly differentiated ectodermal cells (ultimately myoepithelial) develop in direct contact with the developing ducts and mesenchyme. These myoepithelial cells are localised both around and along the developing ductal elements. At term, 15-25 mammary ducts and the major lactiferous ducts are formed, but the ductal and glandular elements require hormonal stimulation at puberty.

Early mammary gland development is independent of hormonal influences, while in the last trimester significant foetal breast development is stimulated by the presence of sex steroids in the foetal circulation. The terminal differentiation of ductal cells is also induced in the last trimester by high foetal prolactin levels.

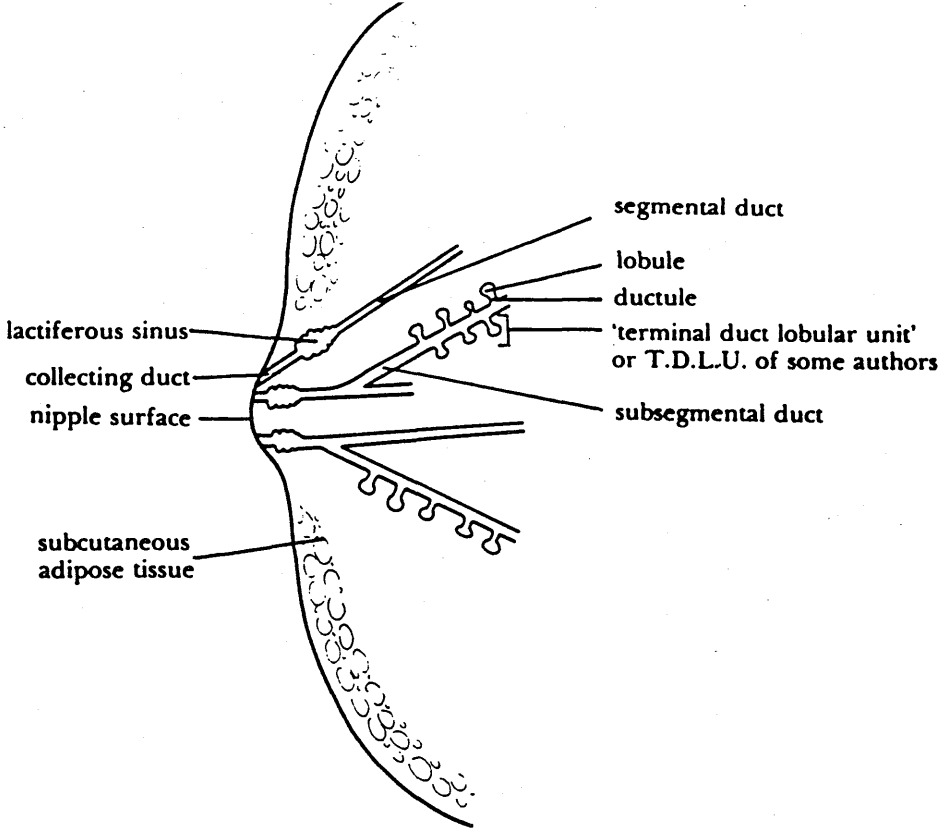
1.42 Structure of the human mammary gland

At puberty the mammary ducts, formed during embryological development, arborise extensively giving rise to 10-15 functional lobes with the remainder being vestigial. Under

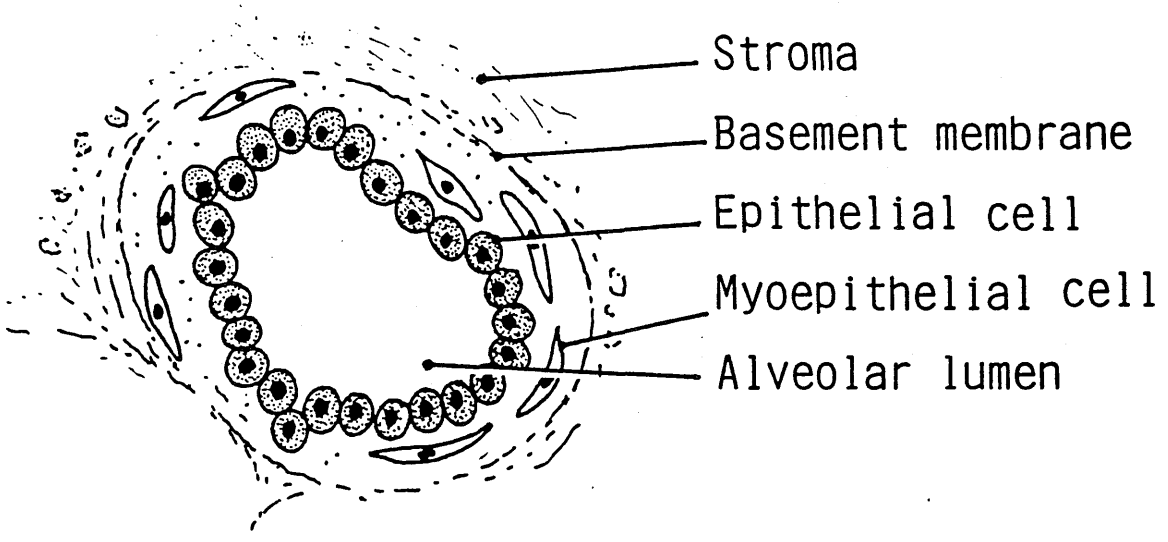
Fig 1.5 A Anatomy of the human mammary gland

B Diagrammatical representation of the normal resting breast. The lobules are lined by a continuous layer of epithelial cells and a peripheral discontinuous layer of myoepithelial cells. The basement membrane follows the contours of the star-like myo-epithelial cells. The adjacent periductal connective tissue contains stromal cells, collagen bundles and elastic fibres.

A


segmental duct
lobule
ductule
'terminal duct lobular unit'
or T.D.L.U. of some authors
subsegmental duct
lactiferous sinus
collecting duct
nipple surface
subcutaneous
adipose tissue

B


Stroma
Basement membrane
Epithelial cell
Myoepithelial cell
Alveolar lumen

the influence of oestrogens, there is a direct or indirect stimulation of ductal proliferation. Both deposition of periglandular adipose tissue and increases in elasticity lead to enlargement of the breast (Vorherr, 1974). Once the menstrual cycle is established, the sex steroids induce changes in breast development and the lobuloalveolar structures undergo limited growth and development.

In the adult female, the human mammary gland is composed of 10-15 lobes disposed radially around the nipple. Each lobe is enclosed within a septum of dense fibrous connective tissue containing ductal elements, blood vessels, lymphatics and nerves. These lobes are embedded in fatty stroma and contain several hundred lobules each. The lobules are linked together by small ducts which converge close to the nipple to form the major lactiferous (segmental) ducts. It is these ducts which open into the lactiferous sinus that communicates with the nipple surface via the collecting duct (see Fig. 1.5a).

The lobules are divided into 10-100 alveoli (acini) enveloped by a collagen sheath which forms the basement membrane, the extension of which surrounds the collecting duct. Mammary alveoli are small circular to ovoid extensions from the alveolar duct. The alveoli represent the basic secretory units of the gland and exhibit significant proliferation and regression during the various phases of mammary gland development.

The ductal-lobular epithelial system of the gland has a highly specialised two cell type lining. The arrangement of these cell types is illustrated in Fig. 1.5b. The terminal ductule of the resting mammary gland contains an inner continuous layer of columnar or cuboidal epithelial cells which line the lumen. The epithelial cells lie either directly on the basement membrane or on myoepithelial cells which form the second discontinuous layer of cells. The luminal surface of the epithelial cells may have a large number of microvilli in contrast to the other surfaces which have few microvilli. The myoepithelial cells are believed to be of ectodermal origin and possess myofibrils which run longitudinally along the interlobular ducts. It is these

specialised cells which form a network around the alveoli and are responsible for the ejection of milk from the alveoli and alveolar ducts.

1.43 Post-pubertal development

The development of the mammary gland after puberty can be subdivided into the following stages: i) inactive (resting) phase; ii) proliferative (active) stage; iii) lactating (secretory) phase and iv) regression phase.

1.431 Inactive phase

This phase of development is observed in the non-pregnant, non-lactating, sexually mature female. Negligible alveolar development has occurred and there is a high ratio of connective tissue to glandular parenchyma. The lobules, which comprise ducts lined by epithelium, are widely separated within the stroma. The ovulatory cycles are associated with premenstrual breast fullness and tenderness as a result of increased blood flow, water retention in connective tissue, ductal alveolar sprouting and perhaps, the production of new alveoli (Vorherr, 1974). During menstruation, however, cellular regression results in the shrinkage of the lobules and alveoli.

Animal studies have shown that although oestrogens result in little lobuloalveolar growth, they induce ductal proliferation and the stimulation of secretory activity (Cowie, 1978). The combined injection of oestradiol and progesterone, however, results in the stimulation of lobuloalveolar development, but inhibition of the secretory activity. Since these steroids were not found to have an effect on hypophysectomised animals (Cowie *et al*, 1966), it has been proposed that a pituitary-derived hormone, possibly prolactin, may account for the growth response to oestrogen and progesterone.

1.432 Proliferative phase

The mammary gland undergoes a dramatic phase of ductal and lobuloalveolar growth during pregnancy. In the early part of pregnancy, hyperplasia of ductal and secretory elements occurs, while in the later trimesters, the changes are associated with alveolar cell hypertrophy and secretory activity. A simultaneous increase occurs in the glandular parenchyma to connective tissue ratio, through an increase in number of alveoli and a reduction in stroma. Alveolar saccules develop by the extensive growth and branching of the extremities of the intralobular ducts. As gestation progresses, the secretory alveoli increase in size. Associated with the apical surface of the alveolar epithelium is a random distribution of secretory microvilli. Other changes during the proliferative stage include the increased level of Golgi bodies and endoplasmic reticulum as well as the appearance of lipid droplets in the alveolar cells by six months gestation. After the first half of pregnancy, adequate growth has occurred to permit lactogenesis following induction by abortion or parturition. As prolactin and placental lactogen have been demonstrated to induce growth in vivo and in vitro, in the absence of sex steroids (Talwalker & Meites, 1961), these hormones have been attributed a major role in mammary growth. The role of the various hormones at the cellular level, in terms of mammary growth, is discussed in Sect. 1.44.

1.433 Lactation phase

Proliferation of the parenchymal cells occurs as part of the dramatic changes to the secretory elements in the first few days after parturition. Within 48 hours rough endoplasmic reticulum has replaced the free ribosomes at the base of the alveolar cells. Lipid droplets have also migrated from the base of the cells to the apical surfaces. Secretory proteinaceous granules are released into the alveolar lumina as part of the increased alveolar secretory activity. The alveoli consequently become distended with milk and the epithelial linings become flattened and distorted. The contents of the alveolar lumina are then passed into the alveolar ducts and ultimately into the lactiferous sinus as a result of the contraction of the myoepithelial cells.

1.434 Regression phase

The cessation of suckling results in the regression of the mammary gland which is associated with the resorption of milk in the alveolar lumina. The gradual reduction in parenchymal tissue is due to lysosomal and autophagic activity with the resulting debris being cleared by macrophages. The regression of the parenchymal tissue is accompanied by an increase in the interlobar and intralobular connective tissue. The post-lactation state of the mammary gland does not involve complete regression to the pre-pubertal state, but to an intermediate stage with the retention of alveoli for development during a subsequent pregnancy.

Involution of the mammary gland, approaching and following the termination of ovarian function, has been regarded as a two-stage process (Vorherr, 1974). Between the ages of 35 - 45, atrophy of the mammary gland commences with the loss of some alveolar and lobular tissue. The later stage, between 45 - 75 years of age, involves virtually complete loss of glandular and ductal elements with a simultaneous rise in fat and connective tissue deposition.

1.44 Hormonal Regulation of Mammary Function

1.441 Lactogenic hormones

Placental lactogen and the pituitary hormones prolactin and growth hormone are the lactogenic hormones involved in mammary development and function. Of these hormones, prolactin is regarded as the most important in mammary growth and differentiation. The action of placental lactogen is unclear in the human, although it appears to be involved in promoting mammary growth and development or the regulation of maternal metabolism during pregnancy.

In structural terms, the amino acid sequence of human placental lactogen (hPL) shown 85% homology with human growth hormone, while prolactin shows only 26% homology (Wallis, 1975).

The identification of human growth hormone, hPL and another growth hormone-like gene on chromosome 17 is consistent with evidence that the three genes have been formed by gene re-duplication (Owerbach et al, 1980).

1.442 Prolactin

Prolactin biosynthesis occurs on membrane bound ribosomes with subsequent storage in granules, prior to release by exocytosis. Both environmental and endogenous stimuli influence prolactin release. Various factors, such as prolactin inhibitory factor (possibly dopamine), prolactin releasing factor, TRH, oestrogens and endogenous opiod peptides regulate prolactin secretion. The internal environment also influences prolactin release, for example, prolactin inhibits its own secretion (Tindal, 1974). Oestrogen administration has been shown to induce pituitary hypertrophy and increase prolactin release (Ratner et al, 1963). Both these effects are reduced after ovariectomy but restored by injection of oestrogen (Macleod et al, 1969). Dopamine agonists have an inhibitory effect on prolactin synthesis which is abolished by preincubation of rat pituitary cells with oestradiol (Raymond et al, 1978) in agreement with the proposal that oestradiol affects dopamine receptor processing (Gudelsky et al, 1981).

Prolactin has been shown to stimulate mammary epithelium, both in vivo and in vitro, resulting in an accumulation of casein mRNA within 60 minutes and increased casein synthesis (Houdebine & Gaye, 1975; Terry et al, 1977). Tight coupling of mRNA levels and casein synthesis does not seem to occur, as prolactin-induced casein synthesis is unaffected when colchicine and prolactin are administered to pseudopregnant rabbits suggesting prolactin may act at a translational level (Teyssot & Houdebine, 1981). Prolactin treatment of explants of lactating tissue for 15-45 minutes has increased both casein release into the medium and milk fat release, with the former increase unaffected by protein synthesis inhibitors

(Daudet et al, 1981). Further evidence for the co-ordinate action of prolactin at multiple sites to increase milk component levels comes from the reduction of casein breakdown in explants from mammary tissue treated with prolactin (Wilde et al, 1980).

Prolactin receptors are subject to "up" and "down" regulation by prolactin itself. The concept of up regulation is in agreement with a rise in receptor levels in pseudopregnant mice mammary glands after prolactin treatment for 48 hours (Djiane & Durand, 1977). Down regulation has been observed within minutes of prolactin administration in vivo, although it is prevented by lysosomotropic and metabolic inhibitors, suggesting internalisation and degradation occurs (Djiane et al, 1980; Costlow & Hample, 1980).

1.443 Placental Lactogen

Increases in placental lactogen levels parallel the growth of the placenta, although the concentration of placental lactogen in the placenta stays constant (Josimowich & Atwood, 1964). Hypoglycaemia is also found to regulate hPL levels, with an inverse relationship between blood glucose and hPL. Placental lactogen is, however, only secreted during pregnancy and plasma hPL levels are 100-fold greater than prolactin during pregnancy, although its physiological role in mammogenesis is unclear.

1.444 Growth hormone

Growth hormone is mainly involved in body growth and tissue metabolism. An essential role in human mammary development and lactation is questionable, as both are normal in dwarfs with a specific deficiency in growth hormone (Rimoin et al, 1968). In some mammalian species, growth hormone has been found to be released at suckling, but this is not the case in humans.

1.445 Steroid Hormone Regulation of Mammary Function

The importance of oestrogens, progestagens and cortical steroids in the co-ordination of mammary function has not been demonstrated by classical endocrine studies in animals. *In vitro* studies, however, suggest that steroid hormones modify the actions of lactogenic hormones and are not regulatory hormones *per se*.

1.4451 Oestrogens

In vivo oestrogens have been found to promote mammary growth and development, while stimulating prolactin production both *in vivo* and *in vitro*. Although milk secretion is inhibited by oestrogens in the lactating gland, there is controversy regarding oestrogen action on cell proliferation (see sect.1.31).

Ductal proliferation and accumulation of secretory material in mammary alveoli in response to oestrogens, is in agreement with the concept that mammary epithelial proliferation is stimulated directly or indirectly by oestrogens. Most *in vivo* studies suggest that the oestrogen effect on mammary growth is exerted either by an indirect action through a pituitary growth factor or synergism with the pituitary hormones (Lyons 1958; Haslam & Shyamala, 1979).

A correlation between plasma prolactin and oestrogen levels has been observed (Hertz *et al*, 1978), indicating that prolactin may exert the oestrogenic effects on mammary growth. In ovariectomised mice, both oestradiol and progesterone have been found to synergise with prolactin in the induction of mammary growth (Traurig & Morgan, 1964). The synergism, in the case of oestrogens, may either occur through direct action or, alternatively, oestrogen-inducible growth factors (Sirbasku, 1978).

In terms of differentiated function, oestrogens seem to sensitise the mammary gland to prolactin as shown by the restoration of mammary sensitivity in ovariectomised mice

injected with oestradiol (Bolander & Topper, 1980). Furthermore, oestrogen treatment of neonatal mice has been found to endow mammary tissue with a greater capacity for further differentiation (Warner et al, 1980).

ER's have been detected in the mammary fat pad and epithelium of virgin mice in similar quantities (Haslam & Shyamala, 1981), in addition to the stroma indicating that the mesenchymal tissue is a site for oestrogen action (see sect. 1.32). Oestrogens have been found to suppress lactation during the post-partum phase, although the mechanism is unclear. Direct action on the mammary gland has been suggested although evidence exists to suggest that interference with prolactin binding may be responsible (Bohnet et al, 1977).

In summary, oestrogens appear to prime mammary tissue to the mitogenic action of prolactin. A synergistic action with prolactin seems to operate, although the mechanism of oestrogen action on cellular proliferation is unclear.

1.4452 Progesterone

Progesterone has an important regulatory role in mammary development during pregnancy and parturition. During pregnancy, complete lobuloalveolar development is induced by the synergistic actions of progesterone with oestradiol and prolactin, while milk release is suppressed. Progesterone, in vitro, does not seem to affect mammary growth during lactation while simultaneously inhibiting the activation of enzymes involved in terminal differentiation.

In most mammals, ductal proliferation is induced by the injection of oestradiol, while oestradiol and progesterone induce lobuloalveolar development characteristic of pregnancy (Topper and Freeman, 1980). In combination with prolactin, progesterone promotes thymidine incorporation in vivo into ductal and alveolar epithelium of hypophysectomised, ovariectomised rats, although prolactin and oestradiol promote growth of ductal cells (Stoudemire et al, 1975).

The inhibition of lactogenesis by progesterone has been shown *in vivo* and *in vitro* to be a direct action of the hormone on the mammary gland, since progesterone administration blocks the lactogenetic changes. Progesterone abolishes those changes associated with terminal differentiation and milk secretion, *in vivo* and *in vitro*, such as increased prolactin-induced levels of α - lactalbumin, casein and casein mRNA's (Turkington & Hill, 1969; Rosen *et al*, 1978), although the mechanism is unclear.

Progesterone action is thought to occur through progesterone receptors (PR) although it has been found to inhibit cortisol binding, thereby preventing glucocorticoid potentiation of prolactin action (Ganguly *et al*, 1982). High affinity mammary gland PR's, which are oestrogen-inducible, have been reported in virgin mice and during various reproductive stages (Haslam & Shyamala, 1979). In mammary fat pads, PR's have been detected, although they could not be regulated by either oestrogens or different stages of lactation (Haslam & Shyamala, 1981). In soluble fractions from lactating rat and mice glands, no specific progestin binding has been observed (Haslam & Shyamala, 1980) resulting in the suggestion that this may explain the absence of a response to progesterone during lactation. High affinity PR's have been identified in rat and mouse mammary epithelial cells cultured in collagen gel matrix (Edery *et al*, 1984 a b). Receptor levels, after 7 days in culture in serum free medium, were similar to levels obtained after dissociation of the gland and were found to be oestrogen-inducible (Edery *et al*, 1984 a).

1.4453 Glucocorticoids

The role of glucocorticoids in mammary function was identified by both *in vivo* and *in vitro* studies which showed that initiation and maintenance of differentiated functions required their presence (Juergens *et al*, 1965; Davis & Liu, 1969). Studies in hypophysectomised animals (Cowie & Lyons, 1959), have suggested that glucocorticoids are permissive factors which potentiate the effects of other lactogenic hormones.

There is controversy regarding the mitogenic effect of glucocorticoids, with various reports of cortisol promoting breast cell proliferation *in vitro* (Stampfer *et al*, 1980; Topper & Freeman, 1980). Other authors, however, have found no effect on normal or malignant breast cells in culture (Klevjer-Anderson & Buehring, 1980). The role of glucocorticoids in differentiated function is unclear since some *in vitro* studies have shown that differentiation may occur in the absence of exogenous cortisol (Bolander & Topper, 1979) while others have shown a specific requirement (Ray *et al*,1981). In explants, prolactin-induced differentiation is stimulated by cortisol while cortisol alone has a negligible effect. This suggests that glucocorticoids have a permissive role in differentiation which may occur via regulation of PR levels (Sakai *et al*, 1979), amplification of prolactin-induced casein transcription or post-translational processing.

Differential effects of glucocorticoids have been observed on differentiated mammary function, for example, the dose response curves for the effects of cortisol on casein or α-lactalbumin synthesis differed significantly in virgin and mid-pregnant mice explants. Physiological levels of cortisol were found to stimulate casein synthesis while α -lactalbumin synthesis is maximal at 10^{-10}M and fully inhibited at greater than 10^{-6}M cortisol (Ona & Oka, 1980).

High affinity glucocorticoid receptors (GR), have been reported in lactating mouse mammary gland (Shyamala, 1973) and steroids with glucocorticoid activity have been found to displace dexamethasone, notably progesterone although it prevents translocation (Shyamala & Dickson, 1976). *In vitro*, high affinity GR's have been characterised in floating collagen gel cultures of mid-pregnant mice mammary epithelial cells (Schneider & Shyamala, 1985). The levels of GR are similar to freshly dissociated rat gland with no significant loss of receptor in culture for ten days in the presence of insulin, prolactin and cortisol (Schneider & Shyamala, 1985). Prolactin was found to be necessary for the maintenance of GR in culture and has been suggested to mediate its effect on GR through the

prolactin receptor (Schneider & Shyamala, 1985). Thus, although it appears that cortisol maintains GR levels through a GR mediated event (Sakai et al, 1979), this action may be mediated indirectly through the maintenance of prolactin receptors by cortisol.

1.446 Spermidine

The intracellular level of polyamines, such as spermidine, rises in the mammary gland during pregnancy and culminates during lactation (Oka & Perry, 1974; Russel & McVicker, 1972). Associated with the rise are increased levels of the enzymes involved in polyamine biosynthesis, notably ornithine decarboxylase and S-adenosyl methionine decarboxylase. This has led to the proposal that polyamines have a regulatory role in mammary growth and milk secretion.

Explant cultures of mid-pregnant mammary gland cultured with insulin, prolactin and cortisol exhibited a rise in spermidine levels prior to increased milk protein synthesis. Insulin and cortisol were found to induce a smaller increase in milk protein synthesis and spermidine levels while other combinations of these hormones had no significant effect (Oka & Perry, 1974).

There is direct evidence for the involvement of spermidine in milk protein synthesis from studies on mammary explants incubated in the presence of combinations of spermidine, insulin, cortisol and prolactin. Spermidine was found to replace cortisol in this system eliciting a similar increase in α - lactalbumin to that produced by prolactin, insulin and cortisol, although casein synthesis was only half that with the three hormones (Oka & Perry, 1974). The differential stimulation of α-lactalbumin and casein synthesis by spermidine was influenced by the effect of cortisol on α - lactalbumin and casein production (Ona & Oka, 1980). Maximal induction of these milk proteins was achieved in vitro by 30nM and 3μM, cortisol respectively. This suggests that cortisol acts through different mechanisms on milk protein synthesis and spermidine is

more effective in mediating the action of cortisol on lactalbumin. Furthermore, inhibitors of spermidine synthesis were found to prevent milk protein synthesis, although the inhibition could be overcome by addition of exogenous spermidine resulting in the suggestion that spermidine mediated the effects of cortisol (Oka & Perry, 1974). The relationship between the regulatory roles of spermidine and glucocorticoids, however, remains unclear.

1.447 Insulin

Insulin is responsible for promoting glucose uptake for lipid synthesis in mammary cells as in adipose cells. It is generally used at supraphysiological levels *in vitro* and has been suggested to play a role in the regulation of mammary cell development. Insulin has been found to promote proliferation in mammary explants (Juergens *et al*, 1965) and is used in excess of 10^{-6}M to maintain mammary cell integrity and hormonal responsiveness *in vitro*.

Little evidence exists for a regulatory role for insulin in mammogenesis with oestrogen and progestin-induced mammary growth in drug-induced diabetic male mice unaffected (Topper & Freeman, 1980). Although insulin is not essential for mammary growth *in vivo*, it is generally required for mammary cell proliferation *in vitro* and is regarded as a mammary growth factor *in vitro*.

During lactogenesis, short-term insulin deficiencies in rats have little or no effect on milk production (Kyriakou & Kuhn, 1973). This suggests that insulin does not directly act on mammary tissue but exerts secondary effects through alterations in substrate availability. Insulin was, however, reported to be a prerequisite for the stimulation of milk protein synthesis by prolactin and cortisol in mid-pregnant mice explants (Topper & Freeman, 1980). In contrast, studies with pseudopregnant rabbit mammary explants have shown that insulin was not required for prolactin-induced casein synthesis (DeLouis & Combaud, 1977) or induced medium chain fatty acid synthesis in mouse explants (Forsyth *et al*, 1972). Topper & Freeman (1980)

have suggested that insulin or a similar growth factor may enable a block in differentiation to be overcome. Supporting evidence comes from primary cultures of pregnant rat alveolar cells, in which insulin promoted α - lactalbumin synthesis.

Although there is no direct evidence for the regulation of glucose transport by insulin into mammary alveolar cells (Robinson et al, 1978), insulin has distinct effects on lipid synthesis in the mammary gland. For example, a reduction in glucose uptake and metabolism via the pentose phosphate pathway and reduced lipid formation, were observed in isolated alveoli from starved rats. In the mammary gland, insulin has been found to regulate the activities of various enzymes involved in glucose metabolism such as pyruvate dehydrogenase and acetyl CoA carboxylase (Robinson & Williamson 1977; Munday & Williamson, 1981). In terms of cell maintenance, insulin, EGF and Somatomedin C can function equally well but only insulin, in combination with cortisol and prolactin can induce accumulation of casein mRNA in mouse mammary tissue in vitro. Insulin has therefore been suggested to have an essential role in the expression of the differentiated phenotype in addition to its metabolic and growth factor role (Bolander et al, 1981). Similarly in explants from pregnant rat mammary glands, casein and α - lactalbumin synthesis have been induced by prolactin, cortisol and physiological levels of insulin (Nicholas et al, 1983). Even with prolonged treatment with melanocyte stimulating activity, cortisol and prolactin, α - lactalbumin synthesis is not induced without the addition of insulin. This suggests that insulin is essential for the induction of rat milk proteins and this effect is independent of the cell-maintenance activity. However, species differences exist since rabbit mammary epithelial cells do not require insulin for phenotypic expression (Topper & Freeman, 1980).

1.448 Other Hormones and Growth Factors

1.4481 Thyroid Hormones

Current evidence suggests that thyroid hormones play a permissive rather than a regulatory role during mammary development and lactation (Lyons, 1958). Animal studies have shown a correlation between lobuloalveolar growth and T_3 plasma levels in mice (Vonderhaar & Greco, 1979), while thyroid hormone levels fluctuated randomly during mammary development in goats treated with oestradiol and progesterone (Hart & Morant, 1980). Inconsistent results have been obtained from *in vitro* studies showing that thyroid hormones have little effect on lobuloalveolar development in serum containing medium (Peters *et al*, 1979). In serum-free media, however, $T_3(10^{-9}M)$ was found to potentiate prolactin-induced α - lactalbumin synthesis in explant cultures (Vonderhaar, 1977).

1.4482 Prostaglandins

In the mammary gland, prostaglandins are produced in large amounts both *in vivo* and *in vitro*. Studies in goats have shown that the mammary gland produces 1ng/min prepartum, with a 100-fold rise postpartum. $PGF_{2\alpha}$ levels in milk also decrease from 100 ng/ml to 0.7 ng/ml after parturition due to rapid metabolism (Maule-Walker & Peaker, 1980). Prostaglandin levels of breast tumours have also been shown to be higher than normal tissue with high levels of PGE_2 often present in invasive tumours (Rolland *et al*, 1980).

1.4483 Epidermal Growth Factor (EGF)

EGF has been isolated as a single chain polypeptide of 53 amino acids (M_r 6045) from mice submaxillary glands, while its human counterpart has a similar sequence and a molecular weight of 5400. The properties of EGF have been reviewed by several authors (Carpenter & Cohen, 1979; Hollenberg, 1979; Gospodarowicz, 1981) who have shown that both mouse and human EGF interact with the same receptor to elicit the same biological activity.

EGF promotes proliferation of several types of cultured cells from different species. It stimulates a cascade of events

such as increased uptake of low molecular weight compounds, phosphatidyl inositol turnover, membrane protein phosphorylation, initiation of DNA synthesis and cell division (Hollenberg, 1979; Carpenter & Cohen, 1979). In terms of its mechanism of action, ^{125}I-EGF has been shown to bind to diffuse mobile membrane receptors which cluster on coated pits and other non-coated areas. These clustered EGF-receptor complexes pinch off to form coated and non-coated vesicles which are processed within the cell by lysosomes, leading to the breakdown of EGF and its receptor.

EGF has been shown to stimulate mammary cell proliferation in various culture systems (Turkington, 1969; Taylor-Papadimitriou et al, 1977 ab; Imagawa et al, 1982). In organ culture, EGF is required for reinduction of lobuloalveolar growth of mouse mammary gland following the previous cycle of in vitro lobuloalveolar growth and regression (Tonelli & Sorof, 1980). In the primary mammary culture system involving collagen gels, in which growth and differentiation occur in the presence of insulin, cortisol and prolactin, EGF (at 0.1-50ng/ml) has been shown to stimulate proliferation but inhibit casein production (Taketani & Oka, 1983). Casein mRNA accumulation was inhibited 55%,but,total mRNA levels increased by 60%, with the total protein synthesis in the presence of EGF almost the same as in the absence. The increased total mRNA population may fulfil the requirements of actively proliferating cells in terms of maintaining the basic cellular activity.

Imagawa et al, (1985) have shown that EGF stimulates the growth of primary mammary cells from virgin mice with the optimal concentration of 10ng/ml. EGF did not stimulate cell proliferation over the maximum levels obtained with progesterone and prolactin and indeed the PR levels decreased 65% with all three agents present (Edery et al, 1984b). It is possible that EGF inhibits growth through its effects on hormone receptor levels (Imagawa et al, 1985). Alternatively, the culture conditions may impose an upper limit on growth which may be obtained by EGF or hormonal stimulation alone.

High affinity EGF receptors have been identified in the mammary gland (Taketani & Oka, 1982; Edery et al, 1985). Using membrane preparations of mammary gland, two classes of receptor have been characterised (Kd's 8.1×10^{-11}M and 5×10^{-10}M). In epithelial cells only one class of high affinity receptor (Kd $\sim 5.7 \times 10^{-10}$M) (Edery et al, 1985) has been found, in contrast to previous findings of two classes of receptor (Taketani & Oka, 1982). Edery et al, (1985) have suggested that the higher affinity receptors are present on the epithelial cells while the lower affinity receptors represent stromal EGF receptors.

EGF receptor levels were found to decrease during maturation of virgin mice (Edery et al, 1985). During pregnancy, receptor levels rose, culminating at mid-pregnancy and declining to very low levels during lactation when prolactin and insulin levels are maximal (Sakai et al, 1978; Inagaki & Kohmoto, 1982). As previously mentioned, EGF has been reported to have a stimulatory effect on cell proliferation and an inhibitory effect on functional differentiation, while reducing prolactin receptor levels in vitro (Taketani & Oka, 1981). The interplay between EGF and prolactin during the various physiological states in vivo and in vitro remains unclear.

Fitzpatrick et al, (1984 a) have shown that 13 breast cell cancer lines specifically bind EGF. The purportedly normal HBL-100 cell line derived from human milk also showed high specific EGF binding while DU-4475, which grows in suspension, had undetectable levels of receptor. In serum-free medium, MCF-7 and T-47D cell lines showed a mitogenic response to 10nM EGF while the MDA-MB-231 and ZR-75-1 lines did not respond by cell proliferation. Within breast tumours, EGF receptors have been detected with a Kd of 2nM for ^{125}I-EGF (Fitzpatrick et al, 1984 b). Specific binding in excess of 1 fmol ^{125}I-EGF/mg membrane protein and 15% specific binding was found in 48% of primary and metastatic breast tumours. However, there is currently debate regarding the relationship between EGF receptor and ER and PR values (Fitzpatrick et al, 1984 b; Sainsbury et al, 1984).

1.4484 Transforming growth factors

Autocrine secretion of growth factors is an important concept in the relationship between the oncogenes and growth factors and has recently been reviewed by Sporn & Roberts, 1985. Two classes of peptide growth factors have been identified which act by autocrine mechanisms. They stimulate phenotypic transformation of normal cells *in vitro* as shown by anchorage-independent growth in soft-agar (Todaro *et al*, 1980). These growth factors are termed transforming growth factors (TGF's). The first class, α-type is structurally related to EGF although it is antigenically distinct and competes for binding to EGF receptors. TGF-β, however, does not compete for EGF binding but requires EGF or TGF-α to induce colony formation in soft agar (Roberts *et al*, 1983). Although TGF-β stimulates the growth of normal fibroblasts (Roberts *et al*, 1983), it has been found to inhibit the growth of many cell types (Tucker *et al*, 1984; Roberts *et al*, 1985)and has,therefore,been proposed as a class of autocrine negative growth factors involved in cell-cycle regulation. TGF-α has been detected in the urine of 18/22 patients with disseminated cancer, including breast cancer but not in controls (Sherwin *et al*, 1983). In breast cancer cells, there has been no report of TGF-α specific receptors although they have been reported in a human epidermal cancer cell line (Massague *et al*, 1982).

According to the autocrine hypothesis, malignant transformation is currently thought to occur through overproduction, expression and action of positive autocrine growth factors and the capacity of cells to respond to their own negative growth factors.

1.45 Conclusions

It is clear that there is a complex interplay between several mammogenic hormones in the development and functioning of the mammary gland. The increasing availability of biochemical and molecular probes for the study of milk secretion and regulation of gene expression combined with defined culture systems should identify the regulatory hormonal mechanisms at the molecular level.

1.5 Breast Cancer

1.51 Aetiology

Breast cancer is the leading type of cancer in women, with about 7% of women in Britain suffering from the disease during the course of their lives (Baum, 1981). The aetiologic agents of breast cancer have yet to be elucidated despite extensive research aimed at identifying and characterising women at high risk. Currently, the risk factors are regarded as early menarche (Staszewski, 1971), late pregnancy, nulliparity (Woods *et al*, 1980), late menopause (Herity *et al*, 1975) and dietary fat intake (Miller *et al*, 1978). However, only one protective factor, an early age at first birth, has been identified (Henderson *et al*, 1974) although it seems that a full term pregnancy is required to confer this effect (Ravnihar *et al*, 1971).

1.52 Premalignancy

The mechanisms underlying the initiation and development of human breast cancer and its malignant progression are poorly understood. Although the pathology of breast cancer is well documented, there remains controversy regarding the nature of a premalignant breast lesion *in vivo*. In a histological survey of non-malignant areas of carcinomatous breast and breast tissue from age matched controls, Wellings *et al* (1975), found an increase in the number of atypical hyperplastic areas in tissue from cancerous breasts over controls. Carcinoma *in situ* has been found among 90% of their patients with infiltrating carcinoma and has led to the suggestion that epithelial hyperplasia gives rise to carcinoma *in situ* (Wellings, 1980). Cells from intraductal or intralobular carcinoma *in situ* were found to form part of a morphological continuum from normal to hyperplastic, and to ultimately invasive cancer cells. Other studies on non-cancerous tissue from malignant breast and morphologically similar non-malignant breast tissue has shown the former to be more angiogenic (Jensen *et al*, 1982). This evidence seems to support the concept that the breast undergoes malignant progression with cancer being the most advanced lesion.

Studies on fibroblast migration into collagen gels by foetal and adult skin fibroblasts have shown differential migratory responses to cell density (Schor *et al*, 1982). Skin fibroblasts from 70% of breast cancer patients have been found to exhibit a foetal-like migratory phenotype. This has led to the proposal that foetal fibroblasts do not undergo the normal developmental isoformic transition in those individuals who have a high risk of developing breast cancer and perhaps, this may occur through a breakdown in normal epithelial-mesenchymal interactions (Schor *et al*, 1985).

1.53 Malignant transformation

In the normal mammary gland, the epithelial component forms only a very small proportion of the mammary tissue. ER's have been detected at very low or negligible levels in less than 10% of normal breast samples (Wittliff *et al*, 1972). Specific binding of radiolabelled oestrogens has been shown in breast tumours *in vitro* (Terenius, 1968) confirming that some breast tumours respond to oestrogens. The mammary gland has an intrinsic ability to synthesise ER when stimulated to grow and differentiate during puberty and pregnancy. Following neoplastic transformation, the mammary cells may retain all, some or even none, of their ability to synthesise ER. It is often assumed that hormone-sensitive tumours retain ER whilst autonomous tumours are ER negative. However, the possibility remains that ER negative breast tumour cells may respond to growth factors released by other cells in response to oestrogen (See Sect. 1.31).

In malignant breast tissue, changes in levels of enzymes involved in hormone metabolism have been observed. For example, decreased levels of 17β -hydroxysteroid dehyrogenase (which can convert oestradiol to the less mitogenic oestrone) have been observed in tumour tissue (Pollow *et al*, 1977). A positive correlation has been reported for ER and Δ^4 - 5α -reductase which is involved in the formation of metabolites that stimulate tumour growth. Elevated 16 α-hydroxylase activity has been reported in many breast cancer patients (Schneider *et al*, 1982).

1.54 Model systems available for the study of hormone action in breast cancer

Various systems have been developed in order to study hormonal regulation of breast cancer. They include: i) animal models; ii) whole gland and organ culture; iii) continuous cell culture and iv) primary cell culture.

1.541 Animal models

1.5411 Carcinogen induced mammary tumours

The first laboratory model for hormone dependent breast cancer was developed by Huggins *et al*, (1959), who demonstrated that dimethylbenz(a)anthracene (DMBA); 3-methylcholanthrene-induced rat mammary tumours were hormone dependent. This model, however, has fundamental disadvantages, in that these tumours are more dependent on prolactin than their human counterparts and also less likely to metastasise. Prolactin administration has been shown to stimulate tumour growth (Leung *et al*, 1975) and increase ER levels *in vivo* (Vignon & Rochefort, 1976) and *in vitro* (Sasaki & Leung, 1975). Oestrogen treatment of hypophysectomised rats with DMBA-induced tumours results in PR induction without any effect on growth of the tumours (Arafah *et al*, 1980).

An alternative carcinogen induced model is the N-nitrosomethylurea (NMU)-induced tumour model in rats (Gullino *et al*, 1975) although the schedule for NMU administration determines hormone dependence (Rose *et al*, 1980). Growth of the NMU-induced tumours is primarily dependent on oestrogens and prolactin (Arafah *et al*, 1982). Furthermore, both these hormones act synergistically in stimulating the *in vivo* growth of the tumours (Arafah *et al*, 1982). This model, therefore, appears more suitable for studying hormonal action in the growth of breast cancer cells.

1.5412 Transplantable rat mammary tumours

There are few reports of hormone dependence in transplantable rat mammary tumours, which generally have the capacity to

metastasise. An example of an oestrogen-sensitive, transplantable breast tumour is the R3230 AC tumour which grows equally well in both intact and ovariectomised rats (Hilf et al, 1965). Although ER's have been identified in this tumour, the ER levels are only a small percentage of those in DMBA-induced tumours.

1.5413 Athymic models

The immuno-suppressed nude mouse offers a system for the transplantation of human tumours (Pantelouris, 1968). The highest success rates after tumour implantation were only 30% with similar rates for benign tissues (Reid et al, 1977; Venter & Reid, 1980). Of the malignant tumours growing in nude mice, it was possible to passage 10% indefinitely. In contrast, it has not been possible to passage benign lesions. Other reports indicate that 20% of breast tumours were successfully hetero-transplanted and all these tumours were undifferentiated (Giovanella et al, 1983). Atypical non-cancerous lobules from malignant breasts were reported to proliferate and dedifferentiate to a greater extent than lobules from normal breast tissue (Jensen & Wellings, 1976). Tumour formation in nude mice by benign and malignant breast lesions in addition to the proliferation and dedifferentiation of normal mammary tissue, demonstrate that the athymic model seems unreliable in discriminating between normal, benign and malignant tissue.

1.542 Whole gland and organ culture

The systemic complexities within animals have limited the assessment of hormonal effects on mammary cells. One approach to overcome these problems has been the development of the whole gland (Ichinose & Nandi, 1966) and organ (explant) culture (Elias, 1959) systems. Studies using the whole mammary gland system have shown that casein gene expression is hormone-inducible and that prolactin and glucocorticoids are prerequisite for the induction of gene expression in the mouse mammary gland (Banerjee et al, 1982). In the explant culture system, small fragments of mammary tissues are maintained in growth

medium (Elias, 1959). An advantage of the explant system is the maintenance of cellular contact with the supporting stroma. In a defined medium supplemented with insulin, cortisol and prolactin were found to stimulate the production of a milk-like secretion and also active casein synthesis (Elias, 1959; Riviera & Bern, 1961). The major criticism of the organ culture system has been the viability of the explants which is generally only a few days (Wellings & Jentoft, 1972; Lagios, 1974) although routine maintenance for up to three months has been reported (Hillman et al, 1983).

1.543 Continuous cell culture

Long term culture systems for human breast cancer cells have been developed in order to overcome some of the problems encountered either in vivo or in organ culture. These clonal systems are devoid of supporting stromal elements which are present in the systems previously described. The established cell lines, which are generally derived from pleural effusions, offer the advantage over the shorter-term culture systems of repeated or sequential experiments on the 'same' tissue.

The only human breast cancer cell lines responding to sex steroids are the MCF-7 (Soule et al, 1973), ZR-75-1 (Engel et al, 1978), CAMA-1 (Leung et al, 1982), T-47D (Keydar et al, 1979) and the PMC 42 line (Whitehead et al, 1983 a). All these cell lines are oestrogen-sensitive in terms of PR synthesis although these effects may not be related to cellular proliferation or thymidine incorporation (Allegra et al, 1981; Eckert & Katzenellenbogen, 1982; Horwitz et al, 1982; Yu et al, 1981).

The continuous cell culture systems have the inherent limitation that variant cell types may be selected which are necessarily not totally representative of the parent tumour population. For example, the responsiveness of different batches of MCF-7 cells to oestradiol has been variable. While early studies showed that oestradiol increased rates of macromolecular synthesis and cell number by more than two-fold (Lippman & Bolan, 1975; Lippman et al, 1976), later reports showed that

oestradiol was unable to stimulate growth in serum-containing medium (Horwitz et al, 1978; Edwards et al, 1980 a). In serum-free medium, MCF-7 cells did not proliferate in response to oestradiol, although morphological changes were observed (Barnes & Sato, 1979). Similarly, growth of MCF-7 cells on extracellular matrix was unresponsive to oestradiol, although tamoxifen was found to inhibit proliferation (Jozan et al,1982).

The CAMA-1 cell line was initially described as being oestrogen responsive in serum-containing medium (Leung et al, 1982) although it became unresponsive after continuous passage (Yu et al, 1981). The original T-47D strain was reported to be unresponsive to oestrogen in serum-containing medium (Keydar et al, 1979) while the cloned subline responds to oestradiol in serum-containing medium (Chalbos et al, 1982).

The most responsive of the human breast cancer lines used to date is the ZR-75-1 line which proliferates in response to oestradiol in both serum-containing and serum-free medium (Engel et al, 1978; Darbre et al, 1983,1984; Allegra et al, 1978). Both basal and oestradiol-stimulated growth was found to be inhibited by tamoxifen under serum-free conditions (Allegra et al, 1981). These observations on oestrogen and antioestrogen action in ZR-75-1 cells have important implications, since they show that hormones act directly on target cells in the absence of serum and growth factors other than transferrin, T_3 and dexamethasone. Also the tamoxifen-induced inhibition of cell growth in the absence of serum suggests that it can exert an effect independent of competing oestrogen.

A new cell line PMC 42 has been described by Whitehead et al, (1983 ab) and has been shown to contain receptors for oestrogen, progesterone, glucocorticoids and EGF (Whitehead et al, 1984). In serum-free medium, these cells do not grow unless supplemented with insulin and cortisol which were shown to have an additive effect when used in combination (Whitehead et al, 1984). Oestradiol and progesterone were also found to stimulate growth, with the combination of these two hormones resulting in a synergistic effect on growth. The stimulatory effect of oestradiol was inhibited by tamoxifen (10^{-6}M) which reduced proliferation of the PMC 42 cell line (Whitehead et al, 1984). Other reports have

shown that tamoxifen, at this concentration, may be directly cytotoxic (Bardon et al, 1984).

Continuous cell culture systems have provided useful biochemical information regarding the subcellular aspects of hormone-dependent breast cancer, but have limitations with regard to their phenotype.

1.544 Primary culture

The main requirements for growth of primary cultures of mammary epithelial cells are conditions which support epithelial proliferation, while suppressing the attachment and subsequent growth of stromal fibroblasts (Whitescarver, 1974; Owens et al, 1976). Various selective procedures have been employed in order to eliminate fibroblastic contamination such as mild trypsinisation (Owens et al, 1976), use of media containing D-valine (Gilbert & Migeon, 1975) use of an anti-fibroblast antibody (Edwards et al, 1980 b) and separation of epithelial and fibroblastic cells on Percoll gradients (Yang et al, 1979, 1980 abc).

Primary cultures are generally prepared from solid tissues by partial enzymic digestion, resulting in breakdown of extracellular matrix and releasing the epithelial component from the stromal cell types. After differential sedimentation of the cell suspension, the resulting organoids (representing alveolar and ductal aggregates) are plated onto the appropriate substrate to which they attach and grow. Some of the epithelial clumps attach to the substrate and two different cell types are apparent within the colony, the inner pavement-like epithelial and the elongated myoepithelial cells.

The importance of the cellular-substrate interactions have been described in Sect. 1.33. Some of the systems available for sustained growth of human mammary epithelial cells may promote epithelial growth by providing basal lamina components for the attachment and subsequent proliferation of these cells, These different systems described previously involve: i) coating of the culture substrate; ii) use of feeder cells; iii) conditioned and defined media and iv) clonogenic systems of growth.

1.5441 Coating of culture substrate

Fibroblastic overgrowth in primary culture has been overcome by coating of the tissue culture substrates with collagen or extracellular matrix.

1.54411 Collagen gel system

The earliest studies with the collagen system analysed growth of mammary epithelial cells grown on or within matrices of reconstituted rat tail collagen (primarily type I collagen). Conflicting results were obtained, with better growth on collagen substrates than within collagen substrates (Yang et al, 1981 b) and vice versa (Yang et al, 1979; 1980 abc; 1981 a). Nandi's group have shown significantly better growth of normal and neoplastic murine cells within the collagen gel matrix (Yang et al, 1979, 1980 abc, 1981 a) and have subsequently shown casein production in floating collagen cultures grown with insulin, cortisol and prolactin (Flynn et al, 1982).

In its current state, the collagen gel system offers a serum-free primary culture system in which cells are embedded within the collagen matrix giving rise to duct-like colonies (Yang et al, 1982 ; Edery et al, 1985; Imagawa et al, 1985). The serum-free growth medium most recently employed was Ham's F12: DMEM (1:1 v/v) supplemented with insulin (0.01 mg/ml), crude soybean lecithin, trace elements, trypsin inhibitor and anti-oxidants.

In previous studies (Yang et al, 1982 ; Imagawa et al, 1982), insulin (0.01 mg/ml), BSA fraction V (0.1%) and EGF (10 ng/ml) were found to be essential for sustained growth of mammary epithelial cells from virgin or midpregnant mice. Mammogenic hormones were not found to consistently stimulate growth. The recent refinements, which include the replacement of albumin with liposomes of crude soybean lecithin, have shown that cell viability is maintained and growth is stimulated in the absence of EGF (Imagawa et al, 1985). In this modified system, both progesterone and prolactin, alone or in combination, stimulated mammary epithelial growth. The stimulatory effect was, however, masked by the presence of EGF in the earlier serum-free system. Analysis of ER and PR in mice mammary epithelial cells cultured in the modified system (Edery et al, 1985) have shown that low levels of ER are present (50-100 fmol/mg DNA). Oestrogen-mediated induction of PR

was reported although this could be inhibited. The relationships between these mammogenic hormones and their receptors have been used to postulate a model for growth induction in the mammary gland (see Section 1.314).

1.54412 Extracellular Matrices

An ECM derived from bovine corneal endothelial cells has been reported to support the growth of normal primary human mammary epithelial cultures in serum-free medium (Biran et al, 1983). This ECM has been found to closely resemble naturally occurring basement membrane, in addition to promoting plating efficiency, growth and differentiation of several cell types (Vlodavsky et al, 1980; Gospodarowicz et al, 1981, 1982). Biran et al, (1983) employed a serum-free medium supplemented with high-density lipoprotein which has previously been shown to promote the growth of normal and transformed cells. Success rates of 80-100% were obtained, with successful subcultures onto ECM at 1:10 split ratios, regardless of the age of the patients.

1.5442 Use of feeder cells

Feeder cells, which are cells that have either been lethally irradiated or treated with mitomycin-C, in order to prevent further cell division, have been used to select for epithelial cell growth (see Sect. 1.334).

1.5443 Conditioned and defined media

During the development of a defined medium to promote the growth of human mammary epithelial cells, commercially available media have been supplemented with hormones, growth factors, foetal calf serum (FCS) and conditioned media (Stampfer et al, 1980, 1982; Smith et al, 1981; Stampfer, 1982; Ham 1982). The enriched medium MM comprised a basal medium supplemented with growth factors, 0.5% FCS and 40% conditioned media from human epithelial and myoepithelial cell lines. The conditioned media were employed since they had been found to promote attachment and growth of mammary epithelial cells (Stampfer et al, 1980).

This MM medium supported rapid growth of normal human mammary epithelial cells in monolayer culture for 3-4 passages, with 1:10 splits although clonal growth occurred only in the presence of a fibroblast feeder layer (Smith et al, 1981).

The optimised basal nutrient medium, MCDB 170, in combination with a serum-free supplement of insulin, cortisol, EGF, transferrin, ethanolamine, phosphoethanolamine and bovine pituitary extract (BPE), supports long term growth of human mammary epithelial cells for 10-20 passages at 1:10 splits (Hammond et al, 1984). The completely defined form, in which BPE is replaced by prolactin and PGE_1, however, supports rapid clonal growth and subculture for only 3-4 passages. Selection of one population of mammary epithelial cells has been observed at passages 2-4 in MCDB 170 supplemented with the BPE-containing supplement (Hammond et al, 1984). Initially two cell populations were observed; a rapidly dividing cell-type and a much larger cell-type which represents a slowly-multiplying population. After 4-5 passages, cultures were found to comprise the smaller cell population since the larger cells were unable to undergo many cell divisions. This defined medium has been reported to support the growth of normal mammary epithelial cells in addition to cells derived from tumour samples (Hammond et al, 1984). It will enable various experiments to be performed, such as drug toxicity testing on cultures of cells derived from primary as well as metastatic tumours.

1.5444 Use of clonogenic systems

A two-layer agar culture system has been reported for the clonogenic growth of human tumour cells (Hamburger & Salmon, 1977 ab). Initial reports indicated the selectivity of the system for several types of tumour cells, including human mammary cancer cells (Hamburger et al, 1978; Von Hoff et al, 1980). Later observations, however, showed that this system is not selective for tumour cell growth since cells from benign lesions, such as gynecomastia, form colonies in soft agar (Von Hoff et al, 1981).

Early reports suggested a relationship between the inhibition of tumour colony formation and a higher probability of clinical response in patients (Salmon et al, 1978; Von Hoff et al, 1981). More recently, in vitro responses to drugs have been lower than would be clinically expected (Sandbach et al, 1982). Maximal clonogenic growth rates have been reported as 75% (Sandbach et al, 1982) regardless of histological grade, patient age, menopausal status and ER status (Sandbach et al, 1982; Smallwood et al, 1984).

Present limitations with this clonogenic system are the low and inconsistent plating efficiencies which do not permit very meaningful drug sensitivity assays.

1.55 Management of breast cancer

Regression of metastatic breast cancer in some premenopausal women after ovariectomy was reported by Beatson (1896). Ablative endocrine therapy is nowadays limited to oophorectomy in selected premenopausal patients and the usual therapies include anti-oestrogen, progestin-, radio-, chemo- and immunotherapy. In order to select the appropriate therapy for patients after the removal of the primary tumour, it is important to determine the extent of hormone dependence of the tumour.

1.551 Assessment of hormone dependence in breast tumours

Early studies to determine the hormone dependent nature of human breast tumours were based on the determination of soluble ER (ERc) levels (Folca et al, 1961). However, only about 50% of patients with positive ERc exhibited objective remission to hormonal therapy (Hawkins et al, 1980). In order to assess more accurately the integrity of the ER system, nuclear ER (ERn) levels have also been measured in conjunction with ERc, resulting in an improvement to the prediction rates for hormonal therapy to 70% (Maas & Jonat, 1980; Leake et al, 1981 b). Analysis of ER levels has shown that up to 20% of breast cancer patients have tumours without functional ERc (Leake et al, 1981 ab; Fazekas & MacFarlane, 1983).

ER assays are generally performed by competitive steroid binding assays (Leake, 1981 a) although immunocytochemical assays with poly- and mono-clonal antibodies have been employed. Polyclonal antibodies against 5S hormone-receptor complexes have shown cytoplasmic staining (Raam et al, 1982; Tamura et al, 1984), while monoclonal antibodies have shown specific staining in the nuclei of selected cell populations in frozen sections (King et al, 1985). Although heterogeneous staining patterns have been observed, there is a good correlation with biochemically determined levels of receptor (King et al, 1985). At present the immunocytochemical assays are being used in conjunction with biochemical assays, until more data is available on the relationship between the staining pattern and the clinical outcome.

As an alternative to the determination of ERn levels, various proteins induced by oestrogens have been measured to assess hormone dependence. These include PR which is rarely found in absence of ER (Barnes et al, 1979). The presence of PR has provided the best index for objective remission to adjuvant hormonal therapy with an improvement in prediction rates to 75-80% (McGuire et al, 1977; Osborne et al, 1980).

The 52K oestrogen-regulated glycoprotein (Westley & Rochefort, 1980) has recently been studied in immunocytochemical assays in primary breast cancers (Garcia et al, 1984). Eighty per cent (20/25) primary tumours showed specific cytoplasmic staining with monoclonal antibodies to the 52K protein while no staining was observed in 'normal' tissue from reduction mammoplasties or normal uteri. The staining intensity, however, did not correlate with ER levels and 2/7 samples with ERc levels below 15 fmols/mg protein showed intense staining. Although 80% positive immunoreactivity to 52K protein was initially reported (Garcia et al, 1984), more recent data suggests that its potential as a hormone-dependent and tumour-associated marker for the detection and monitoring of breast cancer is perhaps limited (C. Coombes, personal comm.).

Primary and metastatic tumours from post-menopausal patients have been shown to have higher ER levels as well as a higher proportion of ER positive tumours than pre-menopausal women (McGuire 1975; Allegra & Lippman, 1980). Correspondingly, lower response rates to endocrine therapy have been observed in pre-menopausal women (25%) than in post-menopausal women (Bertuzzi et al, 1981; McCarty et al, 1983; Leake, 1984).

1.552 Histological grade and tumour heterogeneity

Although there have been some reports of a correlation between ER negative tumours and undifferentiated grade III histology rather than more differentiated I or II (Bloom & Richardson, 1957; McGuire 1975), ER status does not always reflect histological grade (Heuson et al, 1975; Thoresen et al, 1981). Well differentiated tumours are more commonly ER positive suggesting that receptor status is a biochemical feature of cellular differentiation, a reflection of the fact that ER is a "luxury" protein synthesised late in the differentiation pathway (Leake, 1981 b).

Multiple assays for ER within breast tumours have demonstrated intratumoural variation (Braunsberg, 1975; Castagnetta et al, 1983) with up to a 7-fold difference in receptor levels reported (Hawkins et al, 1980). The variation is due to the presence of populations of hormone-dependent and autonomous cells (Woodruff, 1983). Even in hormone-dependent tumours the level of ER detected is dependent on the epithelial cellularity of the tumour (Terenius et al, 1974; Hawkins et al, 1980).

1.553 Receptor status stability

Since breast tumours contain pools of hormone-dependent and autonomous cells, it is implicit that receptor status is likely to be unstable. At second biopsy, about half of the ER +/+ patients were still ER +/+ while about 80% of the ER o/o patients remained unchanged (Leake et al, 1981 ab; Holdaway & Bowditch, 1983). Somewhat surprisingly, the disease in 12% of ER o/o reverted to hormone-dependent disease (Leake, 1984).

This group may account for the 5-10% of ER o/o patients responding to hormonal therapy (Leake 1981 a). The remaining categories of patients with ER o/+ and +/o are generally found to change to o/o at second biopsy (Leake et al, 1981 b).

1.554 Prognosis and response to therapy

High levels of ER in primary disease have been found to correlate with better prognosis (Croton et al, 1981). Receptor status has proved useful in the selection of therapy for breast cancer, whether it is adjuvant endocrine or cytotoxic therapy. Primary ER positive tumours are predisposed to metastasise to bone and lung (Campbell et al, 1981; Stewart et al, 1981) although the potential for nodal metastases is independent of ER status (Heuson et al, 1975; Leake et al, 1981 ab). In lymph nodes, Hawkins et al, (1980) observed ER levels to be twice the levels in the primary tumour and this is perhaps due to increased tumour cellularity in the nodes.

In a recent report on a clinical trial in which patients were randomised to receive hormonal therapy for two years, or systemic therapy at relapse, a significant increase was observed in the disease-free interval in the tamoxifen treated group (NATO, 1985). There were approximately a third fewer deaths in the group given hormonal therapy, regardless of menopausal, ER or nodal status (NATO, 1985). However, there still remain many problems in identifying those patients with ER positive disease who become resistant to adjuvant hormonal therapy.

1.6 The Cytoskeleton

In eukaryotic cells, the filamentous systems of actin-containing microfilaments, tubulin-containing microtubules and intermediate filaments (IF's)form the cytoskeleton. In terms of function, the cytoskeleton provides both a framework for the cell and its organelles,in addition to a mechanism for cell-cell contact and migration. These filamentous systems comprise linear, unbranched protein polymers extending through the cytoplasm. The cytoskeletal proteins represent a highly dynamic group of organelles which have the ability to undergo dramatic reorganisation.

1.61 Microfilaments

Actin filaments of 5-6nm diameter are referred to as micro-filaments. In muscle cells, actin filaments are involved in contractile systems through specific interactions with myosin. In non-muscle cells, actin filaments, however, form cross-linked bundles which provide a supporting framework for the cells and their contents. Actin filaments are present in both dynamic and permanent structures. In microvilli, for example, micro-filaments are relatively stable (Mooseker, 1983) while the dynamic properties of actin filaments are observed in cell surface structures (e.g. filipodia, belt-desmosomes)(Alberts et al, 1983). Microfilaments, therefore, represent the main component of contractile machinery of the cells. In addition, they have been implicated in other processes such as cell movement and movement of intestinal microvilli.

1.611 Stress Fibres

Bundles of actin filaments, termed stress fibres, have mainly been studied in culture. These fibres have been found in either a parallel or convergent arrangement in association with other proteins such as tropomyosin, α-actinin or myosin (Byers et al, 1984). In cells attached to culture substrates as well as stationary cells, stress fibres have been observed although they occur to a lesser extent in migratory cells (Jockush, 1983).

In many cell types, other proteins, such as vinculin, are detectable at the binding sites of the microfilaments and the plasma membrane (Geiger et al, 1980).

Cells transformed by oncogenic viruses, e.g. Rous sarcoma virus, have been associated with reduced levels of stress fibres (Ash et al, 1976; Osborn & Webster, 1975). Mammary epithelial cells, cultured on a collagen substrate, however, were not found to exhibit a relationship between tumourigenicity and the presence of stress fibres (Yang et al, 1980 d). Currently, it is unclear whether there is a specific relationship between transformation and the expression of stress fibres.

1.62 Microtubules

The microtubule system comprises hollow cylindrical elements with an outer diameter of 25nm and a luminal diameter of 15nm. In cross section, it is clear that the microtubules are formed from a polymer of thirteen globular subunits of α-and β-tubulin. The dynamic nature of microtubules within the mitotic spindle and cytoskeleton is apparent although, like the microfilaments, the microtubules in cilia and flagella are relatively stable structures. In terms of function, microtubules act both as structural scaffolds and as part of the machinery for the movement of cells as well as particles within the cells. Evidence to suggest that microtubules have a role in cell shape comes from the parallel arrangement of microtubules at the cell surface.

1.63 Intermediate Filaments

In virtually all differentiated cell types, a major cytoskeletal system of intermediate filaments (IF's) has been identified. These IF's are insoluble cytoplasmic proteins of diameters 7-10nm, intermediate between the microfilaments and the microtubules. Immunological, biochemical and molecular biological studies have revealed the IF's comprise heterogeneous subunits (Lazarides, 1980), in terms of which they are classified into five separate groups: (i) keratins in epithelial tissues;

(ii) vimentin in cells of mesenchymal origin;(iii) desmin in myogenic cells; (iv) glial filaments in glial and related cells and (v) neurofilaments in neurons. Intermediate filaments have been extensively reviewed by Lazarides (1980, 1982) and Steinert et al, (1984).

1.631 Expression of Intermediate Filaments

The generalised cell-type classification for IF's has been outlined above,but, there is a growing list of additions and exceptions. For example, co-expression of vimentin and the cell-type IF has been observed (Lazarides & Hubbard, 1976; Zackroff et al, 1981). The presence of keratin-like IF's has also been reported for some fibroblastic cell lines (Zackroff et al, 1984). Co-existence of subunits of two different IF classes has been observed in vivo (Granger & Lazarides, 1980) and in vitro (Franke et al, 1979; Osborn et al, 1980). Vimentin expression is suggested to occur after release of cells from the in vivo environment (Dairkee et al, 1984). Evidence to support this hypothesis comes from studies on a transformed epithelial cell line which expresses both keratin and vimentin in culture but expresses keratin only in tumours growing in nude mice (Summerhayes et al, 1981).

IF's are useful tools in diagnostic pathology to determine the origin of tumours and metastases, regardless of the differentiated state (Altmannsberger et al, 1982; Osborn, 1983; Moll et al, 1983) with cells in solid tumours expressing the IF of the original cell-type (Osborn, 1983).

1.632 Keratin Filaments

The characteristic class of IF's in epithelial cells are the keratins which comprise a highly complex family of polypeptides. Up to 19 keratins have been identified by high resolution two-dimensional gel electrophoresis although keratins 9 and 11 (according to the nomenclature of Moll et al, 1982a) are not regarded as genuine translation products. Keratin subunits have been shown to form acidic and basic subfamilies with

molecular weights between 40-70K (Tseng et al, 1982; Osborn, 1983). Immunological and electrophoretic studies have shown that in a particular epithelium, between 2-10 keratins are present with co-ordinated doublets of acidic and basic sub-units (Osborn 1983; Sun et al, 1983; Woodcock-Mitchell et al, 1982). The smaller keratin species are expressed in simple epithelia, while terminally differentiated epidermal cells express the largest keratins (see Section 1.643). Keratins comprise a complex multigene protein family with differential expression of keratins dependent on the epithelium and stage of differentiation (Tseng et al, 1982; Wu et al, 1982). A unifying model for keratin expression has been proposed by Sun et al, (1984) and is described below.

1.64 Unifying Model for Classification and Expression of Keratins

1.641 Keratin subfamilies

In terms of their electrophoretic properties and immunological reactivities with the AE1 and AE3 monoclonal antibodies, all human keratins have been shown to form two subfamilies (Tseng et al, 1982; Woodcock-Mitchell et al, 1982; Sun et al, 1983). Keratins 1 to 8 have, both a common AE3 antigenic determinant and isoelectric points above 6.0. This basic subfamily, termed subfamily B has been shown to be structurally similar from peptide mapping studies (Schiller et al, 1982). Moreover, the B subfamily has immunoreactivity to the monoclonal K_G8.13 antibody (Gigi et al, 1982).

Keratins 10 and 12 to 19, which have common AE1 antigenic determinants and pI's below 5.7, form subfamily A. Peptide analysis (Moll et al, 1982 b) and cDNA studies (Fuchs et al, 1981; Kim et al, 1983) have shown that these keratins are similar in structure and sequence. At least one keratin from each subfamily is present in all epithelia in vivo (Schiller et al, 1982; Tseng et al, 1982) and in vitro (Fuchs et al, 1981; Kim et al, 1983).

1.642 Keratin pairs

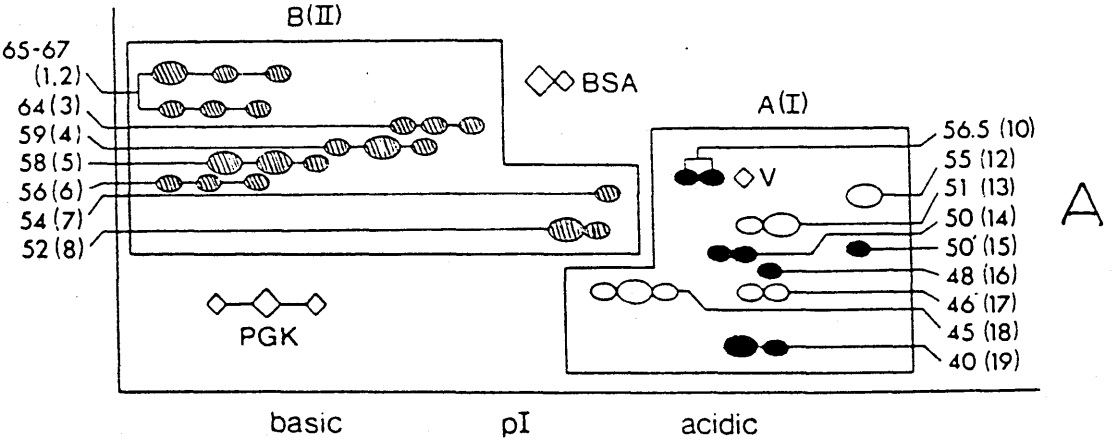
With the exception of the 40-kd keratin 19, which occurs in

Fig. 1.6 Classification of epithelial keratins

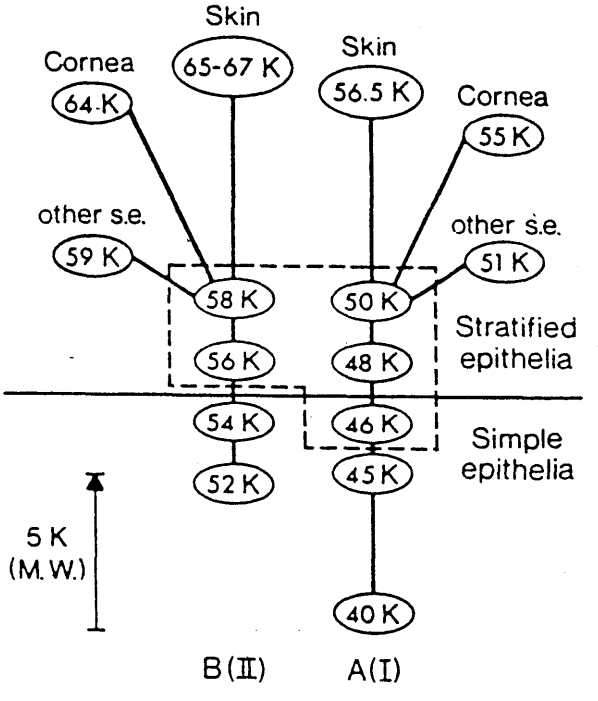
A. Schematic division of human epithelial keratins into acidic (A or type I) and basic (B or type II)subfamilies. Keratins are represented by their molecular weights and numbered according to the classification of Moll et al, (1982a). The keratins represented by dark circles react with AE1 antibody while those identified by the AE3 antibody are hatched circles.

B. A unifying model of keratin expression. Keratins of acidic and basic subfamilies are arranged on the basis of their molecular weights(see 5000 dalton bar).

(Adapted from Sun et al, 1984)



A



B

Keratin subfamilies

virtually all non-epidermal epithelia (Moll et al, 1982 a; Tseng et al, 1982), the remaining subfamily A members have a counterpart in subfamily B (see Fig. 1.6a). Co-expression of keratin pairs is found with the molecular weight of the B subfamily member exceeding that of the A subfamily member by 7-8 Kd (Sun et al, 1984; Tseng et al, 1982).

1.643 Classification of epithelia by keratin composition

On the basis of the keratin composition of various epithelia, it has been shown that keratin expression is dependent on the type of epithelium (see Fig. 1.6b). For example, simple epithelia are generally characterised by the expression of 2-4 of the smaller keratins from the 45-/52-kd and 46-/54-kd pairs in addition to the 40-kd keratin (Moll et al, 1982 a; Schiller et al, 1982). Many monoclonal antibodies have been produced which react specifically with simple epithelial keratins, such as the LE61 antibody which reacts with the 45-kd keratin 18 (Lane, 1982).

1.65 Expression of keratins

In rat mammary epithelial cells cultured in collagen gels, EGF and mammogenic hormones have been reported to alter keratin expression (Palmer et al, 1984). The hormones were found to induce the expression of a 66-kd keratin which is not expressed in the presence of EGF alone. It appears that keratin expression in rat mammary epithelial cells is regulated by hormonal and culture conditions.

Vitamin A is recognised as having an important role in the regulation of epithelial growth and differentiation (Zile & Cullum, 1983). Indeed vitamin A-deficiency induces keratinisation and metaplasia in many secretory and non-keratinised epithelia (Wolbach & Howe, 1925). During epidermal keratinisation, changes in keratin expression have been found (Fuchs & Green, 1980) with basal and suprabasal cells expressing the 50-/58-kd and 56.5-/65-67-kd keratin pairs respectively (Woodcock-Mitchell et al, 1982). The latter keratins are not expressed

in cultured human epidermal cells which form non-keratinised epithelia (Fuchs & Green, 1978) although under vitamin A-deficient culture conditions, expression of these keratins is regained (Fuchs & Green, 1981). Since the 56.5-/65-67-kd keratins have been found in non-epidermal epithelia from vitamin A-deficient rabbits (Tseng et al, 1984) it appears that these keratins are not markers for epidermal keratinisation but for normal and vitamin-A deficiency induced keratinisation.

1.66 Function of intermediate filaments

Since IF's are less soluble than other cytoskeletal proteins, it has been suggested that they have a mechanical rather than a dynamic role in cells (Lazarides,1980, 1982). Studies on cultured cells, which have shown that keratin bundles arise in the perinuclear region and terminate at junctional complexes, have suggested that keratins are involved in the maintenance of cell shape, location of the nucleus and cell-cell contact (Jones et al, 1982). However, more recent studies have shown that microinjection of antibodies, while disrupting the IF network,had no effect on either the cell shape or nuclear centration in culture (Klymkowsky et al, 1983).

Vimentin, in mesenchymal cells has been suggested to have a role in maintenance of cell shape, organelle movement and nuclear centration (Zackroff et al, 1981). Collapse of vimentin filaments into the perinuclear region as a result of colchine treatment indicates a close relationship with the microtubules. This has led to the proposal that there are IF-organising centres which are involved in controlling cytoplasmic distribution and perhaps IF synthesis (Eckart et al, 1982). The presence of different IF's in different cell-types suggests a functional heterogeneity for IF's although the functional role has yet to be elucidated.

1.67 Conclusions

The filamentous systems involved in the cytoarchitecture of eukaryotic cells have been described in this section.

Oncogenic transformation of cells has been found to result in the phosphorylation of various proteins (Hunter, 1984), including vinculin which may link actin bundles to anchor proteins in adhesion plaques. These plaques anchor cells to their substrates and act as internal anchors for bundles of actin filaments. Phosphorylation of vinculin may result in the disruption of the vinculin link and account for the previously described alteration in stress fibres. These and other changes may be related to the process of oncogenesis.

1.68 Aims

The initial objective of these studies was to routinely establish primary cultures of human mammary epithelial cells from breast tumour biopsies. Such cultures were then to be characterised and their responses to hormones and growth factors investigated. The growth responses were assessed, both in terms of cellular proliferation and at the morphological level. More specific aims are presented in the introductory sections of the individual chapters of the results section.

2.0 Materials and Methods

2.1 Tissue Culture Materials

2.11 Plastics

'Nunc' tissue culture flasks (25, 80 and 175 cm^2), 35mm Petri dishes and 4-well plates (16mm diam.) were supplied by Gibco Life Technologies, Paisley, U.K.

'Linbro' Spacesaver Multiwell plates (15mm diam.) and plastic 15mm 'Thermanox' coverslips were obtained from Flow Laboratories, Irvine, U.K.

2.12 Glass

10 and 13mm coverslips were obtained from Chance Propper, Smethwick, U.K. They were boiled for 30 mins. in a 0.1 M Sodium hydroxide solution, rinsed in running tap water overnight and finally given three rinses in distilled, deionised water. Coverslips were separated onto tissue paper, allowed to dry and sterilised in an oven at 160^{o}C for two hours.

2.13 Bacteriological Plastics

90mm plastic Petri dishes, universal containers and bijoux were supplied by Sterilin, Teddington, U.K.

2.2 Media

Ham's F10 (Ham, 1963) and Dulbecco's modification of Eagle's medium (DMEM) (Smith *et al*, 1960) were obtained as 10X stock solutions from Flow Laboratories and Gibco Life Technologies respectively.

2.21 Sera

The culture media were supplemented with foetal calf serum (FCS) which was obtained from Flow Laboratories and Gibco Life Technologies.

2.22 Heat-Inactivated, Charcoal Stripped Serum (HIDCCFCS)

Foetal calf serum was heat-inactivated for 30 mins. at 56°C with constant stirring and then cooled to 4°C. This was added to a pellet of DCC (dextran coated charcoal). DCC was made by stirring 2.5% (w/v) Norit-A charcoal and dextran T-70, 0.025% (w/v) in PBS-A for 18 hours at 4°C before centrifugation at 1000g (5 mins., 4°C). The serum was charcoal stripped for 18 hours at 4°C before pelleting the charcoal by centrifugation. Subsequently, the serum was filtered through Whatman's No. 1 filter paper, 0.45 and 0.2μm filters prior to filter sterilisation and storage at -20°C.

2.3 Tissue culture media

The composition of the media is described below:-

2.31 Basal medium

This comprised a 1:1 (v/v) mixture of Ham's F10 and DMEM buffered with 20mM HEPES.

Deionised, distilled H_2O	400 mls
Ham's F10 (10X)	22.25 mls
DMEM (10X)	22.25 mls
HEPES (1M, pH 7.3)	9.0 mls
Sodium bicarbonate (7.5% solution)	2.5 mls

Sodium hydroxide (1M) was added to adjust the pH of the basal medium to pH 7.2. The sterility of the basal medium was assessed by incubating at 37°C for 2-3 days.

2.32 Dissection Medium

The basal medium was supplemented with the following antibiotics to give the final concentrations shown:-

Penicillin	200 units/ml
Streptomycin	0.1 mg/ml
Kanamycin	0.1 mg/ml
Fungizone	0.0025 mg/ml

2.33 Growth medium for maintenance of cell lines

The basal medium was supplemented with :-

L-glutamine (200 mM)	5 mls (Final concentration 2mM)
Foetal calf serum	50 mls (" " 10% v/v)

2.34 Standard culture medium for growth of primary cultures (SCM)

The basal medium was supplemented with:-

L-glutamine (200 mM)	2mM
Foetal calf serum	10% (v/v)
Insulin	0.005 mg/ml
Penicillin	50 units/ml
Kanamycin	0.1 mg/ml

2.35 PBS-A solution

Dulbecco's PBS-A was prepared by dissolving one tablet of PBS-A (Oxoid) in 100 mls. distilled, deionised water. The solution was then autoclaved.

2.36 Phosphate buffer

A stock 0.2M phosphate buffer (pH 7.4) was prepared in distilled water as follows:-

Solution A : $NaH_2 PO_4 2H_2O$ 31.2 g/l

Solution B : $Na_2 HPO_4$ 28.4 g/l

This stock buffer was prepared by mixing 19ml of solution A with 81 ml of solution B and filtered through a 0.2 µm membrane filter. This buffer was further diluted in filtered, distilled water.

2.37 McIlvaine's buffer

This buffer, which comprises 0.2M Na_2HPO_4 and 0.1M citric acid, was prepared by dissolving 28.4g of Na_2HPO_4 and 21.0g of citric acid in distilled water. The buffer was made up to total volume of one litre with the pH adjusted to 5.5.

2.4 Fine chemicals

These were obtained as follows:-

Acetone	Fisons, Loughborough, U.K.
Charcoal (Norit-A)	Sigma, Poole, U.K.
Chrome alum	BDH, Poole, U.K.
Citifluor mountant	City University, London, U.K.
Dextran T-70	Pharmacia, Hounslow, U.K.
Dimethyl Sulphoxide	BDH, Poole, U.K.
Disodium hydrogen phosphate	BDH, Poole, U.K.
D.P.X. mountant	BDH, Poole, U.K.
EDTA (Ethylenediamine tetraacetic acid)	BDH, Poole, U.K.
Ethanol (Burrough's)	Fisons, Loughborough, U.K.
Formaldehyde	BDH, Poole, U.K.
Gelatin	Sigma, Poole, U.K.
Glacial acetic acid	BDH, Poole, U.K.
L-glutamine (200 mM)	Gibco Life Technologies, Paisley, U.K.
Glutaraldehyde	Sigma, Poole, U.K.
Hank's balanced salt solution	Flow Laboratories, Irvine, U.K.
HEPES 4-(2-hydroxyethyl-1-piperazine-ethanesulphonic acid)	BDH, Poole, U.K.
Hydrochloric acid	BDH, Poole, U.K.
Methanol	May & Baker, Dagenham, U.K.

Mitomycin C	Sigma, Poole, U.K.
Osmium tetroxide	Johnson Matthey, Royston, U.K.
PBS-A tablets	Oxoid, Basingstoke, U.K.
Sodium bicarbonate (7.5% solution)	Gibco Life Technologies, Paisley, U.K.
Sodium dihydrogen phosphate	BDH, Poole, U.K.
Sodium hydroxide	BDH, Poole, U.K.
Sucrose	Farmachem, Strathaven, U.K.
Tannic acid	Mallinckdrot, Paris, France
Trichloroacetic acid	BDH, Poole, U.K.
Uranyl acetate	BDH, Poole, U.K.

All other fine chemicals were BDH 'AnalaR' grade or equivalent.

2.41 Stains

Giemsa	BDH, Poole, U.K.
Hoechst 33258 (2-[2-(4-Hydroxyphenyl)-6-benzimidazolyl] -6-(1-methyl-4-piperazyl) benzimidazole trihydrochloride.)	Sigma, Poole, U.K.

2.42 Hormones and growth supplements

These were obtained as follows:-

Oestradiol -17β	Sigma, Poole, U.K.
Insulin (bovine)	Sigma, Poole, U.K.
Prolactin (ovine)	Sigma, Poole, U.K.
Cortisol (hydrocortisone)	Sigma, Poole, U.K.
Tamoxifen	ICI, Macclesfield, U.K.
Megestrol acetate (Megace)	Bristol Myers, New York, U.S.A.
Retinoic acid	Sigma, Poole, U.K.
Epidermal Growth Factor	Collaborative Research, c/o Uniscience, Cambridge, U.K.

Stock solutions of all steroids (1×10^{-3}M) and retinoic acid (2×10^{-3}M) were prepared in absolute ethanol (A.R).
Crystalline bovine insulin (2mg/ml) was dissolved in 10mM HCl

Crystalline ovine prolactin was prepared in methanol (A.R) acidified with 10mM HCl (final concentration).
Lyophilised EGF was reconstituted in distilled water and diluted to 0.01 mg/ml in Hank's balanced salt solution.
All solutions were stored at -20°C.

2.43 Enzymes

Worthington collagenase, CLS type III, chromatographically purified from Clostridium histolyticum (100-125 U/mg) was obtained from Lorne Diagnostics, Reading, U.K. A stock solution (2000 U/ml) was prepared in PBS-A, sterilised through a 0.2µm filter and stored at -20°C.
Crude trypsin (2.5% solution) came from Gibco Life Technologies.

2.44 Radiochemicals

(Methyl-^{3}H) Thymidine 49 Ci/mmol	Amersham International, Amersham, U.K.

2.45 Antibiotics

These were obtained as follows:-

Penicillin	Glaxo, Middlesex, U.K.
Streptomycin	Glaxo, Middlesex, U.K.
Kanamycin (10 mg/ml)	Gibco Life Technologies
Gentamycin (10 mg/ml)	Flow Laboratories
Fungizone (0.25 mg/ml)	Gibco Life Technologies

Penicillin and streptomycin were prepared as stock solutions of 10^4U/ml and 10 mg/ml respectively in PBS-A.

2.46 Photographic materials

Ilford K2 nuclear emulsion was purchased from Hamilton Tait, Glasgow U.K.
D19 developer and Kodafix fixative from Kodak, Manchester, U.K.
Films: Light microscopy Ilford FP4 (125 ASA) and Kodak Panatomic -X (32 ASA)
Electron microscopy Ilford FP4 (125 ASA)

Fluorescence microscopy Ektachrome
160/400/800/1600 ASA Kodak

2.47 Miscellaneous

Gillette scalpels were supplied by Macarthy's Surgical, Cumbernauld, U.K.
Disposable sterile 'Millex' 0.22μm filtration units were from Millipore, London, U.K.
Nylon mesh (0.2mm) was purchased from John Staniar & Co., Manchester, U.K.

2.471 Gelatin chrome alum solution

5g of $CrK(SO_4)_2 \cdot 12\ H_2O$ was dissolved in 800mls water containing 5 mls of 40% formaldehyde and 1ml photoflo. This solution was mixed with 5g gelatin previously dissolved in 200 mls distilled water.

2.48 Antibodies

LE61 mouse monoclonal to keratin 18 was kindly supplied by Dr. E.B. Lane, Imperial Cancer Research Fund, London, U.K.
Anti-desmoplakin guinea pig antibody was kindly provided by Dr. D. Garrod, Dept. of Medical Oncology, University of Southampton, U.K.
Mouse monoclonal antibody M8 was provided by Dr. P.A.W. Edwards, Ludwig Institute for Cancer Research, Sutton, U.K.
Anti-vimentin mouse monoclonal antibody was purchased from Amersham International, Amersham, U.K.
Mouse monoclonal antibodies HMFG-1 and HMFG-2 were purchased from Seward Laboratory, Bedford, U.K.
Polyclonal rabbit anti-keratin was obtained from Dako Ltd., High Wycombe, U.K.

FITC - (fluorescein isothiocyanate conjugated) antibodies were purchased as follows:-

Swine anti rabbit immunoglobulins	Dako Ltd.
Rabbit anti mouse immunoglobulins	Dako Ltd.
Rabbit anti guinea pig immunoglobulins	Dako Ltd.

2.49 Normal sera

Normal mouse serum (Balb/c strain) and normal rabbit serum were kindly provided by Dr. A.H. Lope Pihie, Dept. of Biochemistry, University of Glasgow.

2.5 Human tissues

Breast tumour tissue was kindly supplied by surgeons at Stobhill General Hospital, Victoria Infirmary and the Western Infirmary, Glasgow.

2.51 Cell lines

NIH - and Swiss - 3T3 mouse embryo fibroblast cell lines were kindly provided by Dr. D. Morgan, Beatson Institute for Cancer Research, Glasgow.

The STO mouse embryo fibroblast line (Ware & Axelrad, 1975) was provided by Miss S. Chuah, Dept. of Biosciences, Strathclyde University, Glasgow, U.K.

Normal rat kidney fibroblast, NRK, (DeLarco & Todaro, 1978), Madin Darby Canine Kidney, MDCK (Gausch *et al*, 1966) and EILU, a human foetal lung epithelial cell line, were provided by Dr. R.I. Freshney, Dept. of Medical Oncology, University of Glasgow.

EWLU, a human foetal lung fibroblast strain was established after collagenase dissociation of foetal lung tissue.

2.6 Microscopy

2.61 Phase contrast

A Leitz Diavert microscope, equipped with a Polaroid camera was used to examine cells growing in culture.

2.62 Light microscopy

Cultures of fixed cells were examined on an Olympus IMT microscope equipped with an OM2 35mm camera.

2.63 Fluorescence microscopy

Immunofluorescence studies were performed on a Polyvar fluorescence microscope (Reichert Jung, Vienna) with epi-illumination.

2.64 Scanning electron microscopy

Primary cultures grown on 10mm glass coverslips in multiwell plates were fixed at 37°C after removal of most of the culture medium but ensuring that the coverslips remained covered by medium. Prewarmed fixative (at 37°C), comprising 2.5% (v/v) glutaraldehyde in 0.1M phosphate buffer was added for an hour. Further processing was performed at ambient temperatures. The fixative was removed and the coverslips washed three times with a 0.1M phosphate/2%(w/v) sucrose rinse (pH 7.4). Cultures were postfixed with 1% tannic acid (w/v) in 0.05M phosphate/1% sucrose buffer (pH 7.4) for one hour and washed three times with 0.05M phosphate/1% sucrose buffer (pH 7.4). Further postfixation was performed with a 2% (w/v) aqueous solution of osmium tetroxide for one hour. Coverslips were washed three times with distilled water and transferred into a 0.5% (w/v) aqueous solution of uranyl acetate for 30 minutes after which they were thoroughly washed with distilled water.

For dehydration by critical point drying (CPD), coverslips were dehydrated through graded acetones (30,50,70,90% and absolute) and critical-point dried from liquid CO_2 in a Polaron E3000 CPD. In contrast, for freeze drying, coverslips were rapidly frozen in liquid propane prior to transfer into the chamber of the freeze drier.

Dehydrated coverslip cultures were mounted onto 13mm aluminium stubs and gold sputter coated for 8 mins. on a high resolution

Polaron E5000 instrument. The stubs were examined in a Philips PSEM 500 instrument with a manual stage at accelerating voltages up to 24 kV.

2.7 Tissue Culture Methods

2.71 Tissue collection and dissection

Human mammary tissue samples were collected fresh from the operating theatre and transported, in sterile containers, on ice to the laboratory.

2.72 Preparation of primary cultures of human mammary epithelial cells from biopsy samples

A small piece of tumour (at least 150mg) was retained for subsequent ER analysis. The remainder of the tissue was transferred into a sterile 90mm Petri dish containing ice-cold dissection medium.

Fatty and necrotic areas of the biopsy were removed and the tissue transferred to a fresh Petri dish containing dissection medium. The tissue was carefully minced into small pieces, approximately $2mm^3$, using closely apposed scalpel blades to avoid applying excessive mechanical pressure on the tissue. The fragments of tissue were suspended in 10mls. medium and transferred to a universal container. They were allowed to settle under gravity, then washed twice with fresh dissection medium. Finally, the fragments were centrifuged in a bench-top MSE Centaur 1 centrifuge (200g, 5 mins.), resuspended in standard culture medium and transferred into a $25cm^2$ tissue culture flask. Collagenase was added to a final concentration of 200 U/ml and the flasks incubated for 1-5 days at 37^oC depending upon the nature of the tissue. Disaggregation of the fragments was aided by gentle pipetting of the suspension in 10ml pipettes to disperse the cell clumps and/or resuspending the fragments in fresh standard culture medium containing collagenase (200 U/ml).

PREPARATION OF PRIMARY CULTURES

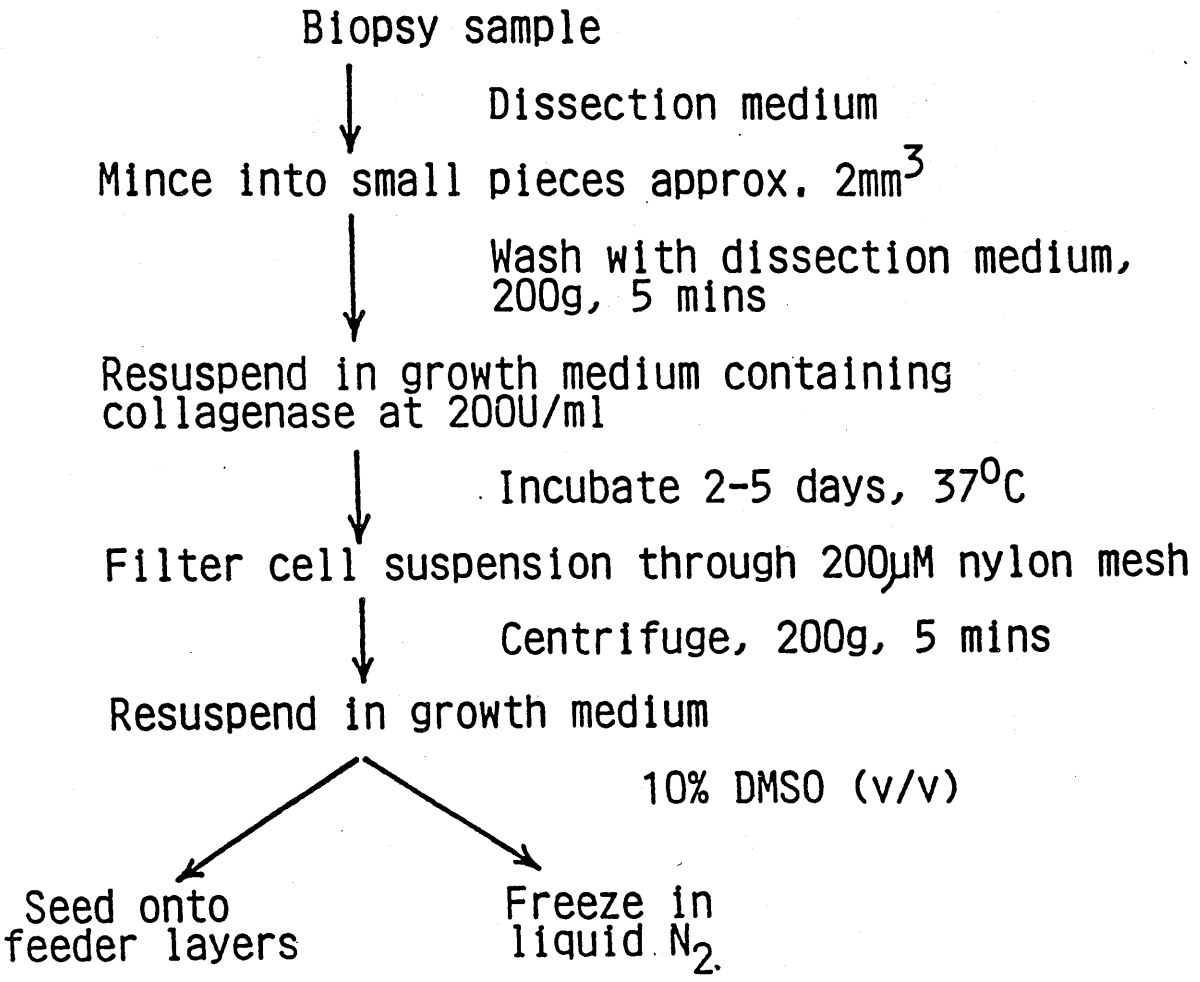


Fig. 2.1 Methodology for the establishment of primary cultures.

Following enzymatic disaggregation, the suspension was filtered through a0.2mm nylon mesh, centrifuged (200g, 5 mins.) and resuspended in standard culture medium. The resultant cell suspension, comprising clumps of cells (organoids) and single cells was seeded onto feeder layers on flasks or coverslips or alternatively frozen down in liquid nitrogen with 10% (v/v) dimethylsulphoxide. The flasks of cells were maintained in a 37°C, Leec incubator while coverslip cultures in multiwell plates were maintained in a humidified Heraeus incubator with a 98% air - 2% CO_2 atmosphere. The culture medium was replaced every two days and the cells examined regularly using an inverted phase-contrast microscope. The standard methodology is summarised in Fig. 2.1.

2.73 Preparation of confluent feeder layers

Feeder cells were grown to subconfluence in either 80 or 175cm^2 flasks in the growth medium for cell lines. Parallel flasks of feeder cells were set up and one of the flasks trypsinised at subconfluence. Mitomycin C was added to the remaining flasks at a concentration of 2$\mu g/10^6$ cells for all cell lines except STOcells for which 4$\mu g/10^6$ cells was required to stop cell division. Following 24 hours exposure to the drug, the medium was removed, the cells were washed twice with PBS-A and fed with fresh medium. After a further 24 hours, the medium was removed and the cells washed with PBS-A before subculturing with 0.25% trypsin in PBS-A. The feeder cells were resuspended in fresh medium and counted prior to seeding onto either culture flasks or coverslips in multiwell plates at a density of 10^5 cells/cm^2 for all cell lines except STO in which case $2x10^5$ cells/cm^2 were required to obtain a confluent feeder layer. After overnight attachment, the disaggregated epithelial cells from the biopsy were seeded onto the feeder layers.

2.74 Maintenance of cultures

2.741 Subcultivation

The growth medium was removed from the flasks of cells to be subcultured and the monolayers washed twice with PBS-A and covered with 0.25% trypsin in PBS-A for 30 seconds. The solution was aspirated off and the flasks incubated at 37°C for 10-15 minutes until the cells had rounded up and detached from the substrate. Fresh growth medium was added to the flask and the cells resuspended by gentle pipetting. The cells were then counted on a Model ZB1 Coulter Counter and transferred to fresh culture vessels as required.

2.75 Cryopreservation of cells

Cultured cells were harvested while still subconfluent (as described in Sect. 2.741) and resuspended in growth medium. Alternatively, cells were obtained directly after enzymic disaggregation and resuspended in the appropriate culture medium. In the case of the cell lines, the minimum cell count was 1×10^6 cells/ml. Dimethylsulphoxide (DMSO) was added to the resuspended cells to provide a final concentration of 10%(v/v). Aliquots of 1 ml were transferred to ampoules which were stored in a thick-walled expanded polystyrene container at -70°C for at least three hours in order to ensure gradual freezing. The ampoules were then transferred to liquid nitrogen and stored until required.

Frozen cells were recovered from liquid nitrogen by rapidly thawing the ampoules in water at 37°C. The contents of the ampoule were diluted dropwise, over two minutes, in fresh medium and transferred to a culture flask. The residual DMSO and any unviable cells were removed on the following day by aspirating off the medium and replacing with fresh culture medium.

2.76 Check for contamination by mycoplasma

Cultures were tested monthly for contamination by mycoplasma using the fluorescent Hoechst 33258 staining technique of Chen (1977). Only mycoplasma-free cultures were used for further culture experiments.

2.77 Cell fixing and staining

The growth medium was removed from the cultures to be fixed and the cell surfaces washed twice with PBS-A in order to remove all traces of the growth medium and its serum. The cell cultures were fixed with methanol for 10 mins.at room temperature. The fixative was poured off and the cultures allowed to dry before staining.

1 volume of Giemsa stain was added to cover the culture surface. Five minutes later, the stain was diluted with 9 volumes of distilled water. After a further ten minutes, the stain was removed and the culture washed extensively with water until no more stain could be washed off. When the cultures had dried, they were examined on an Olympus IMT microscope.

2.78 Indirect immunofluorescence studies

In order to perform the immunofluorescence studies, it was necessary to grow the primary cultures on glass 13mm coverslips which did not autofluoresce, unlike the plastic Thermanox coverslips. Primary cultures were established on feeder layers and maintained on these coverslips in multiwell plates for up to four weeks. Prior to fixation, the medium was removed and the coverslips washed twice with PBS-A at room temperature. The cultures were fixed either in acetone or methanol for 10 mins. at $0^{o}C$. All subsequent procedures were performed at ambient temperatures.

After the fixative was removed, the coverslips were washed in PBS-A for 5 mins. before incubating with the primary antibody (at the appropriate dilution in PBS-A) for 30 mins.. The primary antibody was washed off and the coverslips given three washes in PBS-A (5 mins. each wash). The coverslips were incubated with conjugated secondary antibody diluted in PBS-A for 30 mins., generally at twice the concentration of the primary antibody. After the secondary antibody was removed, the coverslips were washed twice in PBS-A before counter-staining with Hoechst 33258 (0.05µg/ml in PBS-A) for 10 mins.

in the dark. The coverslips were washed twice with PBS-A before mounting onto a microscope slide (with the cells facing downwards into the Citifluor mountant). These slides were viewed on the fluorescence microscope and photographed on an Ektachrome 160, 400 or 800/1600 ASA film.

2.79 Methodology for autoradiography

Primary cultures were grown on plastic or glass coverslips for autoradiography. The growth medium was replaced with fresh medium containing (^{3}H)-methyl thymidine, 5μCi/ml and the cultures labelled for three hours. The medium was removed and the coverslips washed with two changes of PBS-A (0°C, 5 mins.) before fixation with methanol (0°C, 10 mins.). Coverslips were washed twice for 5 minutes with 5% trichloroacetic acid (w/v) and distilled water (0°C) respectively. Coverslips were rinsed in ethanol and air dried before mounting the coverslips (with cells uppermost) onto microscope slides with DPX mountant. After overnight drying, the slides were dipped into gelatin chrome alum solution (sect. 2.471) and dried before dipping into Ilford K2 nuclear emulsion (1:2 parts water (v/v), 45°C). The emulsion was drained and the slides were dried in a horizontal position with a fan (15-20 mins.) before storing in a light-proof box with dessicant at 4°C for 3-4 days.

After exposure, the light-proof boxes were brought to ambient temperature at which subsequent processing was performed. Slides were developed for 5 mins. in Kodak D19 developer and washed with distilled water for 2 mins. The slides were fixed in Kodafix (1:3 parts distilled water) and washed four times with tap water. Finally, the slides were stained with undiluted Giemsa for one minute before diluting 1: 9 parts water for 10 minutes and subsequently washing off the stain with tap water.

2.80 Experiments with conditioned medium

Conditioned medium was pooled from subconfluent flasks of a breast fibroblast strain BMAMF (between passages 6 to 9) 48 hours after feeding with fresh growth medium. This cell strain

was established from an ER negative breast tumour from a pre-menopausal woman. The conditioned medium was filter sterilised and stored at -20°C prior to use.

Pooled conditioned medium was diluted 50:50 with fresh culture medium and the supplements were added to give the appropriate final concentrations. For the control flasks, medium was incubated for 48 hours at 37°C in tissue culture flasks prior tomixing with fresh growth medium. The final concentration of ethanol was made up to 0.02% (v/v).

In these experiments with pooled conditioned medium, organoids were seeded onto feeder layers in either the diluted conditioned medium or control medium, with or without steroids. The colony numbers and areas were measured after the establishment of the primary cultures.

3. Growth of human mammary epithelial cells in primary culture.

3.1 Introduction

The hormone dependent nature of many breast cancers and the clinical significance of the oestrogen and progesterone receptor indices are well documented (Osborne et al., 1980; Leake et al., 1981 ab). There is however a significant proportion of patients whose response to antioestrogen therapy does not correlate with the receptor status. The precise role of oestrogens in human breast carcinogenesis is unclear. The direct and indirect mechanisms of oestrogen action on cellular proliferation have been previously described (sect. 1.3). Primary cultures of human mammary epithelial cells have, therefore, been established in order to study hormone dependence and growth regulation in vitro.

Growth of mammary epithelial cells in primary culture has proved difficult, with the major problem being the rapid overgrowth of the epithelial cells by stromal fibroblasts. The approaches adopted to overcome this problem have been described in Sect. 1.33. The studies described below have been based on a modification of the technique reported by Rheinwald & Green, (1975) who grew human epidermal keratinocytes on specific feeder layers.

3.11 Aims

The objectives of these studies were:

i) to develop a reproducible system for the establishment of primary cultures of human breast epithelial cells from tumour biopsies.

ii) to study the responses of these primary cultures to hormones and growth factors as assessed by the measurement of colony

ii) area/number and (^{3}H) thymidine incorporation.

iii) to investigate the possible existence of a paracrine-type growth regulatory mechanism mediated by stromal breast fibroblasts.

3.2 Results

Human breast tumours were disaggregated by enzymatic treatment with collagenase (see Sect. 2.72) which results in the breakdown of the extracellular matrix and release of the epithelial component and the stromal cell types. The disaggregated cell suspension (see Fig. 3.1) was found to comprise both single cells and clumps of cells (organoids) which represent epithelial alveolar and ductal aggregates. This cell suspension was plated out onto either plastic tissue culture substrates or onto feeder layers in order to establish primary cultures of human mammary epithelial cells. Cells grew as distinct colonies which arose, not from single cells, but from the organoids (see Fig. 3.2) The latter became attached to the substrate within 12-18 hours of initial plating.

3.21 Growth on plastic substrates.

When the disaggregated breast tumour cell suspension was plated onto standard tissue culture substrates, it was found that the epithelial cells were rapidly overgrown by stromal fibroblasts. Even when stromal cells were selectively allowed to attach onto one culture flask and the epithelial organoids transferred to a fresh flask after two hours, fibroblastic overgrowth was the end-result. It was, however, possible to grow some epithelial-like cultures for 3-4 days as colonies (Fig. 3.3a) which degenerated and progressed into fibroblastic cultures within a further week (Fig. 3.3 b & c). Two strains of breast stromal cells, BABF and BMAMF were established and could be cultured indefinitely with standard growth medium. These breast fibroblast strains, which grew rapidly in culture, were not contact-inhibited and ultimately resulted in the piling up of these cells. The stromal cell strain BMAMF was later used for the experiments to investigate

Fig. 3.1 Cell suspension of disaggregated human breast tumour.

Breast tumour biopsies were dissociated, filtered through a 0.2 mm nylon mesh, centrifuged and resuspended in the standard culture medium (as described in Sect. 2.72). The dissociated cell suspension contained both single cells and clumps of epithelial cells(organoids).

Phase contrast (Mag.x 120).

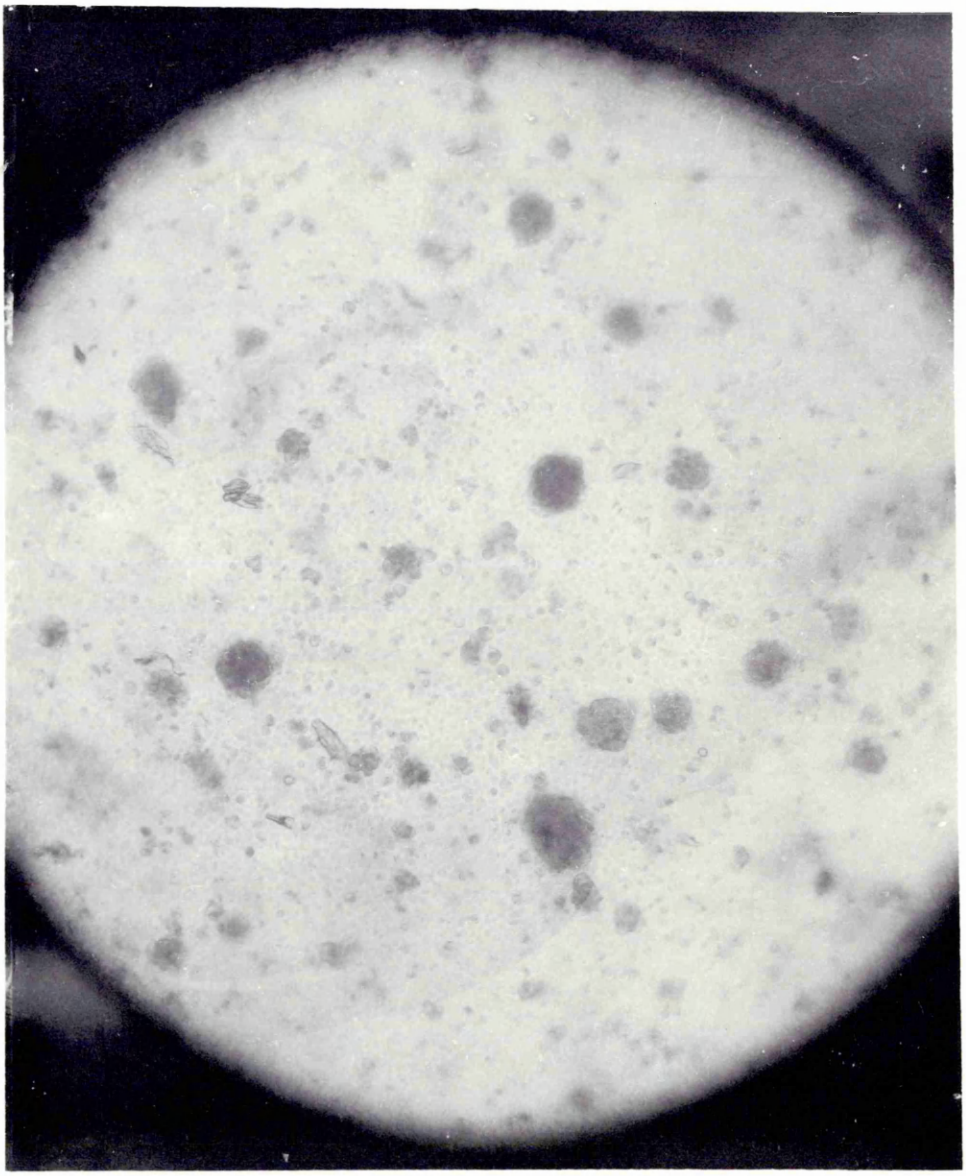


Fig. 3.2 Growth pattern of primary human breast culture on the NIH - 3T3 feeder layer.

Primary cultures were established as in Sect. 2.72 and the growth of these cultures was followed over the first ten days in culture.

A Day 1: organoid has attached to the feeder layer.

Phase contrast (Mag. x100).

B. Day 4: organoid has spread out onto the feeder layer and started to grow.

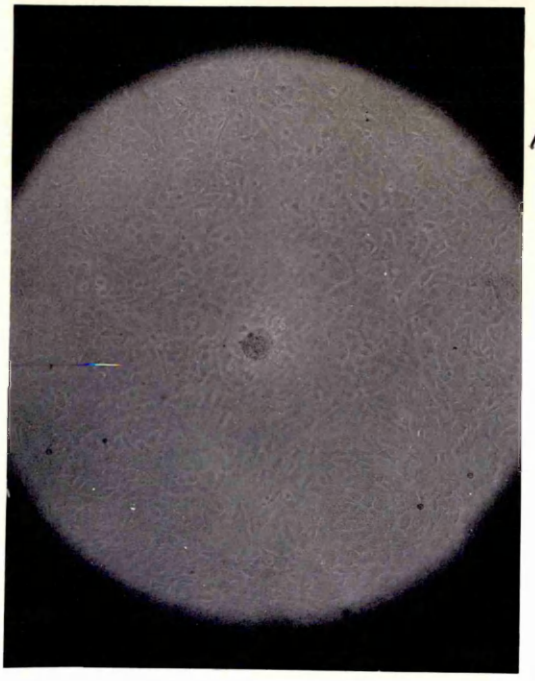
Phase contrast (Mag. x100).

C. Day 7: growth of breast organoid is well established.

Phase contrast (Mag. x100).

D. Day 10: outgrowth of cells from organoid extends underneath the feeder layer which is pushed aside.

Phase contrast (Mag. x100).



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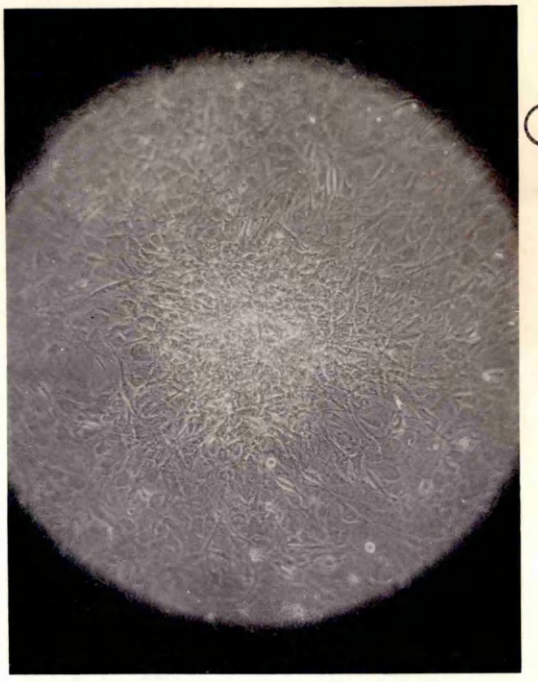

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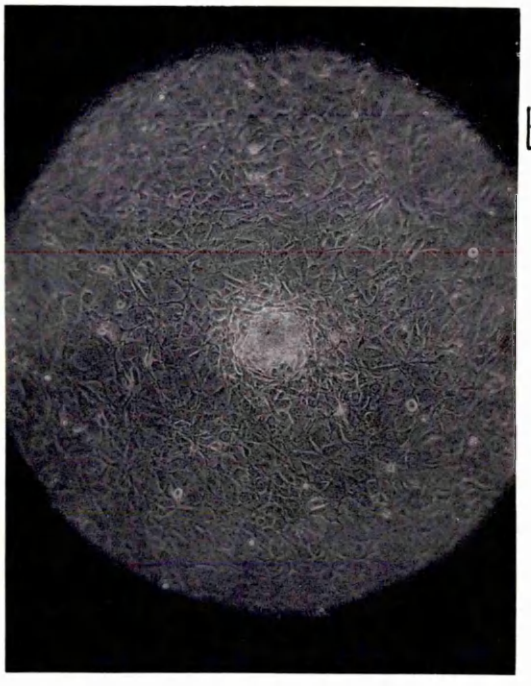

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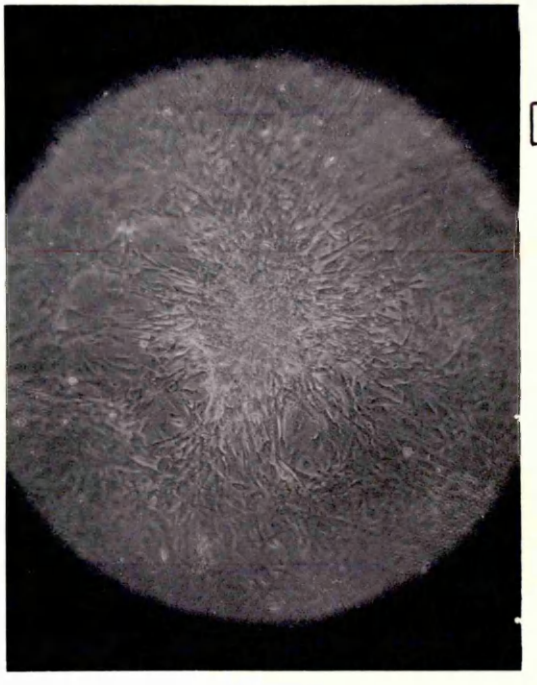


D

Fig. 3.3 Growth of a primary breast culture on a plastic substrate.

A human breast tumour biopsy was dissociated into a cell suspension (see Fig.3.1) and plated onto a conventional plastic tissue culture substrate. Cultures were stained with Giemsa (Sect. 2.77).

A. Epithelial colony from breast tumour, BED, after two days in culture.

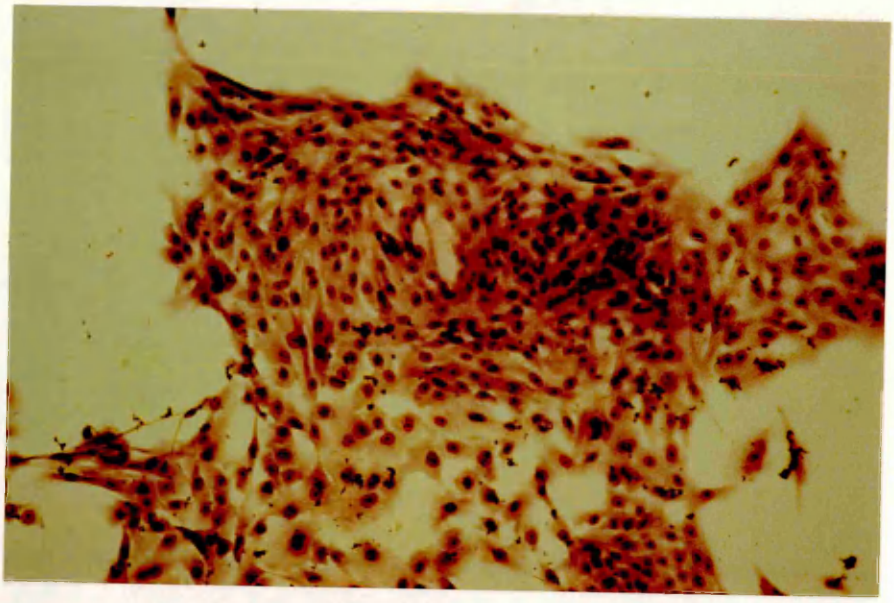
Mag. x450.

B. Stromal fibroblasts have rapidly taken over the epithelial culture after five days in culture.

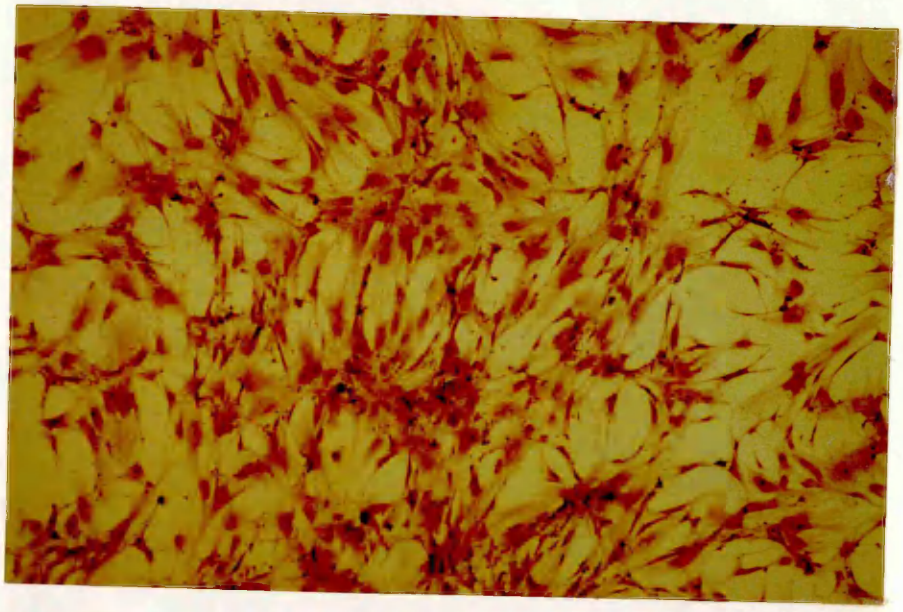
Mag. x180.

C. The primary culture is almost purely stromal fibroblasts after nine days in culture.

Mag. x180.



A



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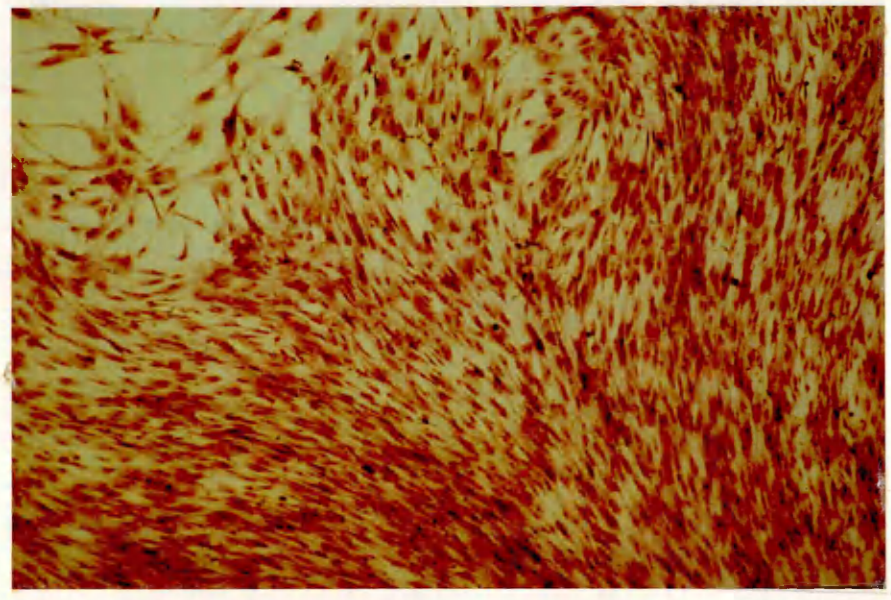


C

the possible existence of a paracrine mechanism for mammary epithelial cell growth (see Sect. 2.80).

3.22 Growth of primary cultures on feeder layers

Selective growth of human mammary epithelial cells was attempted on a variety of feeder layers. Previous studies from my laboratory have employed an undifferentiated epithelial FHI-4 feeder layer derived from embryonic human intestine by collagenase treatment (Freshney *et al.*, 1984). Unfortunately, stocks of this cell line were no longer available and therefore alternative feeder layers were tested for their ability to support mammary epithelial cell growth.

Epithelial feeder layers of mitomycin-C treated canine kidney MDCK cells and human embryonic lung cells EILU were unable to support the growth of breast epithelial cells. A bovine endothelial cell line CPAE was also unable to sustain mammary epithelial cell growth. Various fibroblast cell lines including NRK normal rat, NIH-, Swiss-3T3 and STO mouse embryo fibroblasts were also tested as feeder layers. The most successful of these feeder layers were the mitomycin-C treated mouse embryo fibroblast feeder layers, although the different cell lines supported mammary epithelial cell growth to varying extents (see Table 3.1). Growth and maintenance of primary breast cultures has been achieved most rountinely using STO (41/52 successful cultures) and NIH-3T3 (26/35 successful cultures) feeder layers. The 3T3 cell line from NIH mice proved three times more successful than that from Swiss mice (7/30 successful cultures) in supporting the growth of primary breast cultures.

3.221 Mode of growth on feeder layers.

After the initial attachment and spreading of breast organoids onto the feeder layers, their growth was followed for several weeks. The initial pattern of growth of a breast colony is shown in Fig. 3.2. These colonies were characteristically ring shaped (Fig. 3.4a). The organoids have been observed to push aside the feeder layer after initial attachment, with the subsequent

Table 3.1 Growth of primary breast epithelial cultures on different feeder layers.

Primary human mammary epithelial cells were established as in Sect. 2.72 on Mitomycin-C treated mouse embryo fibroblast feeder layers (Sect. 2.73). Success rates for the growth of these primary cultures were determined.

Feeder layer	Total number of cultures	Successful cultures	Percentage of successful cultures
NIH-3T3	35	26	74
Swiss-3T3	30	7	23
STO	52	41	79

Fig. 3.4 Growth of a primary breast culture on the NIH-3T3 feeder layer.

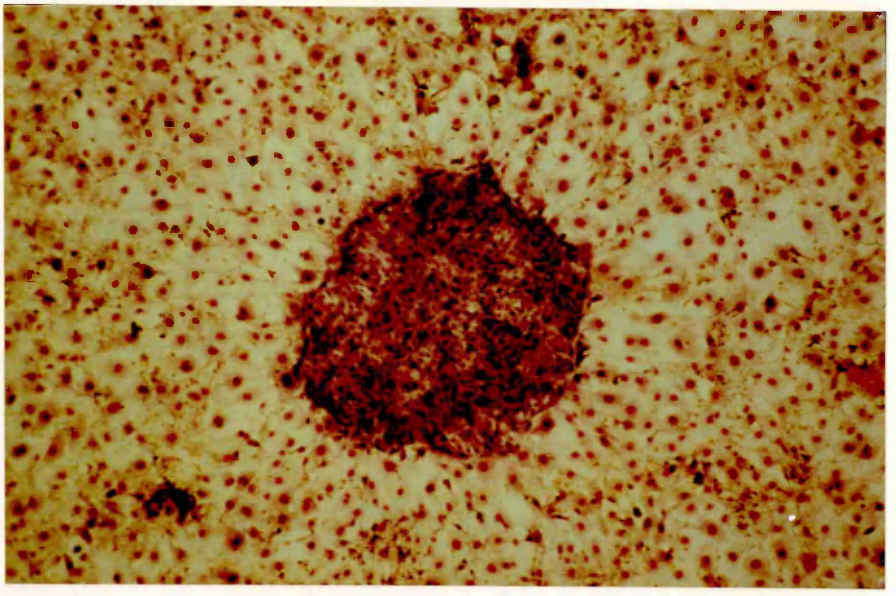
Primary cultures were established as in Sect. 2.72, fixed and stained after four weeks as described in Sect. 2.77.

A. The characteristic ring-shaped colony is surrounded by the feeder cells.

Mag. x180

B. High magnification view of the characteristic pavement-like epithelial cells of the primary breast colony.

Mag. x450.



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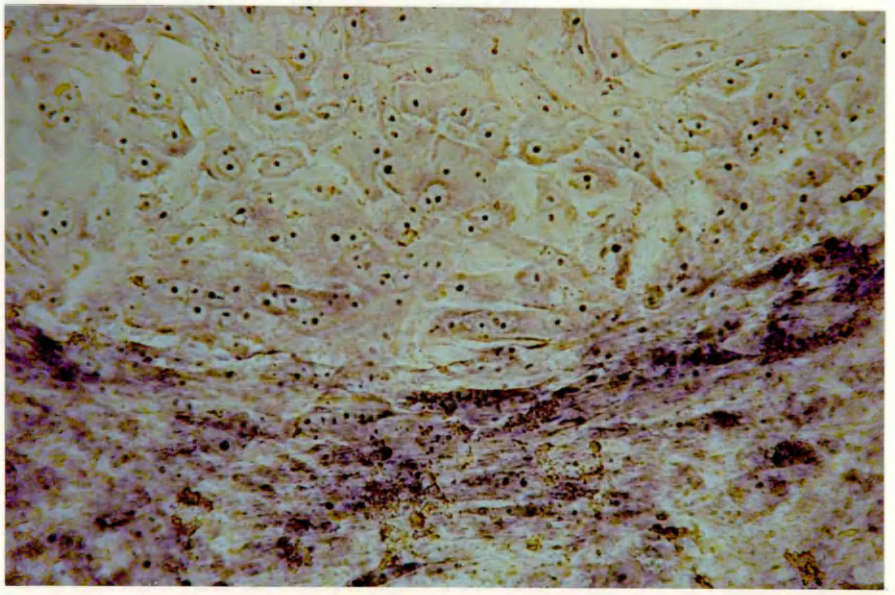


B

outgrowth of cells extending underneath the feeder layer. This has been confirmed by the immunofluorescence studies (Fig. 4.2b) and scanning electron microscopy studies (Fig. 5.3b).

The primary breast colonies contained two morphologically distinct cell types. Flatter cobblestone-like epithelial cells were present in the central region, while the somewhat elongated cells were located towards the periphery of these colonies (Fig. 3.4b). These elongated cells may represent the myoepithelial cells which have not been observed in culture for more than ten days (B. Gusterson, pers. comm.) The immunofluorescence studies with the LE61 antiserum (see Sect. 4.33) however, identified all the cells within the colonies as epithelial cells and not as basal myoepithelial cells.

3.23 Growth regulation of primary cultures by hormones and growth factors.

Growth regulation experiments were conducted using the standard culture medium supplemented with full foetal calf serum. The levels of steroid hormones in the foetal calf serum used for cell culture have been measured both prior and subsequent to heat inactivation and charcoal stripping (see Sect. 2.22). HIDCCFCS contained depleted levels of steroid hormones. Only oestradiol was detectable. It was present at 77pM in comparison to 185pM in full foetal calf serum. The concentration of endogenous oestradiol in the standard culture medium (containing 10% (v/v) foetal calf serum) was 18.5 pM with the full serum or 7.7 pM if HIDCCFCS was used. Since the level of oestradiol employed for the growth stimulation experiments was 1nM, it was decided to use the full serum for supplementation of the growth medium. Additionally, heat inactivation and charcoal stripping have been reported to remove proteins and attachment factors which play an important role in the growth of primary cultures (Munir, pers. comm).

These growth experiments were performed by establishing primary cultures on feeder layers in 25cm^2 tissue culture flasks (see Fig. 3.5). Cultures were grown for four weeks with feeding on alternate days. Growth was assessed by determination of both the colony area and number. There was no evident difference between the cell density of small or large breast colonies in either the control or hormone stimulated cultures. It was, therefore, assumed that an increase in the colony area was paralleled by a corresponding increase in cell number. The hormones and growth factors did not appear to mediate their effects directly through the mitomycin-C treated feeder layers since conditioned medium from feeder cells did not prolong or stimulate the growth of primary cultures on conventional plastic tissue culture substrates.

Due to the small size of the breast biopsies, it was not possible to construct dose response curves for the hormones and growth factors studied. These were therefore used either at physiological concentrations or at levels previously described for breast cell culture. The results of the growth stimulation experiments on the mouse embryo fibroblast feeder layers are detailed in the following section.

3.231 Growth stimulation on the NIH-3T3 feeder layer

Table 3.2 provides a detailed breakdown of the hormonal stimulation experiments performed with the primary breast cultures using cortisol (10^{-7}M) and oestradiol (10^{-9}M) either alone or in combination. From the data it is evident that the standard deviations are very large. The magnitude of the standard deviations may be attributed to variations, both in the size and heterogeneity of the parent tumours and the number of breast cells and organoids seeded onto the feeder layers.

Taking into account the variations described for colonies grown under control conditions, the mean total colony area,

Fig. 3.5 The effect of steroid hormones on the growth of breast colonies

Breast colonies were established using the standard methodology (Sect. 2.72) and grown for four weeks on the NIH-3T3 feeder layer. The parent tumours shown as ER +/+ were found to contain more than 30 fmol/mg protein in the cytosol and 250 fmol/mg DNA in the nuclear fraction as determined by an exchange assay. Oestradiol (E) and cortisol (C) were added to give final concentrations of 10^{-9}M and 10^{-7}M respectively. Ethanol was added to the control cultures to give a final concentration of 0.02% (v/v).

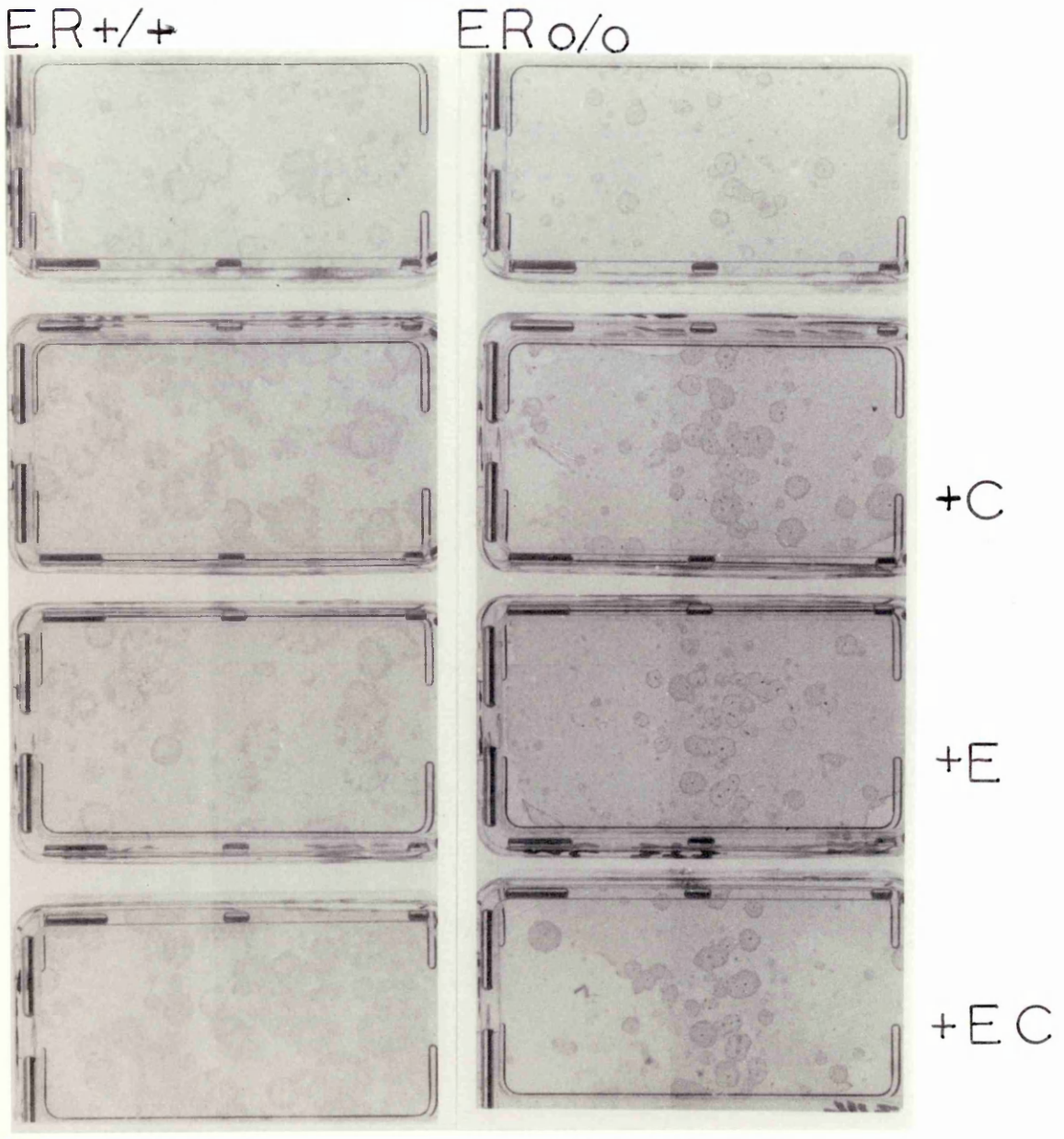

ER+/+
ER o/o
+C
+E
+EC

Table 3.2 The effect of oestradiol and cortisol on the growth of primary human mammary epithelial cultures on NIH-3T3 feeder layers.

Table A. Hormonal effects on the mean total colony area (MTCA) of breast colonies grown on $25cm^2$ flasks.

Table B. Hormonal effects on plating efficiency as assessed by the mean total colony number (MTCN) of breast colonies grown on $25cm^2$ flasks.

Patients were divided according to menopausal status (pre- and post-menopausal). Oestrogen receptor positive (ER +/+) tumours contained more than 30 fmol/mg protein in the cytosol and 250 fmol/mg DNA in the nuclear fractions. Cultures were established as described in Sect. 2.72 and maintained for four weeks with oestradiol (10^{-9}M), cortisol (10^{-7}M) or 0.02% (v/v) ethanol for the control cultures.

Absolute values for the mean total colony area (MTCA) and the mean total colony number (MTCN) ± standard deviations are presented. Percentage changes are expressed (in brackets) with respect to the control flasks. Growth of the cultures was compared by the student's 't' test(NS=no significant difference, i.e. $p > 0.1$; ND= not determined). The magnitude of the standard deviations was very large as a result of the differences in size and cellularity of the parent tumours, as well as the number of cells seeded.

A

Status	Number of experiments	Mean Total Colony Area (cm^2)			
		Control	Cortisol	Oestradiol	Cortisol & Oestradiol
Postmenopausal ER +/+	6	2.31 ± 3.35	3.38 ± 4.52 p<0.1 (+46.1%)	2.56 ± 3.53 p<0.1 (+10.8%)	3.48 ± 3.57 p<0.001 (+50.7%)
Postmenopausal ER o/o	10	4.22 ± 3.69	4.34 ± 5.74 NS (+2.9%)	4.40 ± 5.34 NS (+4.3%)	5.40 ± 5.47 NS (+28.0%)
Premenopausal ER +/+	4	5.35 ± 1.04	10.18 ± 1.48 p<0.001 (+90.3%)	7.41 ± 1.22 p<0.001 (+38.5%)	9.54 ± 1.46 p<0.001 (+78.3%)
Premenopausal ER o/o	4	3.19 ± 0.63	3.74 ± 0.51 NS (+17.1%)	2.97 ± 0.35 NS (-7.0%)	4.62 ± 0.81 NS (+44.6%)
Postmenopausal ER +/o	1	7.87	3.24 ND (-58.8%)	4.34 ND (-44.9%)	4.78 ND (-39.3%)

B

Status	Number of experiments	Mean Total Colony Number			
		Control	Cortisol	Oestradiol	Cortisol & oestradiol
Postmenopausal ER +/+	6	24.0 ± 26.5	30.0 ± 27.1 p<0·02 (+25.0%)	22.7 ± 21.1 NS (+-5.4%)	37.3 ± 26.0 NS (+55.4%)
Postmenopausal ER o/o	10	37.1 ± 30.9	38.8 ± 27.3 NS (+4.6%)	35.6 ± 27.8 NS (-4.0%)	39.8 ± 31.0 NS (+7.2%)
Premenopausal ER +/+	4	48.0 ± 8.1	52.0 ± 12.7 NS (+8.3%)	50.0 ± 10.4 NS (+4.2%)	70.0 ± 15.0 p<0.01 (+45.8%)
Premenopausal ER o/o	4	92.5 ± 4.0	90.0 ± 5.7 NS (-2.7%)	70.0 ± 4.9 p<0.01 (-24.3%)	71.5 ± 2.9 p<0.001 (-24.3%)
Postmenopausal ER +/o	1	65	58 ND (-10.8%)	72 ND (+10.8%)	58 ND (-10.8%)

MTCA, for postmenopausal ER o/o derived cultures was 1.8x that of the postmenopausal ER +/+ cultures. In contrast, the premenopausal ER +/+ derived cultures had a mean total colony area 1.7x that of the premenopausal ER o/o cultures under same growth conditions.

Cortisol (10^{-7}M) alone generally elicited a marked mitogenic response in terms of both colony area and colony number in premenopausal and postmenopausal ER +/+ derived primary cultures, while there was a smaller response from the ER o/o derived cultures. In terms of the mean total colony number, MTCN, there was a significant 25% increase in the postmenopausal and a lesser 8.3% increase in the premenopausal ER +/+ groups, respectively. Cortisol had little effect on the colony number of either postmenopausal and premenopausal ER o/o derived cultures. In the cultures derived from the postmenopausal ER +/o sample, there was a 58% decrease in colony area but an 11% increase in the total colony number in response to cortisol.

In response to oestradiol, both postmenopausal and premenopausal ER +/+ derived cultures showed statistically significant increases in MTCA of 11 and 39% respectively. Oestradiol resulted in a slight increase (non-significant) in the MTCA of postmenopausal ER o/o derived cultures and a slight decrease for premenopausal ER o/o biopsies. A significant reduction in MTCN was observed in premenopausal o/o cultures but this decrease was of a smaller magnitude for postmenopausal ER o/o derived cultures. The postmenopausal +/o derived cultures showed a 45% decrease in MTCA and an 11% increase in MTCN.

With the exception of the one postmenopausal +/o biopsy established in primary culture, there was a 28-78% increase in the MTCA in response to cortisol and oestradiol in combination regardless of the menopausal and receptor status. Postmenopausal and premenopausal +/+ derived cultures exhibited significant MTCA increases of 51 and 78% respectively in response to the combination of steroids. A similar increase

in MTCN was observed for postmenopausal +/+ derived cultures but there was only a 46% increase in the premenopausal +/+ MTCN relative to the 78% increase in MTCA.

The premenopausal ER o/o derived cultures showed a 45% increase in MTCA with a 24% decrease in MTCN in response to the combination of cortisol and oestradiol. The postmenopausal ER o/o derived cultures, however, responded to the combination of cortisol and oestradiol by a 28% increase in MTCA but only a 7% increase in MTCN.

The results of stimulation by oestradiol and cortisol in combination, have been further analysed with regard to menopausal status or receptor status alone (Table 3.3). There was an increase of 25% in the MTCA of postmenopausal derived cultures but a more significant 66% increase in the MTCA of premenopausal derived cultures. There were also large increases in the MTCA of 67 and 32% for ER +/+ and o/o cultures respectively, although these were not statistically significant. With regard to colony number, there was a 17% increase over control values for postmenopausal derived cultures while there was no change for premenopausal derived cultures. For ER +/+ derived cultures, a significant 50% increase in MTCN was observed. In contrast, there was an 8% reduction in the MTCN of ER o/o derived cultures. The one postmenopausal +/o derived culture was found to have a 39% reduction in MTCA in response to the oestradiol and cortisol combination and an 11% reduction in MTCN.

3.232 Growth stimulation on the STO feeder layer

Table 3.4a shows that the MTCA of control premenopausal derived cultures is significantly greater (2.44X) than the control postmenopausal cultures when grown on the STO feeder layer. Similarly, the control ER +/+ derived cultures have a significantly greater (2.67X) MTCA than the control ER o/o derived cultures. Stimulation of both pre- and post-menopausal derived cultures by the cortisol and oestradiol combination was found to increase the colony area by 36-37%. The effect of the cortisol and oestradiol combination on MTCN of pre- and post-menopausal cultures

Table 3.3 The effect of menopausal and receptor status on the growth of primary breast cultures on the NIH-3T3 feeder layers.

Table A. The effect of the cortisol (10^{-7}M)/oestradiol (10^{-9}M) combination on the growth of primary cultures with regard to the mean total colony area (MTCA).

Table B. The effect of the cortisol (10^{-7}M)/oestradiol (10^{-9}M) combination on the growth of primary cultures in terms of the mean total colony number (MTCN).

Patients were divided according to menopausal status (pre- and post-menopausal). Oestrogen receptor positive (ER +/+) tumours contained greater than 30 fmol/mg protein in the cytosol and 250 fmol/mg DNA in the nuclear fractions.

Absolute values for the mean total colony area (MTCA) and the mean total colony number (MTCN) are presented for the control cultures ($\pm$ standard deviations) of the experiments. Percentage changes are expressed with respect to the control flasks. Growth of the cultures was compared by the student's 't' test (NS= not significant, i.e. $p > 0.1$; ND=not determined).

A

Status	Number of experiments	Mean Total Colony Area (cm^2)		
		Control	Cortisol and oestradiol	Percentage change
Postmenopausal	17	3.76 ± 3.62	4.68 ± 4.66 NS	+24.6
Premenopausal	8	4.27 ± 1.34	7.08 ± 2.79 ($p<0.05$)	+65.7
ER +/+	10	3.52 ± 2.99	5.90 ± 4.16 NS	+67.4
ER o/o	14	3.93 ± 3.12	5.18 ± 4.58 NS	+31.9
ER +/o	1	7.86	4.78 ND	-39.3

B

Status	Number of experiments	Mean Total Colony Number		
		Control	Cortisol and oestradiol	Percentage change
Postmenopausal	17	34.1 ± 29.3	40.0 ± 23.9 NS	+17.3
Premenopausal	8	70.3 ± 24.5	70.8 ± 10.0 NS	+0.7
ER +/+	10	33.6 ± 23.8	50.4 ± 19.5 ($p<0.001$)	+50.0
ER o/o	14	52.9 ± 36.6	48.9 ± 29.8 NS	-7.6
ER +/o	1	65	58 ND	-10.8

Table 3.4 The effect of menopausal and receptor status on the growth of primary breast cultures on the STO feeder layers.

Table A. The effect of the cortisol (10^{-7}M)/oestradiol (10^{-9}M) combination on the growth of primary cultures with regard to the mean total colony area (MTCA).

Table B. The effect of the cortisol (10^{-7}M)/oestradiol (10^{-9}M) combination on the growth of primary cultures in terms of the mean total colony number (MTCN).

Patients were divided according to menopausal status (pre- and post-menopausal). Oestrogen receptor positive (ER +/+) tumours contained more than 30 fmol/mg protein in the cytosol and 250 fmol/mg DNA in the nuclear fractions.

Absolute values for the mean total colony area (MTCA) and the mean total colony number (MTCN) are presented for the control cultures ($\pm$ standard deviations) of the experiments. Percentage changes are expressed with respect to the control flasks. Growth of the cultures was compared by the student's 't' test (NS= not significant, i.e. $p > 0.1$; ND= not determined).

A

Status	Number of experiments	Mean Total Colony Area (cm^2)		
		Control	Cortisol and oestradiol	Percentage change
Postmenopausal	15	1.36 ± 1.11	1.85 ± 1.73 NS	+35.9
Premenopausal	11	3.33 ± 3.19	4.57 ± 3.76 NS	+37.3
ER +/+	10	3.48 ± 3.15	4.25 ± 5.39 NS	+22.3
ER o/o	14	1.26 ± 1.42	1.68 ± 1.81 NS	+34.3
ER +/o, o/+	2	2.26 ± 0.93	6.02 ± 5.08 ND	+166.2

B

Status	Number of experiments	Mean Total Colony Number		
		Control	Cortisol and oestradiol	Percentage change
Postmenopausal	15	54.4 ± 54.1	76.7 ± 94.1 NS	+41.0
Premenopausal	11	93.5 ± 69.7	79.6 ± 70.5 NS	-14.9
ER +/+	10	90.4 ± 50.8	111.4 ± 69.3 NS	+23.2
ER o/o	14	56.6 ± 74.6	60.3 ± 92.3 NS	+6.5
ER +/o, o/+	2	70.5 ± 13.4	34.0 ± 17.0 ND	-51.8

was variable in that there was a 41% increase for postmenopausal cultures and a 15% reduction for premenopausal cultures (see Table 3.4b).

The combination of cortisol and oestradiol increased the MTCA and MTCN of ER +/+ derived cultures by approximately 23%. The MTCA of the ER o/o derived cultures was found to increase by 34% while the MTCN rose by only 7%. The cultures derived from the abnormal receptor status group showed a 166% increase in the MTCA while there was a 52% decrease in MTCN.

The overall responses of primary breast cultures to cortisol and oestradiol, alone and in combination, as well as to EGF and EGF/cortisol are summarised in Table 3.5. Cortisol alone elicited a small increase in both the MTCA and MTCN. Oestradiol, however, resulted in a 10% reduction in MTCA which was statistically significant. This was accompanied by a slight, but not statistically significant, (3.5%) increase in the MTCN. The cortisol/oestradiol combination gave rise to a significant 37% increase in MTCA but a smaller 10% increase in MTCN.

EGF, alone, and together with cortisol resulted in a dramatic increase in MTCA and a smaller effect on MTCN. EGF elicited a 146% increase in MTCA and a smaller (25%) increase in MTCN. In the presence of the cortisol/EGF combination, there is an even greater (265%) increase in MTCA and a 31% increase in MTCN.

The detailed analysis of the effects of cortisol and oestradiol on primary breast cultures grown on mitomycin-C treated STO feeder layers is shown in Table 3.6a and 3.6b. The MTCA of the premenopausal ER +/+ colonies was found to be 3.3X that of the postmenopausal ER +/+ derived colonies. Similarly, the MTCA of premenopausal ER o/o derived cultures was 1.7X that of those cultures derived from postmenopausal o/o biopsies.

Cortisol alone resulted in an 8-10% decrease in MTCA of the ER +/+ derived cultures. A contrasting effect of cortisol was observed on MTCN of pre- and postmenopausal derived cultures.

Table 3.5 The effect of hormones and growth factors on the growth of primary mammary epithelial cultures on STO feeder layers.

Primary cultures were established as in Sect. 2.72 and grown for four weeks with cortisol (10^{-7}M), oestradiol (10^{-9}M), EGF (10ng/ml) or 0.02% (v/v) ethanol in the control cultures.

Absolute values are presented for the mean total colony area (MTCA) and the mean total colony number (MTCN) of the control cultures ($\pm$ standard deviations) of the experiments. Percentage changes are expressed with respect to the control flasks and compared by the 't' test (NS= no significant difference, i.e. $p > 0.1$).

	CONTROL	CORTISOL	OESTRADIOL	CORTISOL & OESTRADIOL	EGF	CORTISOL & EGF
Number of experiments	26	26	26	26	10	4
Mean Total Colony Area	2.19 ± 2.41	2.29 ± 2.09 NS (+4.9%)	1.97 ± 1.94 p<0.05 (-9.9%)	3.00 ± 3.03 p<0.02 (+37.3%)	5.39 ± 1.83 p<0.002 (+146.5%)	7.99 ± 4.55 p<0.1 (+265.7%)
Mean Total Colony Number	70.7 ± 64.0	75.3 ± 77.4 NS (+6.5%)	73.2 ± 69.1 NS (+3.5%)	77.9 ± 83.4 p<0.01 (+10.2%)	88.4 ± 46.7 p<0.1 (+25.0%)	93.0 ± 51.1 p<0.05 (+31.5%)

Table 3.6 The effect of oestradiol and cortisol on the growth of primary mammary epithelial cultures on the STO feeder layers.

Table A. Hormonal effects on the mean total colony area (MTCA) of breast colonies grown on $25cm^2$ flasks.

Table B. Hormonal effects on plating efficiency as assessed by the mean total colony number (MTCN) of breast colonies grown on $25cm^2$ flasks.

Patients were divided according to menopausal status (pre- and post-menopausal). Oestrogen receptor positive (ER +/+) tumours contained more than 30 fmol/mg protein in the cytosol and 250 fmol/mg DNA in the nuclear fractions. Cultures were established as described in Sect. 2.72 and maintained for four weeks, with oestradiol ($10^{-9}M$), cortisol ($10^{-7}M$) or 0.02% (v/v) ethanol for the control cultures.

Absolute values for the mean total colony area (MTCA) and the mean total colony number (MTCN) are presented for the control cultures ($\pm$ standard deviations) of the experiments. Percentage changes are expressed with respect to the control flasks. Growth of the cultures was compared by the student's 't' test (NS=no significant difference, i.e. $p > 0.1$; ND=not determined). The magnitude of the standard deviations was very large as a result of the differences in size and cellularity of the parent tumours, as well as the number of cells seeded.

A

Status	Number of experiments	Mean Total Colony Area (cm^2)			
		Control	Cortisol	Oestradiol	Cortisol & oestradiol
Postmenopausal ER +/+	6	1.80 ± 1.40	1.63 ± 1.20 NS (-9.8%)	1.65 ± 1.31 NS (-8.8%)	2.01 ± 1.38 p<0.1) (+11.4%)
Premenopausal ER +/+	4	5.99 ± 3.54	5.49 ± 2.35 NS (-8.3%)	4.75 ± 2.87 NS (-20.6%)	7.61 ± 2.48 p<0.1 (+27.2%
Postmenopausal ER o/o	8	0.98 ± 0.95	1.59 ± 1.45 p<0.05 (+63.4%)	1.25 ± 1.37 NS (+28.6%)	1.66 ± 2.13 NS (+70.7%)
Premenopausal ER o/o	6	1.63 ± 1.92	1.40 ± 1.12 NS (-14.3%)	1.31 ± 1.14 NS (-19.7%)	1.70 ± 1.47 NS (+4.6%)
Postmenopausal ER +/o o/+	1	1.60	1.16 ND (-27.4%)	1.05 ND (-34.3%)	2.42 ND (+51.2%)
Premenopausal ER o/+ +/o	1	2.92	5.64 ND (+93.0%)	3.42 ND (+17.2%)	9.61 ND (+229%)

B

Status	Number of experiments	Mean Total Colony Number			
		Control	Cortisol	Oestradiol	Cortisol & oestradiol
Postmenopausal ER +/+	6	60.0 ± 39.4	76.0 ± 61.4 NS (+26.7%)	64.0 ± 40.8 p<0.02 (+6.7%)	81.7 ± 61.9 p<0.1 (+36.2%)
Premenopausal ER +/+	4	136.0 ± 23.1	116.0 ± 18.5 NS (-14.7%)	154.0 ± 48.5 NS (+13.2%)	156.0 ± 60.0 NS (+14.7%)
Postmenopausal ER o/o	8	48.5 ± 71.3	78.3 ± 123.3 NS (+61.4%)	68.5 ± 97.5 p<0.1 (+41.2%)	76.8 ± 121.8 NS (+58.4%)
Premenopausal ER o/o	6	67.3 ± 84.3	57.0 ± 47.6 NS (-15.3%)	38.3 ± 25.1 NS (-43.1%)	38.3 ± 19.9 NS (-43.1%)
Postmenopausal ER +/o o/+	1	61	47 ND (-27.4%)	54 ND (-34.3%)	46 ND (-24.6%)
Premenopausal ER +/o o/+	1	80	24 ND (-70.0%)	72 ND (-10.0%)	22 ND (-72.5%)

Postmenopausal derived cultures showed a 27% increase in MTCN while the premenopausal derived cultures showed a 15% decrease. Postmenopausal ER o/o derived cultures showed a significant (63%) increase in MTCA and a similar increase in MTCN. Premenopausal o/o derived cultures, however responded with a 14% reduction in MTCA and a similar decrease in MTCN. The postmenopausal abnormal receptor status derived culture showed a comparitive 23-27% decrease in both MTCA and MTCN in the presence of cortisol. However, the premenopausal abnormal receptor status sample showed a different result with a 93% increase in MTCA and a 70% reduction in MTCN.

Oestradiol was found to reduce the MTCA of ER +/+ post- and premenopausal derived cultures by 9 and 21% respectively. However, the MTCN was increased by 7 and 13% for post- and premenopausal ER +/+ derived cultures respectively. The ER o/o derived cultures varied in that the MTCA for postmenopausal derived cultures rose by 29% while there was a decrease of 20% for the premenopausal category. For both groups of ER o/o derived cultures there was a 41-43% decrease in the MTCN after treatment with oestradiol alone.

The combination of cortisol/oestradiol resulted in a 5-229% increase in the MTCA of the primary cultures. Post- and premenopausal ER +/+ derived cultures showed 11 and 27% increases in MTCA respectively. Both groups of ER +/+ derived cultures also exhibited a rise in the MTCN although the positions were reversed with the postmenopausal ER +/+ derived cultures showing a 36% increase while the premenopausal ER +/+ cultures showed a 15% increase in the MTCN.

The ER o/o derived cultures showed a greater variation in the MTCA increases in response to cortisol/oestradiol. The MTCA of the postmenopausal ER o/o groups rose by 71% , while a slightly smaller increase (58%) occurred in the MTCN. The premenopausal ER o/o group, however, only showed a rise of 4.6% in MTCA and a simultaneous 43% decrease in MTCN.

The cultures established from the postmenopausal abnormal receptor status biopsy were found to respond to cortisol and oestradiol individually by reduced total colony areas of 27 and 34% respectively. The combination of cortisol/oestradiol, however, increased the total colony area by 51%. The total colony number was reduced in the presence of cortisol, oestradiol or their combination by 23, 12 and 25% respectively.

Stimulation of the premenopausal abnormal receptor status derived cultures resulted in increases in total colony area of 93, 17 and 229% with cortisol, oestradiol and their combination respectively. The addition of these steroids also resulted in a reduction in the total colony number of 70, 10 and 73% respectively.

3.2321 Growth stimulation by prolactin

The effect of prolactin (5µg/ml) on the growth of primary breast cultures established on STO feeder layers was assessed in the presence and absence of cortisol and oestradiol. Table 3.7 shows that prolactin resulted in a significant (88%) increase in the MTCA and a 19% reduction in the MTCN over control values. Cortisol in combination with prolactin only resulted in a 24% increase in MTCA over control values. This represents a 65% reduction over the prolactin value without any effect on the MTCN.

Oestradiol in combination with prolactin elicited a 61% increase in the MTCA over control values. The reduction in MTCA by oestradiol was smaller than that induced by cortisol. With regard to the MTCN, there did not appear to be any additional effect over and above that produced by prolactin alone.

The combination of oestradiol, cortisol and prolactin resulted only in a 6% increase in the MTCA. This combination of hormones caused a reduction in MTCA (compared to prolactin alone) and was less than the additive reductions elicited by cortisol or oestradiol on MTCA. The combination of the three hormones elicited a 30% reduction in MTCN in comparison to the 21% decrease with prolactin and one steroid hormone and the 19% reduction by prolactin alone.

Table 3.7 The effect of prolactin on the growth of primary breast epithelial cultures on STO feeder layers.

Absolute values are presented for the mean total colony area (MTCA) and mean total colony number (MTCN) and standard deviations. Percentage changes are expressed (in brackets) with respect to control values and have been compared by the paired 't' test (NS= not significant $p > 0.1$).

Primary cultures were prepared as in Sect. 2.72 and grown for four weeks. Hormones were added to the experimental flasks as follows, cortisol (10^{-7}M), oestradiol (10^{-9}M) and prolactin (5µg/ml).

Supplement	Number of experiments	Mean Total Colony Area	Mean Total Colony Number
Control	6	0.39 ± 0.28	86.7 ± 85.4
Prolactin	6	0.74 ± 0.52 p<0.02 (+88.3%)	70.7 ± 72.8 p<0.1 (-18.5%)
Cortisol and Prolactin	6	0.49 ± 0.41 NS (+23.7%)	68.3 ± 73.9 p<0.05 (-21.2%)
Oestradiol and Prolactin	6	0.63 ± 0.44 NS (+60.5%)	68.0 ± 73.2 p<0.05 (-21.6%)
Oestradiol, Cortisol and Prolactin	6	0.42 ± 0.26 NS (+6.4%)	60.7 ± 66.9 NS (-30.0%)

3.24 Does a paracrine mechanism operate for mammary epithelial cell growth?

Experiments with conditioned medium from breast stromal fibroblasts (see Sect. 2.80) were performed in order to ascertain whether stromal fibroblasts are involved in a paracrine-type mechanism with regard to growth regulation of mammary epithelium. Table 3.8 shows the effects of conditioned medium, from stromal fibroblasts grown in the presence (CME) or absence (CM-) of oestradiol (10^{-9}M), on the growth of primary breast cultures.

Conditioned medium from breast stromal fibroblasts, (not treated with oestradiol), was found to reduce the MTCA by 7% and MTCN by 13%. Addition of exogenous oestradiol to the conditioned medium was only found to have a slight effect on the growth of the primary breast cultures over and above that elicited by CM- alone. The combination of CM- and oestradiol (CM-/E) resulted in a 12% reduction in the MTCA which represents an additional 4% decrease over CM- alone. In terms of MTCN, the reduction by CM-/E was only about half that elicited by CM- alone.

Conditioned medium from breast stromal fibroblasts grown in the presence of oestradiol (CME) was found to increase the MTCA by 18% and the MTCN by a similar amount. The addition of cortisol to CME in two experiments was found to decrease the MTCA by 36% while simultaneously reducing the MTCN by 17%. In the presence of CME, cortisol reduced the MTCA and MTCN by 36 and 17% respectively, relative to the control values.

3.25 Autoradiography of primary cultures.

An attempt was made to assess the effect of hormonal stimulation on the (^{3}H) thymidine labelling patterns of the primary breast colonies. Fig. 3.6a shows a typical primary culture derived from a large organoid which has stained intensely with Giemsa. Within the labelled colonies, there were areas of labelled and unlabelled cells, giving rise to a heterogeneous labelling pattern. These labelled cells exhibited a secondary

Table 3.8 The effect of breast stromal fibroblast conditioned medium on the growth of primary breast epithelial cultures on STO feeder layers.

Absolute values are presented for the mean total colony area (MTCA) and mean total colony number (MTCN) and standard deviations. Conditioned medium was obtained from human breast stromal fibroblasts (BMAMF) grown in the presence (CME) or absence (CM-) of exogenous 10^{-9}M oestradiol (see Sect. 2.80). Primary cultures were established as in Sect. 2.72 using 50% conditioned medium (v/v). Cortisol (C) and oestradiol (E) were added to give final concentrations of 10^{-7}M and 10^{-9}M respectively. In the absence of exogenous steroid hormone, ethanol was added (0.02% final concentration). Results were compared by the paired 't' test (NS = not significant, i.e. $p > 0.1$; ND = not determined).

The values marked with an asterisk (*) are expressed with respect to the control values for these particular experiments only.

Supplement	Number of experiments	Mean Total Colony Area	Mean Total Colony Number
Control	8	1.34 ± 1.26	86.5 ± 84.0
CM-	7	1.24 ± 1.57 NS (-7.1%)	75.3 ± 79.2 NS (-12.9%)
CM-/E	8	1.18 ± 1.35 NS (-11.5%)	81.4 ± 85.7 NS (-5.9%)
CME	7	1.70 ± 1.89 NS (+18.1%)	100.1 ± 90.4 NS (+15.7%)
CME/C	2	2.13* ± 1.39 ND (+35.8%)	147.0* ± 18.4 ND (-17.4%)

Fig. 3.6 Labelling patterns in primary human mammary epithelial cultures grown on the NIH-3T3 feeder layer.

Primary cultures were prepared as described in Sect.2.72. After two weeks, cultures were labelled with (^{3}H) thymidine (5μCi/ml) for three hours as in Sect. 2.79, subject to autoradiography and then stained with Giemsa.

A. Heterogeneous labelling of epithelial colony.

Mag. x116

B. Focal areas of labelled cells.

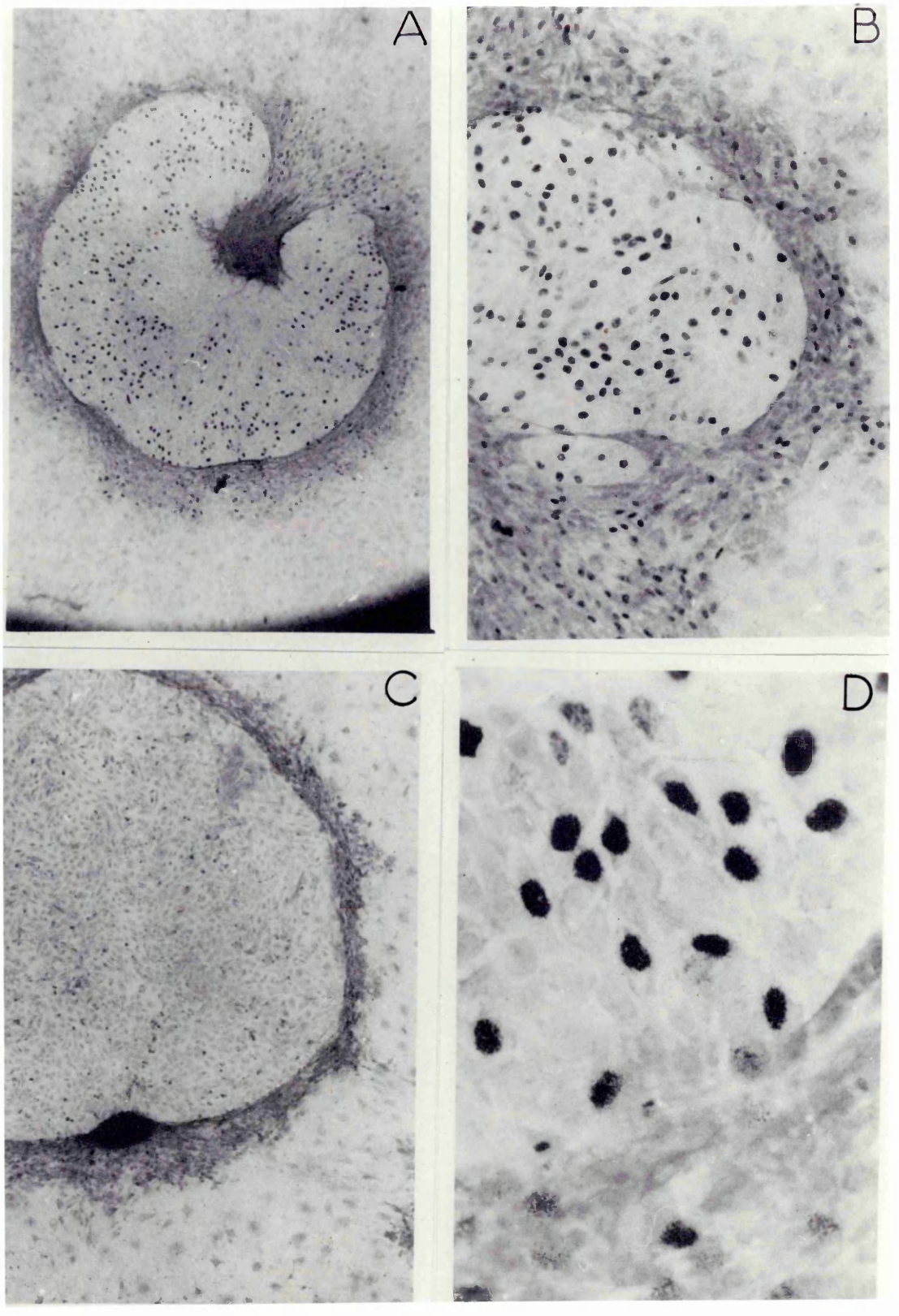
Mag. x288

C. Epithelial colony with no labelled cells.

Mag. x288

D. High magnification view of differing intensities of labelling within the primary cultures.

Mag. x1160


A
B
C
D

level of organisation, in that they formed both foci and whorls. The intensity of labelling was found to vary within the breast colony (Fig. 3.6d) and is probably a reflection of the exposure time of those cells in S phase. The proportion of labelled cells in the primary cultures varied from zero to 20%. Individual cultures showed no labelling at all(Fig. 3.6c), intermediate labelling and also extensive labelling (Fig. 3.6b).

The background feeder layer itself did not incorporate any (^{3}H)-thymidine during the labelling period, regardless of hormonal treatment. In the absence of hormonal supplementation, the labelling of the epithelial cells was similar to that in Fig. 3.6a. Addition of cortisol ($10^{-7}M$) slightly increased the proportion of labelled cells, while oestradiol ($10^{-9}M$) appeared to have either no effect or reduced the number of labelled cells (data not shown). The combination of cortisol and oestradiol resulted in an increase similar to that with cortisol alone (data not shown).

Due to heterogeneity of intercolony labelling, random sampling of breast colonies was not considered to provide a representative analysis of hormonal effects on the growth of the primary cultures.

3.3 Discussion

Primary cultures of human mammary epithelial cells have routinely been established from tumour biopsies with 74 and 79% success rates on mitomycin-C treated NIH-3T3 and STO feeder layers respectively. The differences in the ability of the NIH- and Swiss-3T3 cell lines to select for the growth of breast epithelial cells (74% and 24% success rates respectively), are unclear. Both the NIH- and Swiss-3T3 cell lines have been established in the same manner although from different strains of mice. The improved success rate with the NIH-3T3 cell line may reflect, for example, the ability of different feeder layers to provide a good ECM for attachment and sustained growth of the breast colonies.

3.31 Growth regulation on the NIH-3T3 feeder layer

Cultures from premenopausal ER +/+ biopsies were found to grow better in culture than those from postmenopausal ER +/+ biopsies since they had a greater MTCA than the latter group. In contrast, the relative colony area formation for the post- and pre-menopausal ER o/o groups was reversed.
It is generally accepted that ER negative tumours are histologically poorly differentiated while the ER positive tumours are, generally better differentiated (see sect 1.552). For the well differentiated ER positive group, cultures from the premenopausal patients grew better than those of the postmenopausal group. However, it is interesting that in the poorly differentiated ER negative group, cultures from the more aggressive premenopausal tumours grew less well than the cultures from postmenopausal tumours. These results seem somewhat contradictory in that there is selection of cultures from the poorly differentiated postmenopausal ER negative group as well as the more differentiated premenopausal ER positive group. Selection of cultures from tumours in these states of differentiation, perhaps suggests that similar cell populations were selected from tumours within the different groups.

3.311 Response to cortisol and oestradiol.

Primary cultures derived from the more differentiated ER +/+ biopsies showed a significant response to the addition of cortisol or oestradiol in terms of MTCA regardless of menopausal status. In the ER o/o group, these steroids had little effect when used alone, except that cortisol increased the MTCA for the premenopausal ER o/o category by 17%. Individually, these steroids had only a slight effect on the MTCN with the exception of a 25% increase for the postmenopausal ER +/+ group and a 24% decrease for the premenopausal ER o/o group. These results suggest that those cultures which have been derived from the more differentiated ER +/+ biopsies have a greater ability to respond to cortisol or oestradiol. Even so, the level of response is low relative to the effects *in vivo*, indicating that much of the mitogenic

effect may be indirect.

3.312 Response to cortisol and oestradiol in combination.

The steroid hormone combination resulted in a significant response in the cultures derived from the more differentiated biopsies while it evoked a variable response from the receptor-poor derived cultures. Dramatic changes occurred in the MTCA and MTCN for the ER +/+ groups regardless of the menopausal status. Cultures from the ER +/+ premenopausal biopsies showed a greater increase than those from postmenopausal ER +/+ biopsies. The increases appeared to be less than additive for MTCA but were synergistic for the MTCN of the cultures from the more differentiated ER +/+ biopsies. Primary cultures derived from poorly differentiated ER o/o biopsies showed a synergistic response to the hormone combination in terms of MTCA. The effect of the cortisol/oestradiol combination on the MTCN of cultures from ER o/o biopsies was, however, variable. A slight (7%) increase in MTCN occurred for the postmenopausal ER o/o group but the premenopausal ER o/o group showed a 24% decrease. The reasons for the variations in plating efficiency and subsequent growth elicited by the hormone combination are unclear.

Interestingly, there was no overlap in the responses of cultures, from the ER +/+ and o/o biopsies, to either cortisol alone or the combination of oestradiol/cortisol. The MTCA of ER +/+ derived cultures rose by 51-78% in response to the hormone combination while that of the ER o/o group increased by 28-45%. Similarly, the increases in MTCA for the ER +/+ and o/o groups to cortisol were 46-90% and 3-17% respectively. There was also no overlap for the MTCN's of these groups in response to cortisol alone or in combination with oestradiol. If the receptor status of the parent biopsy is maintained in short-term primary culture, these responses may represent a potential discriminant between cultures derived from ER +/+ and o/o biopsies.

The combined results of the growth experiments on the NIH-3T3

feeder layer (Table 3.3) have shown that the MTCA of premenopausal derived cultures is increased by 66% in response to the cortisol/oestradiol combination. This contrasts with the 25% increase with the postmenopausal derived cultures. Further analysis of the MTCA has shown that the receptor status influences the increase in MTCA since cultures from ER +/+ and o/o biopsies showed 67 and 32% increases respectively. However, some of the responses may not be mediated by the ER since, although the ER o/o derived cultures do not show any dramatic responses to oestradiol and cortisol alone, they respond dramatically to the hormone combination. These responses may, perhaps, be mediated by an autocrine-type mechanism. The results of an investigation into a possible paracrine mechanism, such as might operate in vivo are described in sect. 3.24.

In terms of MTCN a 17% increase was found for cultures from the postmenopausal biopsies in response to the cortisol/oestradiol combination while there was no change for cultures from the premenopausal group. This suggests that these steroids promote the plating efficiency of the postmenopausal derived cultures. In the well differentiated ER +/+ category, a 50% increase in MTCN was found while an 8% reduction occurred in the ER o/o group. This indicates that cortisol and oestradiol dramatically enhance plating efficiency for the well differentiated ER +/+ group of biopsies. Indeed, external signals such as hormones and growth factors may well be expected to exert differential effects on plating efficiency and on subsequent growth.

3.32 Growth regulation on the STO feeder layer.

The results in Table 3.4a and 3.4b have shown that cultures from premenopausal biopsies grow better than those from the postmenopausal biopsies. Similarly, cultures from ER +/+ grow better than those from the ER o/o groups. These results are particularly interesting in that both the premenopausal (often less well differentiated) and the ER +/+ (usually well differentiated) groups grew well in culture. These somewhat contradictory results suggest that the subpopulations of cells, selected by the culture conditions provided, may not always be typical of the parent tumour.

The response to the cortisol/oestradiol combination seems to occur independently of the menopausal status, but is of a greater magnitude in cultures from ER o/o biopsies than ER +/+ biopsies. This indicates that some proportion of the response may occur independently of the ER.

In the small sample of abnormal receptor status biopsies (a group normally considered to be oestrogen insensitive), the largest increase in MTCA (166%) was found in response to the cortisol/oestradiol combination. This is consistent with the greater response to the combination being in the ER o/o group. In contrast, the MTCN for the abnormal receptor group decreased by 52% which was the largest decrease for any of the groups classified according to receptor status. It is interesting that the greatest change in both the MTCA and MTCN should occur in the same group and again indicates that factors which promote plating and colony growth are different.

In terms of MTCN, there is a fairly good correlation with MTCA for those cultures from postmenopausal and ER +/+ biopsies. The remaining premenopausal and ER o/o groups showed little correlation. Interestingly, these groups showed the largest increases in MTCA in terms of menopausal and receptor status.

The combined results in Table 3.5 have shown that cortisol alone elicits slight increases in MTCA and MTCN, and oestradiol increases MTCN but reduces MTCA by 10%. This reduction in MTCA by oestradiol is surprising in that it suggests that oestradiol may result in the inhibition of cell proliferation.

The combination of cortisol and oestradiol has elicited significant increases in both the MTCA (37%) and MTCN (10%). These results imply that the overall effect of the hormone combination induces increases in both cellular proliferation and plating efficiency.

3.321 Response to EGF.

EGF alone dramatically increased both cellular proliferation (146%) and plating efficiency by 25%. The effect of EGF was potentiated by cortisol with respect to the MTCA. The combined effect of EGF and cortisol on plating efficiency was at least additive. The results of this latter experiment suggest that cortisol and EGF may elicit their effects on cellular proliferation through different mechanisms.

The detailed analysis of growth regulation on the STO feeder layer has shown that growth of the premenopausal cultures was better than that of the postmenopausal group regardless of receptor status (Tables 3.6a and 3.6b). These results contrast with growth on the NIH-3T3 feeder layer which showed best growth in the premenopausal ER +/+ and postmenopausal ER o/o categories. However, these detailed results for the STO feeder layer suggest that cultures from the less well and least differentiated biopsies grew better suggesting selection of different cell populations by the different feeder layers. The differences in the response of the breast cells to particular signals, when grown on different feeder layers, emphasises the importance of the stromal-epithelial interaction in modulating growth responses <u>in vivo</u>.

3.322 <u>Response to cortisol and oestradiol</u>.

With the exception of postmenopausal ER o/o group and the premenopausal abnormal receptor groups, cortisol and oestradiol were individually found to reduce the MTCA. With regard to plating efficiency, cortisol and oestradiol had differing effects. Cortisol reduced the plating efficiency in all groups except the postmenopausal ER +/+ and o/o groups. However, oestradiol in common with the cortisol/oestradiol combination increased the plating efficiency of the ER +/+ groups while reducing that of the remaining groups. The data, therefore, supports the view that oestradiol does not have a direct mitogenic effect on breast epithelium.

3.323 <u>Response to the cortisol/oestradiol combination.</u>

There was an overall increase in the MTCA in response to

the hormonal combination, regardless of the menopausal or receptor status. With the exception of the abnormal receptor groups, the greatest responses occurred in the postmenopausal ER o/o and premenopausal ER +/+ groups. These results indicate that the least differentiated and poorly differentiated groups have the greatest ability to respond to the hormonal combination by increased cellular proliferation.

In response to the cortisol/oestradiol combination, the MTCN was increased in the ER +/+ and the postmenopausal ER o/o groups while it was reduced in the remaining groups. Plating efficiency was therefore greatly enhanced in the postmenopausal ER o/o and ER +/+ groups and to a lesser extent in the premenopausal ER +/+ group. Interestingly, both postmenopausal ER +/+ and o/o groups showed an increased plating efficiency with both cortisol and oestradiol alone. The effects of the hormonal combination on plating efficiency were additive for the postmenopausal ER +/+ group but only similar to cortisol alone in the postmenopausal ER o/o group.

3.3[24] Growth regulation by prolactin.

Prolactin alone elicited a highly significant increase in MTCA of the primary cultures. The 88% increase in MTCA was accompanied by a 19% reduction in plating efficiency. The reduction in plating efficiency by prolactin occurred regardless of additional steroid hormone stimulation. The increase in cell proliferation by prolactin was antagonised by the addition of either or both cortisol and oestradiol. The total reduction by both cortisol and oestradiol was almost additive which suggests that these steroids may antagonise the action of prolactin on cellular proliferation by separate mechanisms. However, since full dose response curves for the effects induced by either hormone were not constructed, an unequivocal statement cannot be made.

There was little effect of either steroid on MTCN but in combination the three hormone cocktail resulted in a 30% decrease in plating efficiency. This suggests that cortisol and oestradiol act synergistically in the presence of prolactin to reduce the plating efficiency.

3.33 Growth regulation by conditioned medium.

Interestingly, these experiments showed that the only category of conditioned medium which increased the MTCA was the conditioned medium from breast fibroblasts grown in the presence of exogenous 10^{-9}M oestradiol (CME). Somewhat surprisingly, the enhancement of cellular proliferation by CME was inhibited when cortisol was also added and indeed the MTCA and MTCN were reduced by 36 and 17% respectively. This observation is surprising considering the previously described effects of cortisol on primary breast epithelial cell growth. The results of these experiments with conditioned medium have raised the possibility that a paracrine-type mechanism may, at least in part, operate in the growth regulation of mammary epithelium. This preliminary data merits further investigation to elucidate the exact mechanism involved,in addition to the isolation of the factor(s) responsible for the increase in cell proliferation.

3.34 Autoradiography of primary cultures.

Labelling of the primary breast cultures with (^{3}H) thymidine revealed that these cultures were not uniformly labelled. The presence of foci of both labelled and unlabelled cells, in those colonies which were labelled, raises the possibility of local synchronisation of those cells which are either actively synthesising DNA or in a quiescent state.

Hormonal treatment was found to have a slight effect on the (^{3}H) thymidine labelling pattern of the primary breast cultures. In those colonies which were labelled, addition of cortisol was found to slightly increase the proportion of labelled cells. Oestradiol, however, appeared to have little

or no effect on the number of labelled cells. The cortisol/oestradiol combination increased the proportion of labelled cells to a similar level as the stimulation by cortisol alone. However, since, there was such a marked heterogeneity of labelling,it was decided that an estimation of the fraction of labelled cells by random sampling would not be a very accurate representation and therefore be statistically invalid.

3.35 Conclusions.

Primary cultures have routinely been established from breast tumour biopsies with approximately 80% success rates on feeder layers of NIH-3T3 and STO mouse embryo fibroblasts. Cultures have followed two different patterns of growth, either growing on or underneath the feeder layer. Autoradiographic studies have highlighted the extensive variation in the labelling patterns of the breast colonies. The heterogeneity within the labelling appears to be due to areas of quiescent cells and other areas of actively synthesising cells. It has invalidated the use of autoradiography to assess hormone dependence in the primary cultures.

Growth on the NIH-3T3 feeder layer.

The culture conditions provided by the NIH-3T3 feeder layer selected for the growth of the more differentiated ER +/+ premenopausal and the less differentiated postmenopausal ER o/o groups. This suggests,perhaps,that cell populations with some common characteristics were selected from tumours within the different groups. The ER +/+ groups showed the greatest response to cortisol or oestradiol alone in terms of MTCA regardless of menopausal status. Little response to individual steroids was observed from the ER o/o group in general. Similarly, there was generally little effect of cortisol or oestradiol alone on the MTCN.

The well differentiated ER +/+ groups showed significant increases in MTCA and MTCN in response to the cortisol/oestradiol combination. The poorly differentiated ER o/o categories

showed a synergistic response to the hormone combination in terms of MTCA although the effects on MTCN were variable. There was no overlap in the extent of the response of the ER +/+ and o/o groups to the hormone combination and cortisol alone for either MTCA or MTCN. The MTCA increased by 67 and 32% for the ER +/+ and o/o groups respectively, indicating that almost half of the response may be independent of the ER, perhaps involving an autocrine-type mechanism.

Growth on the STO feeder layer.

There was selection for the premenopausal (less-well differentiated) and the ER +/+ (well differentiated) groups on this feeder layer. Individually, the steroids cortisol and oestradiol reduced the MTCA in all the groups except the postmenopausal ER o/o and the abnormal premenopausal group. Differing effects of the individual steroids were observed on plating efficiency. These steroids alone do not generally appear to have a direct mitogenic effect on cell proliferation.

The response of cultures to the cortisol/oestradiol combination seemed to be independent of menopausal and, perhaps, receptor status since the magnitude of response was greater in cultures from the ER o/o group than the ER +/+ group. The greatest responses to the hormone combination were observed in the premenopausal ER +/+ (i.e. the naturally proliferating group) and the postmenopausal ER o/o group (i.e. the poorly differentiated group).

EGF alone elicited a significant increase in cell proliferation and plating efficiency. This mitogenic effect of EGF was potentiated by cortisol although, in terms of plating efficiency the effects were only additive. The synergistic mitogenic actions of cortisol and EGF suggest that they may mediate their effects on cellular proliferation by different mechanisms.

Prolactin was the only non-steroidal mammogenic hormone used in these studies. It induced a most significant increase in MTCA and a simultaneous reduction in plating efficiency. The steroids, alone or in combination, antagonised the mitogenic effect of prolactin and reduced the plating efficiency. An antagonistic effect of oestradiol on prolactin has not previously been reported for human breast cells.

The experiments with conditioned medium from breast stromal fibroblasts suggested the possible existence of a paracrine-type mechanism. This was, however, inhibited by cortisol in terms of both colony area and number.

The results presented in this section suggest that external signals, such as hormones and growth factors exert differential effects on both plating efficiency and growth. The magnitude of the growth responses to hormones and growth factors has been found to be relatively small in comparison to the effects *in vivo*. This suggests that a large proportion of the mitogenic effect *in vivo* may be indirect. The combination of cortisol/oestradiol has indicated the possible existence of an autocrine-type mechanism for growth regulation, since on the STO feeder layer the ER negative group showed a 50% greater response than the ER positive group to the same cortisol/oestradiol combination. Additionally, the experiments involving conditioned medium have raised the possibility of a paracrine-type mechanism. The data presented, therefore, suggest that growth regulation of breast epithelium *in vivo* may be subject to endocrine, autocrine and paracrine regulatory mechanisms.

4. Characterisation of Primary Cultures.

4.1 Introduction.

4.11 Identification of The Mammary Origin.

Markers for breast tissue include various enzymes which are elevated in malignant tissues although they are present in normal or benign tissues. These include mitochondrial malate dehydrogenase, lactate dehydrogenase isozyme 5 and the K4 - pyruvate dehydrogenase isozyme (Balinsky et al, 1983). Recently, a tissue specific marker for human mammary epithelium, in the form of thioesterase II, has been identified (Smith et al, 1984) and should prove useful in diagnostic pathology.

In the studies outlined below, the identification of the mammary origin of the primary cultures has, however, relied on the presence of antigens associated with the human milk fat globule membrane. The three monoclonal antibodies employed were the HMFG -1, HMFG-2 (Taylor-Papadimitriou et al, 1983) and M8 (Foster et al, 1982) antibodies. HMFG - 1 and HMFG - 2 react with different epitopes of a large molecular weight glycoprotein, immunologically related to the epithelial membrane antigen, EMA, (Burchell et al, 1983) and M8 reacts directly with the EMA antigen itself (Sloane + Ormerod, 1981). Partial purification of the antigen has shown that it comprises a heterogeneous glycoprotein(s) of high molecular weight. The carbohydrate moiety is regarded as a major antigenic determinant with the principal sugars being galactose and N-acetyl-glucosamine (Ormerod et al, 1983). The identification of EMA in tumour tissue is highly indicative of its epithelial origins while the absence of the antigen almost certainly excludes breast derivation.

The antigenic determinants detected by HMFG - 1 and HMFG - 2

are strongly expressed on the lactating breast and many primary cancers although they are expressed to a lesser extent in the glandular epithelium of the resting breast. HMFG - 1 and HMFG - 2 reactivity has been reported to be similar in cultured cells and tissue sections although HMFG - 1 reacts more intensely with cultured milk epithelial cells and lactating gland sections than HMFG - 2 which reacts more strongly with ductal carcinomas and cell lines derived from them or their metastatic lesions (Burchell et al, 1983). The M8 antibody binds to both apical and lateral membranes of mammary epithelial cells, but not to myoepithelial or stromal cells (Foster et al, 1982). The specificity of the M8 antibody is such that it stains only some of the mammary epithelial cells like other similar antibodies (Arklie et al, 1982). The significance of this heterogeneous staining pattern, however, remains unclear.

4.12. Identification of The Epithelial Origin.

4.121 Desmosomal Junctions.

One criterion to establish the epithelial nature of the primary cultures is the presence of desmosomes which are cell membrane organelles involved in intercellular adhesion. They comprise parallel cell membranes separated by an intercellular space of 25 - 35nm. Dense rigid plaques (10 - 15nm thick) are connected to the cytokeratin bundles (Henderson & Weber, 1981). These desmosomes may be identified by either ultrastructural or immunological studies.

4.122 Expression of Intermediate Filaments.

The intermediate filaments characteristic of epithelial cells are the cytokeratins. The pattern of keratin expression is, moreover, a characteristic feature of a specific epithelial cell type. Within the breast, there is differential keratin expression in the myoepithelial and the glandular or luminal cells (Lane & Klymkowsky, 1981). For example, the myoepithelial cells do not express keratins 18 and 19 which are both however expressed by luminal mammary epithelial cells.

In cultured epithelium, co-expression of keratin and vimentin

intermediate filament types has been reported (Franke et al, 1979). However human tumour epithelial cells, which co-express vimentin and keratin in culture, do not co-express vimentin when these tumour cells develop into solid tumours in nude mice (Ramaekers et al, 1983.) It has been proposed that the co-expression of vimentin is not an artefact of cell culture, but, is induced by the release of the epithelial cells from their neighbouring cells in the solid tumours (Ramaekers et al, 1983; Dairkee et al, 1984).

4.13. Aims.

The aims of these studies were to characterise the mammary and the epithelial origins of the primary cultures established using the methodology described in Sect. 2.72. An attempt was also made to assess the effects of retinoic acid and various steroids on the expression of keratin 18 which is a marker for simple epithelium.

4.2 Results.

4.21 Expression of human mammary antigens.

Immunofluorescent staining of primary cultures with HMFG - 1 revealed very little if any specific binding of the antibody (data not shown). In contrast, staining with HMFG - 2 resulted in high levels of specific staining as seen in Fig. 4.1a. The staining appears to be cytoplasmic and is most intense in the outer cells of the colony. The feeder layer of mouse embryo fibroblasts showed only background staining similar to that obtained with normal mouse serum.

Immunofluorescence studies with the M8 antibody revealed that some of the cells within the colonies were stained by the antibody (Fig. 4.1b.) The staining was heterogeneous although no particular characteristics could be ascribed to the cells which bound antibody.

4.22 Identification of desmosomal junctions.

A polyclonal antiserum against high molecular weight

Fig. 4.1 Expression of human mammary epithelial antigens in primary culture.

Primary cultures were grown for four weeks on 13mm glass coverslips on STO feeder layers, as described in Sect. 2.72 and immunocytochemistry was performed as in Sect. 2.78 on five separate occasions.

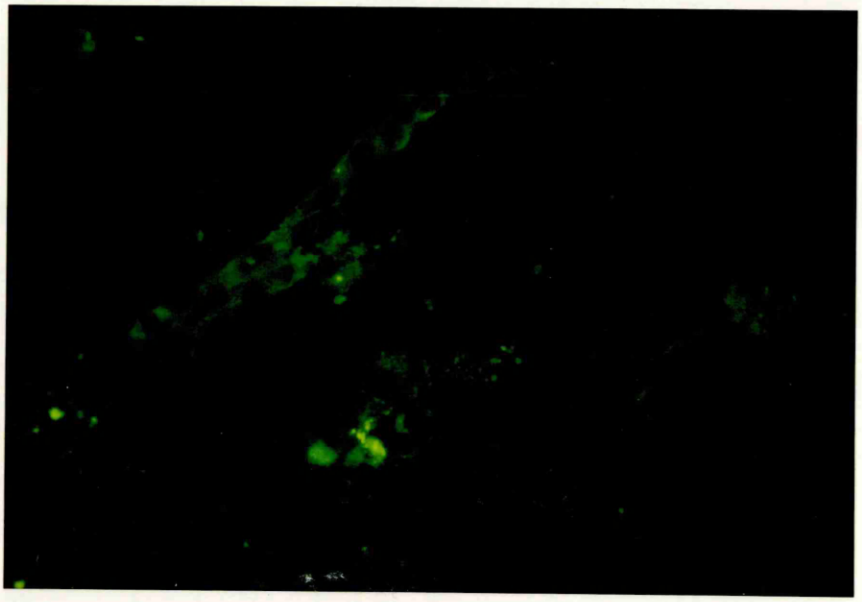
A. Specific immunofluorescence staining of a human mammary fat globule membrane antigen using HMFG-2 antisera (Mag.x144).

Uniform staining of the breast colony was observed.

Primary and secondary antibodies were used at $^{1}/_{10}$ dilutions.

B. Specific immunofluorescence staining of an epithelial membrane antigen with the M8 antiserum (Mag. x180). A heterogeneous staining pattern was obtained with this antibody.

Primary and secondary antibodies were employed at $^{1}/_{10}$ dilutions.



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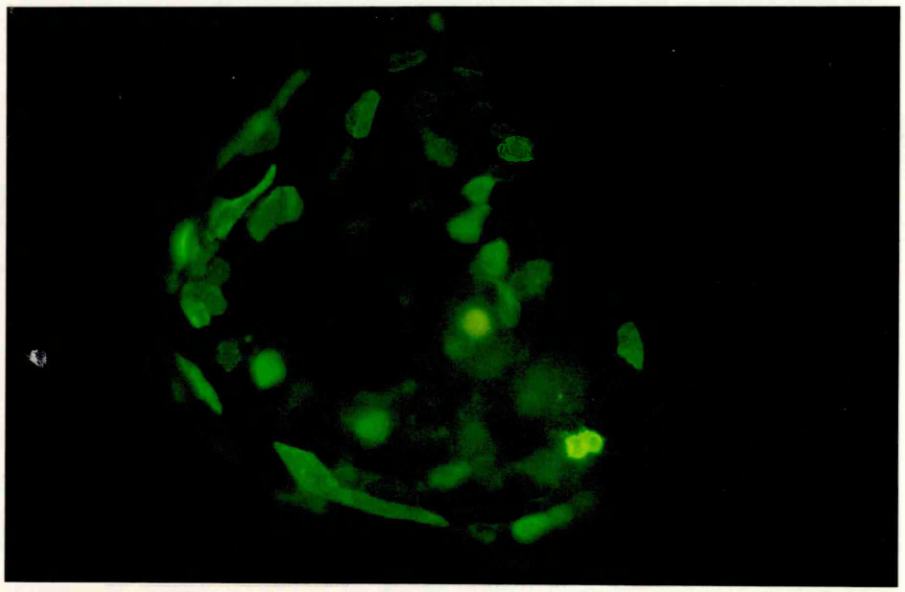


B

desmosomal components, referred to as desmoplakins (Cowin & Garrod, 1983) has been employed in immunofluorescence studies on primary cultures. Intense staining of cell interfaces was observed in the epithelial colonies (Fig. 4.2a), particularly at the epithelial - stromal junction. Staining was often punctate (data not shown) as would be expected from the interdigitating nature of the desmosomes.

4.23 Expression of Intermediate Filaments.

Specific staining of primary human mammary epithelial cultures was obtained with wide spectrum keratin (Fig. 4.2b), LE61 (anti-keratin 18) (Fig 4.3a) and vimentin antisera (Fig. 4.4c). The feeder layer showed only background staining comparable to control staining with normal serum. Studies with the wide spectrum keratin antibody revealed that the characteristic "ring" colonies may grow underneath the feeder layer since they extended beyond the apparent edge of the colony visible under phase contrast microscopy (see Figs 4.2b and 4.2c). Counterstaining with Hoechst 33258 showed that the specific staining was associated with the colonies of breast cells which have the small nuclei, rather than the feeder cells with the somewhat larger nuclei (compare Figs 4.3a and 4.3b).

The expression of keratin 18, detected by the LE61 monoclonal antibody was generally found to be quite uniform (Fig. 4.3a and 4.4a). Occasionally, however, heterogeneous staining was observed in some colonies (Figs. 4.4b). Intense staining was associated with either localised areas within the colony (Fig. 4.4b) or less frequently with single cells (data not shown). The accessibility of the antibody does not seem to be responsible for the heterogeneous staining obtained with the LE61 since the elevated central portions of the colonies are not those regions with the most intense staining. The reason for the heterogeneity in the staining with the LE61 antibody remains unclear but almost certainly reflects the heterogeneous nature of the primary cell cultures.

Co-expression of keratin and vimentin was observed in the primary

Fig. 4.2 Expression of epithelial specific antigens in primary breast cultures.

Primary cultures were established as in Sect. 2.72 on 13mm glass coverslips. Cultures were processed after two weeks as described in Sect. 2.78. Primary cultures were grown on STO (Fig.A) or NIH-3T3 (Figs. B and C) feeder layers.

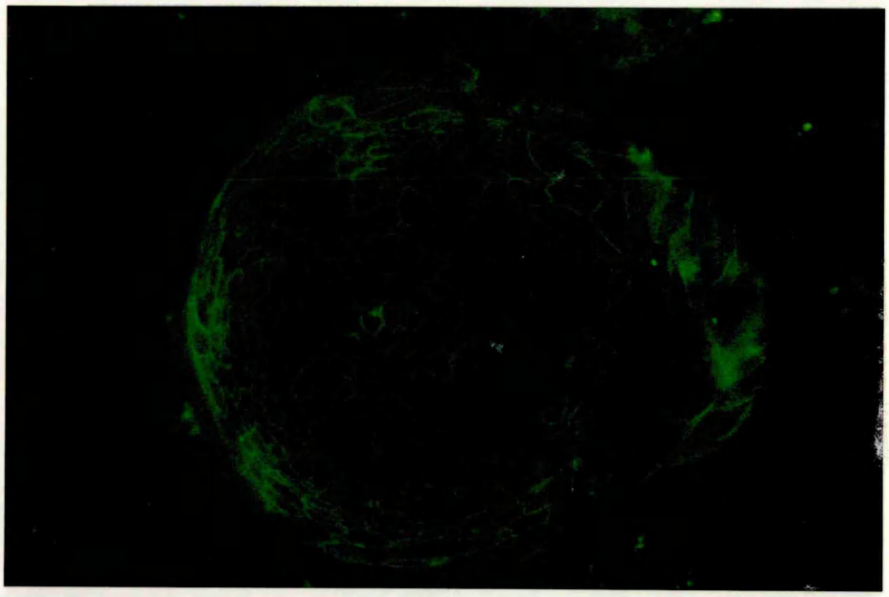
A. Specific immunofluorescence staining of desmosomes with desmoplakin (desmosomal plaque protein) antiserum (Mag. x324). Primary and secondary antibodies were used at $^{1}/_{50}$ dilutions in PBS-A.

B. Specific immunofluorescence staining of keratins with polyclonal human keratin antiserum (Mag. x260). This is the same field of view as Fig. C and shows the epithelial cells extending beyond the apparent edge of the ring colony visible under phase contrast.

Primary and secondary antibodies were used at $^{1}/_{40}$ and $^{1}/_{20}$ dilutions in PBS-A respectively.

C. Same field of view as Fig. B. Phase contrast (Mag. x260).

Immunocytochemistry was performed on three separate occasions.



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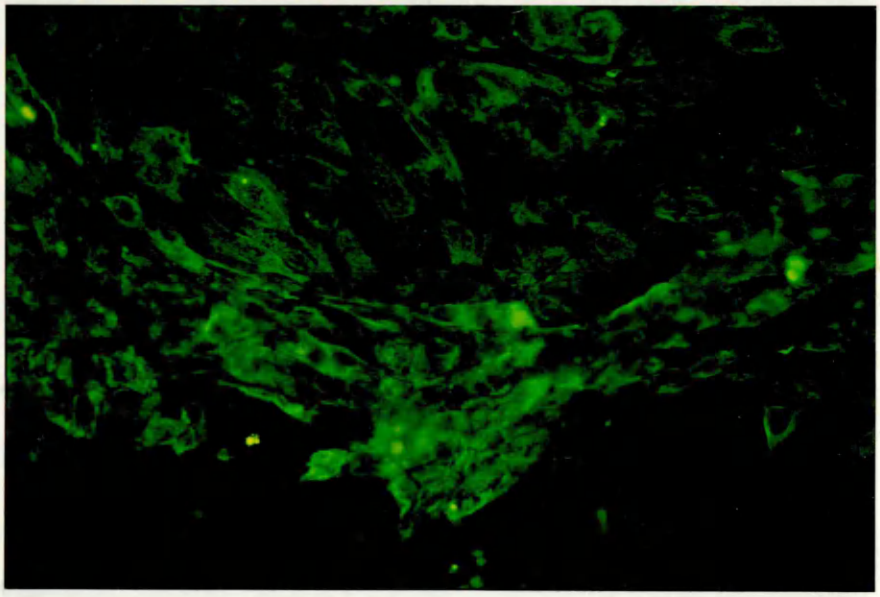

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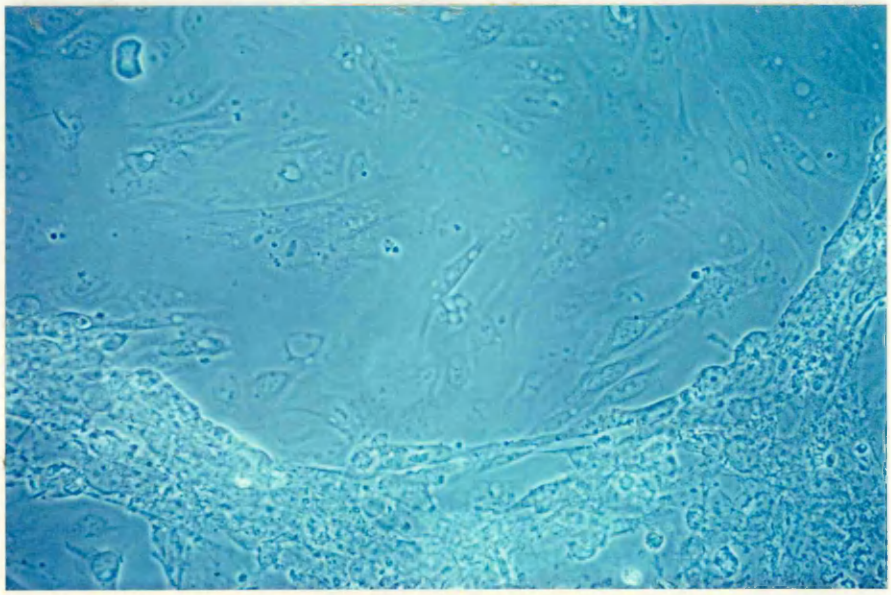


C

Fig. 4.3 Staining of primary human mammary cultures with LE61 antiserum.

Epithelial cultures were established using the standard procedure on STO feeder layers (Sect. 2.72). Immunocytochemistry and Hoechst 33258 staining were conducted (as in Sect. 2.78) after four weeks in culture on over 20 occasions.

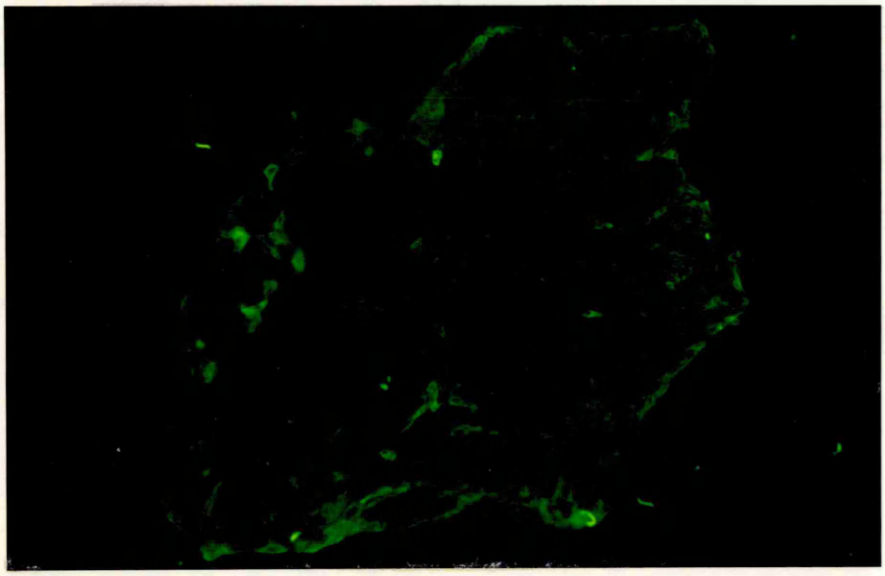
LE61 and the secondary antisera were employed at $^{1}/_{20}$ dilutions in PBS-A.

A. Specific immunofluorescence of keratin 18 (Mag. x64).

The breast epithelial cultures were stained with LE61 (keratin 18) antiserum.

B. Hoechst 33258 counterstaining of primary breast cultures (Mag. x64).

Same field of view as Fig. A. The cell nuclei were stained by Hoechst 33258. The breast epithelial cells were visualised as a concentrated mass of smaller nuclei in comparison to the larger nuclei of the feeder cells.



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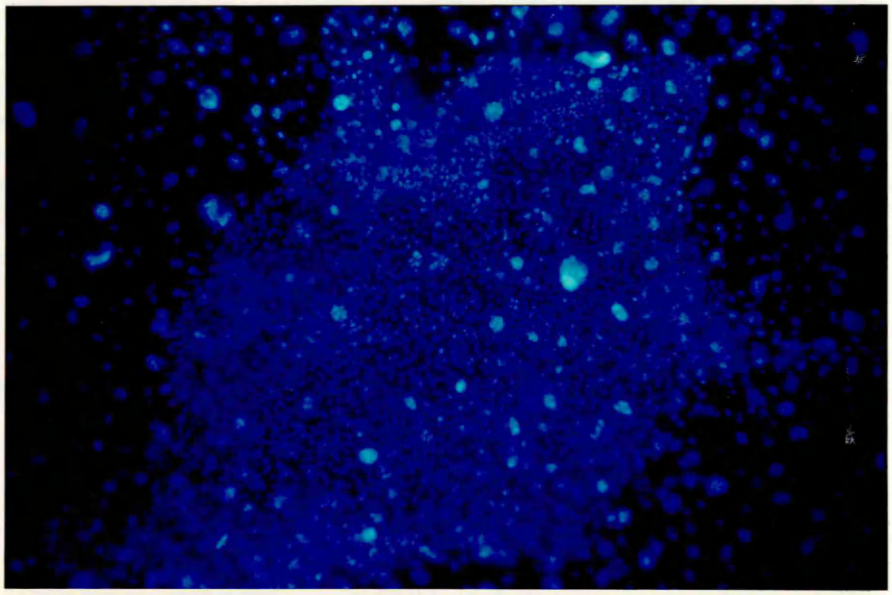


B

Fig. 4.4 Expression of intermediate filaments in primary breast epithelial cultures.

Primary cultures were grown on STO feeder layers for four weeks (as described in Sect. 2.72) and immunocytochemistry was conducted as in Sect. 2.78 on over 20 occasions with LE61 and on three occasions with vimentin antiserum.

A. Specific immunofluorescence of keratin 18 (Mag. x1012).

The primary breast epithelial culture, BMAM, was stained with LE61 antiserum and examined at high magnification.

Primary and secondary antibodies were used at $^{1}/_{20}$ dilutions in PBS-A.

B. Specific immunofluorescence of keratin 18 (Mag. x81).

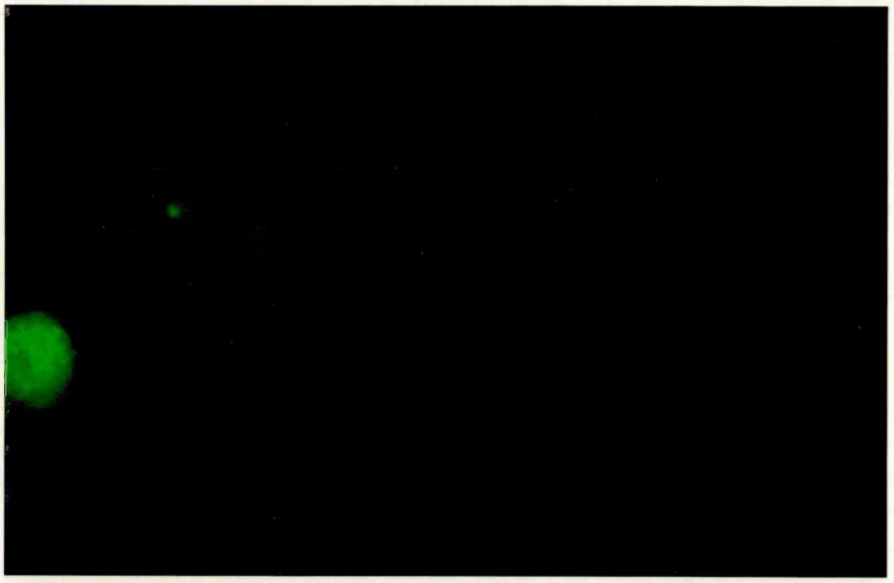
Staining of the MTII culture with LE61 antiserum revealed the presence of intense staining groups of cells amongst the remaining uniformly stained epithelial cells.

Primary and secondary antibodies were used at $^{1}/_{20}$ dilutions in PBS-A.

C. Specific immunofluorescence of vimentin (Mag. x260).

The primary breast epithelial culture, BMAM, was stained with vimentin antiserum which highlighted both the epithelial breast colony and the mesenchyme derived feeder cells.

Vimentin and secondary antisera were used at $^{1}/_{10}$ dilutions in PBS-A.



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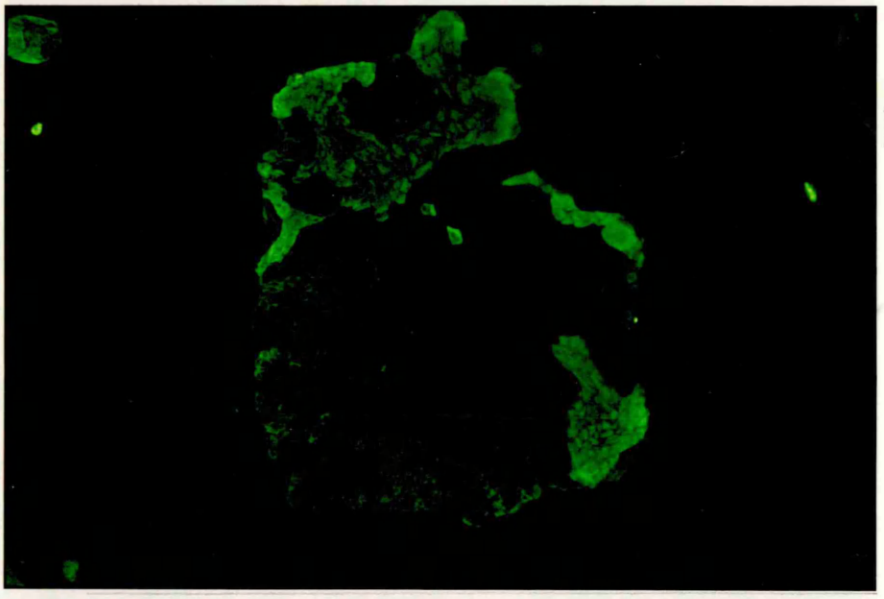

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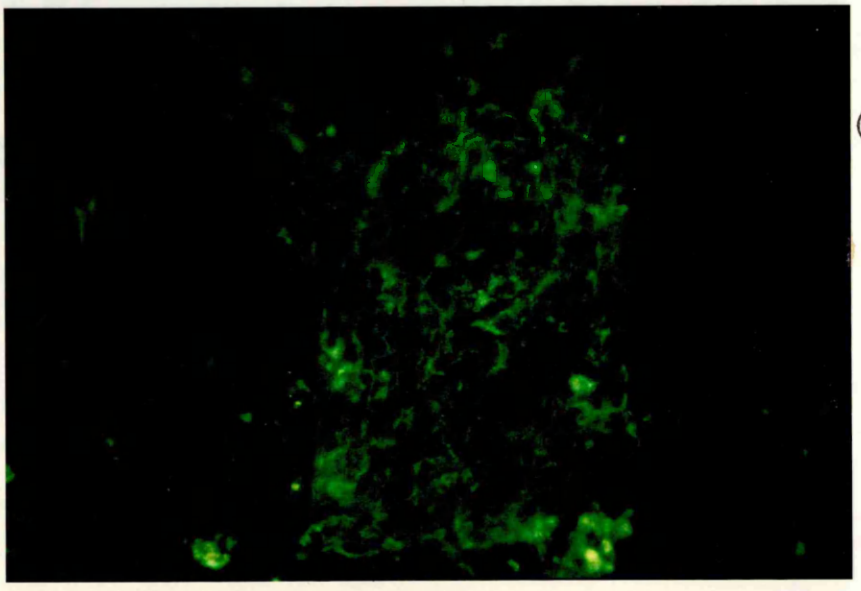


C

human mammary epithelial cultures (Fig. 4.4c). The feeder cells were also stained with the anti-vimentin antibody as vimentin is the characteristic intermediate filament class of mesenchyme-derived cell types.

4.24 Effects of retinoic acid and steroids on the expression of keratin 18.

The effects of retinoic acid (2×10^{-6} M) and various steroids on the expression of keratin 18 were investigated by immunofluorescence studies with the LE61 antiserum. In various epithelia, retinoic acid has previously been reported to regulate differentiation and keratin expression (see sect. 1.65). Cells cultured under control conditions exhibited a characteristic fine radial array of keratin filaments (see Fig 4.4a). These keratin filaments were found to emanate from the perinuclear region and extended to the plasma membrane. Retinoic acid (2×10^{-6}M) at greater than physiological levels, appeared to induce a phenotypic alteration in the expression of keratin 18. This alteration had two effects on the pattern of keratin 18 staining. Firstly, the intensity of the fluorescence signal was diminished (Fig. 4.5a). There was also a modification in the keratin network with the keratin filaments appearing to be much thicker.

In the presence of either cortisol or oestradiol, two different types of staining patterns were observed with the LE61 antiserum (see Fig. 4.5b). The first type of staining was similar to the characteristic fine filamentous network observed in cultures grown under control conditions (Fig. 4.4a). The other pattern of staining was a very intense staining over the whole surface of the cell. In combination, cortisol and oestradiol resulted in the same type of staining patterns although the majority of cells exhibited the characteristic filamentous network (data not shown).

The effects of tamoxifen (10^{-7}M) and megestrol acetate (10^{-7}M) on the expression of keratin 18 were also investigated

Fig. 4.5 Staining of primary human mammary epithelial cultures with LE61.

Cultures were prepared as described in Sect. 2.72 in the standard culture medium. Retinoic acid and steroids were added to give the final concentrations shown in the figure legends. Cultures were grown, with change of medium and additive each 48 hours, for four weeks(unless otherwise stated) and processed for immunofluorescence (Sect.2.78). LE61 and secondary antisera were used at $^{1}/_{20}$ dilutions in PBS-A.

A. Specific immunofluorescence of keratin 18 using the LE61 antiserum (Mag. x1012).

Primary breast cultures, BMAM, were grown with 2×10^{-6}M retinoic acid. The intensity of fluorescence was generally significantly greater in control cultures.

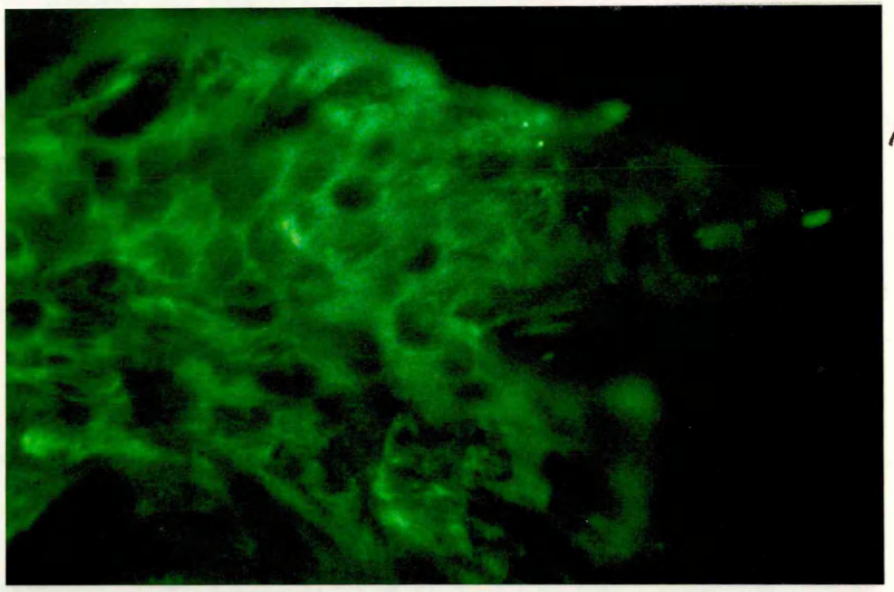
B. Specific immunofluorescence of keratin 18 using the LE61 antiserum (Mag. x 648).

The primary breast cultures, MTII, were grown for 24 days in the presence of 10^{-9}M oestradiol. Two types of keratin 18 staining were observed.

C. Specific immunofluorescence of keratin 18 using the LE61 antiserum (Mag. x1012).

The primary cultures, BMAM, were grown in the presence of cortisol (10^{-7}M) for fourteen days. Tamoxifen (10^{-7}M) was added to the cortisol for the next seven days and the cultures allowed to recover for three days in medium supplemented with 10^{-7}M cortisol. A change in the morphology of the epithelial cells was observed.

These experiments were performed on three different occasions.



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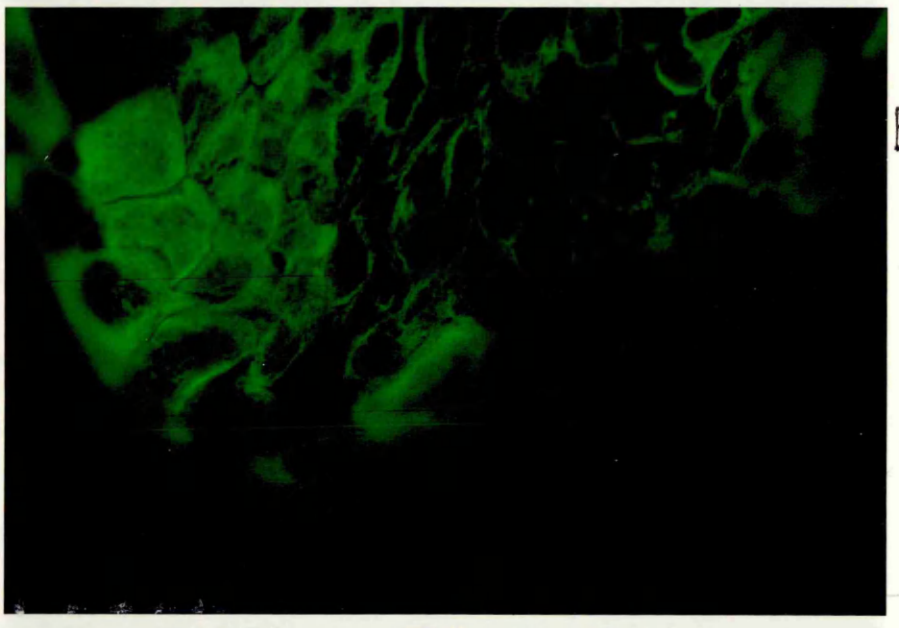

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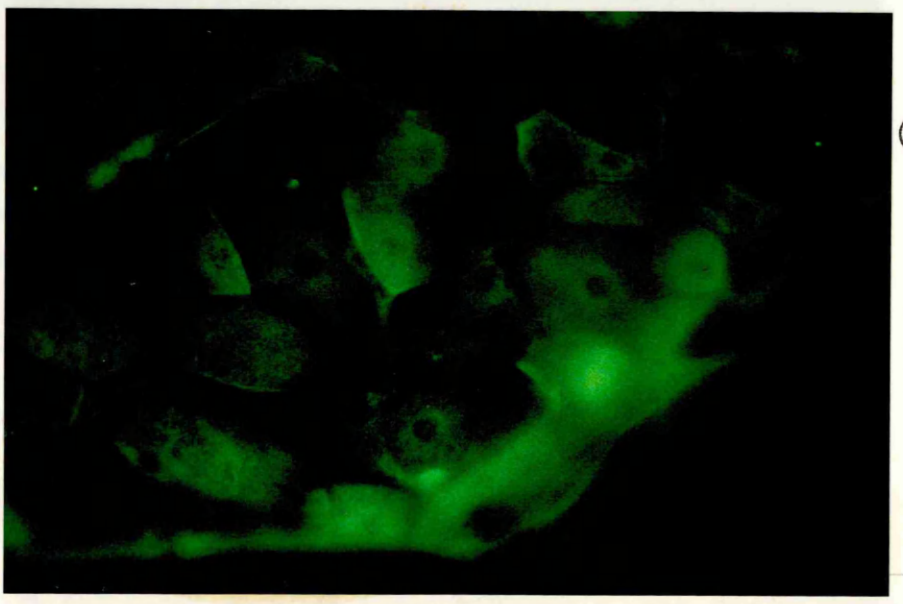

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on cultures grown for 14 days with cortisol (10^{-7}M). Tamoxifen or megestrol acetate was added for seven days along with cortisol and the cultures were allowed to recover for three days in medium supplemented with cortisol. In those cultures exposed to tamoxifen, the cells were found to have a very irregular shape (Fig.4.5c). The expression of keratin 18 was similar for both types of treatment and the predominant staining pattern was similar to that of the control cells.

The results of these steroid experiments have shown that there is little change in the pattern of keratin 18 expression. Tamoxifen, in conjunction with cortisol, was found to elicit a change in cell shape which did not occur with any of the other steroids.

4.3 Discussion.

4.31. Expression of Human Mammary Antigens.

Immunofluorescent studies have shown differential binding of the HMFG - 1, HMFG - 2 and M8 monoclonal antibodies to primary cultures derived from human breast tumour biopsies. HMFG - 2 was bound most strongly while there was little binding of HMFG - 1. This observation correlates with the findings that HMFG - 2 reacted more intensely with cells derived from ductal carcinomas than HMFG - 1 (Burchell *et al*, 1983). Heterogeneous binding of the M8 antibody is in agreement with the report from Edwards & Brooks, (1984), who have observed this characteristic heterogeneous antigen expression. This heterogeneity has been suggested to be due either to random antigen expression or perhaps related to the different physiological states of the mammary epithelial cells. The studies with these antibodies have confirmed the human mammary origin of the primary cultures and also possibly their malignant nature.

4.32 Desmosomal junctions.

Intense staining with the anti-desmoplakin antibody has identified the presence of desmosomal junctions within the

epithelial colonies. This has in turn confirmed the epithelial nature of the colonies derived from the human breast tumour biopsies.

4.33 Expression of intermediate filaments.

The immunofluorescence studies have shown that primary breast cultures grow either on (Fig 4.3a) or underneath the feeder layer (Figs. 4.2b and 4.2c). The specific staining with the wide spectrum and the LE61 (anti-keratin 18) antisera have confirmed the epithelial nature of the primary breast cultures.

Staining of the primary breast cultures with the LE61 antiserum has shown that the colonies arise from luminal cells and not the basal myoepithelial cells. The heterogeneity in staining pattern with the LE61 antiserum was unlikely to represent an artefact and provides evidence to suggest the heterogeneous nature of the cells within the primary cultures.

These studies have shown the co-expression of vimentin in primary breast cultures. Vimentin is the intermediate filament class characteristic of mesenchymal derived cells and is not expressed in solid epithelial tumours *in vivo*. The coexpression of vimentin in primary culture confirms other previous reports of vimentin coexpression in epithelial cells *in vitro*.

4.34 Effects of retinoic acid and steroids on keratin 18 expression.

Retinoic acid appeared to elicit a phenotypic alteration in the expression of keratin 18 in primary human mammary epithelial cultures. Although there was a reduction in the fluorescence signal and a more linear organisation of the keratin filaments (see Fig 4.5a), these effects suggest either a reduction had occurred in the expression of keratin 18 or there was a masking of its antigenic determinants. These observations are similar to previous findings with human

endometrial primary cultures in response to retinoic acid (1×10^{-7}M) (Field, 1985). These preliminary observations on the effects of retinoic acid on the expression of keratin 18, a marker of simple epithelia, merit further investigation using immunological or biochemical techniques.

In the presence of all the steroids employed, two different types of staining pattern were observed. In addition to the characteristic fine keratin network, an intense type of staining pattern was observed. There was no obvious difference in the keratin staining pattern with cortisol and oestradiol alone or in combination. Neither tamoxifen nor megestrol acetate had any effect on the staining pattern with the LE61 antiserum although an irregular morphology was induced by the cortisol/tamoxifen combination. Although little difference was observed in the LE61 staining pattern, the change in morphology with the cortisol/tamoxifen combination merits further investigation.

4.4 Conclusions.

The human mammary epithelial nature of the primary cultures established from human breast tumour biopsies has been confirmed. The presence of desmosomes, breast cell surface antigens and the expression of keratins (characteristic of epithelial cells) has been verified by immunofluorescence studies. Vimentin, although not expressed in solid epithelial tumours *in vivo*, was co-expressed in primary cultures. An attempt was made to assess the effects of retinoic acid and various steroids on epithelial differentiation. Retinoic acid appeared to reduce both the intensity and the pattern of keratin 18 expression. Tamoxifen, in conjunction with cortisol had a significant effect on cellular morphology although there was little effect on the expression of keratin 18.

5. Surface Morphology of Primary Cultures.

5.1. Introduction.

The effects of oestradiol on primary cultures of organoids, derived from reduction mammoplasties, have been described with regard to both ultrastructural alterations in chromatin structure and cell surface morphology (Chambon et al, 1984). Treatment with 10nM oestradiol was found to induce dispersion of chromatin in 80% of the clear epithelial cells at the organoid surface and in the epithelium bordering the lumina. The surface morphology of these clear cells cultured on either glass or floating collagen gels has been further examined by scanning electron microscopy (SEM). It was observed that the control organoids had a relatively smooth apical surface and were both small and spherical. Oestrogen-treated organoids, however, were heterogeneous with 31% similar to controls and the remainder being polylobular. Of the control organoids, 20 - 29% were homogeneously flat with few short microvilli while 42 - 100% of oestradiol-treated organoids had a dense array of long and short microvilli. Other surface features of oestrogen-treated organoids included blebs or knobs on the cell surface.

5.11. Aims.

The objective of these studies was to observe the surface morphology of primary human mammary epithelial cultures and to assess any effects produced by hormones.

5.2. Results.

5.21. Fixation Procedures.

Glutaraldehyde is universally employed as a primary fixative for whole cells as it has been shown to terminate all movements within seconds of addition to the cultures (Brunk

et al, 1975). It was therefore chosen as the primary fixative for primary human breast epithelial cultures. Primary fixation was carried out at the standard culture temperature of 37°C since Brunk et al,(1981), have shown that cultured cells cease to move without any effect on morphology when fixed at this temperature. Post fixation was performed with osmium tetroxide since it both stabilises lipids against extraction by organic solvents (Brunk et al, 1981) and acts as an electron dense stain.

The most important aspects of the fixative vehicle are the buffering capacity and osmolarity. A standard pH of 7.4 was employed and fixation buffers of differing osmolarities (each containing 2.5% v/v glutaraldehyde) were compared. Fixative vehicles comprising 0.2, 0.1 and 0.05M phosphate buffers (pH 7.4) were assessed for their effects on the surface morphology. Somewhat surprisingly, the different buffers did not appear to elicit swelling or shrinkage artefacts as assessed by phase contrast and scanning electron microscopy (data not shown). Nevertheless, the isotonic 0.1M phosphate buffer (290 mOsm/kg) was used as the vehicle for the primary fixative, glutaraldehyde.

There remains controversy regarding the effective contribution of glutaraldehyde to the osmolarity of the fixative (Brunk et al, 1975; Lee et al, 1979). When the culture medium and the fixative vehicle are isotonic, glutaraldehyde, in the commonly used range (2 - 2.5%), does not make a contribution to the effective osmolarity (Brunk et al, 1975;1981; Bell, 1984). The cell cultures, however, remain osmotically sensitive until they are post fixed with osmium tetroxide.

Primary fixation procedures were also varied in order to determine the best conditions for primary fixation. Initial fixation conditions involved the addition of an equal volume of prewarmed fixative (37°C) to the growth medium for five minutes with subsequent replacement of the thus diluted

fixative by "full" fixative for another 55 minutes. A comparison of the addition of full fixative to a minimal volume of growth medium required to cover the coverslips showed no apparent effect on the surface morphology of the cultures (data not shown). The final primary fixation procedure involved direct addition of prewarmed 2.5% (v/v) glutaraldehyde in 0.1M phosphate buffer to the growth medium for an hour at $37^{o}C$.

Preservation of the surface morphology was also compared by both critical point drying (CPD) and freeze drying (FD) dehydration procedures. Dehydration by CPD resulted in extensive cracking commonly associated with cell shrinkage (see Fig 5.1a) while freeze drying produced thermal cracking (see Fig 5.1b). Tannic acid was used as a mordant and primary post-fixative prior to treatment with osmium tetroxide (see sect. 2.64) in order to improve membrane stability. Thermal cracking with freeze dried preparations remained a problem but there was a significant improvement in the preservation of surface morphology. Therefore, despite these technical problems, it was possible to study the surface morphology as well as the hormonal effects on primary human mammary epithelial cultures.

5.22 Surface morphology.

SEM was performed on eight specimens of human mammary tumours established in primary culture. In terms of their surface morphology, CPD preparations (Figs. 5.1a, 5.2a and 5.2b) have shown that there are two categories of epithelial cells. Firstly, there are epithelial cells with a homogeneous distribution of microvilli and secondly, there are those epithelial cells which have a heterogencous surface morphology. The heterogenous morphology is illustrated by the cells at the centre right of Fig. 5.2a. In the more central cell, the microvilli are present in the upper right hand corner and the lower half of the cell

Fig. 5.1 Scanning electron microscopy of primary human breast cultures.

Primary cultures were established as described in Sect. 2.72 on 10mm glass coverslips and maintained for two weeks under the standard conditions(Sect. 2.34). Cultures were fixed and processed for SEM according to the methodology in Sect. 2.64 unless otherwise stated.

A. Low magnification scanning electron micrograph of primary breast culture (Mag.x62)

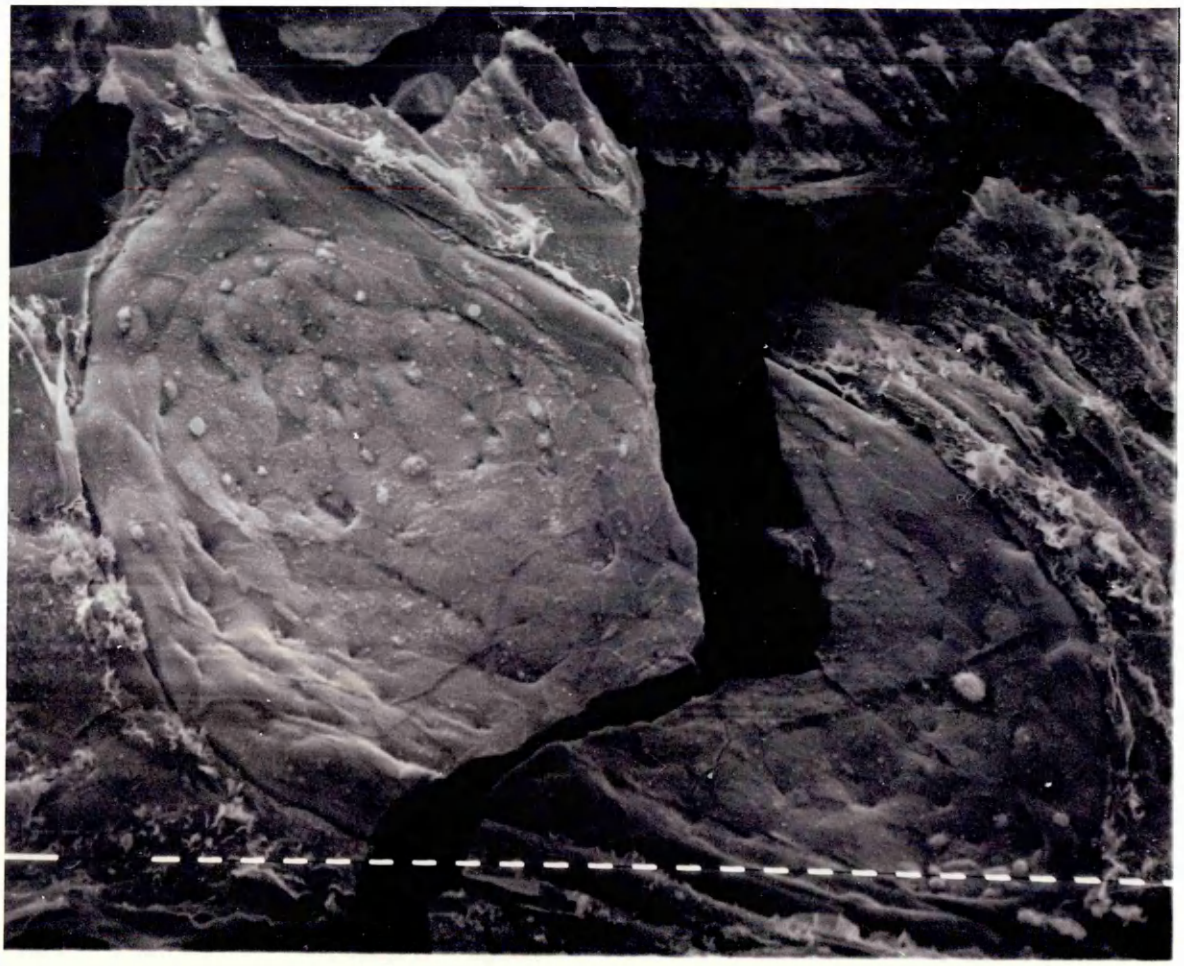
The primary culture was processed as in Sect. 2.64 without tannic acid postfixation and dehydrated by critical point drying. Extensive cracking of the primary culture has occurred as a result of cell shrinkage associated with critical point drying. The mammary epithelial cells can be seen growing underneath the surrounding feeder layer.

B. Low magnification scanning electron micrograph of primary breast culture (Mag.x520)

The primary culture was processed as described in Sect. 2.64 using tannic acid and dehydrated by freeze drying. Thermal cracking of the cultures was observed across the coverslip as a result of the differential cooling rates of the cells and their substrate. The morphology of the epithelial cells is preserved to a greater extent than the CPD preparations. The various blebs, which are present on the epithelial and fibroblast surfaces, probably represent artefacts due to tannic acid postfixation.

Cultures were prepared using CPD and FD techniques on three and six occasions respectively.

A



B

Fig. 5.2 Scanning electron microscopy of primary human breast cultures.

Primary cultures were prepared as in Sect. 2.72 on 10mm glass coverslips and grown for two weeks. They were fixed and processed for SEM as described in Sect. 2.64 without tannic acid postfixation. Dehydration was performed by CPD from liquid CO_2.

A. Scanning electron micrograph of primary breast culture (Mag. x2080).

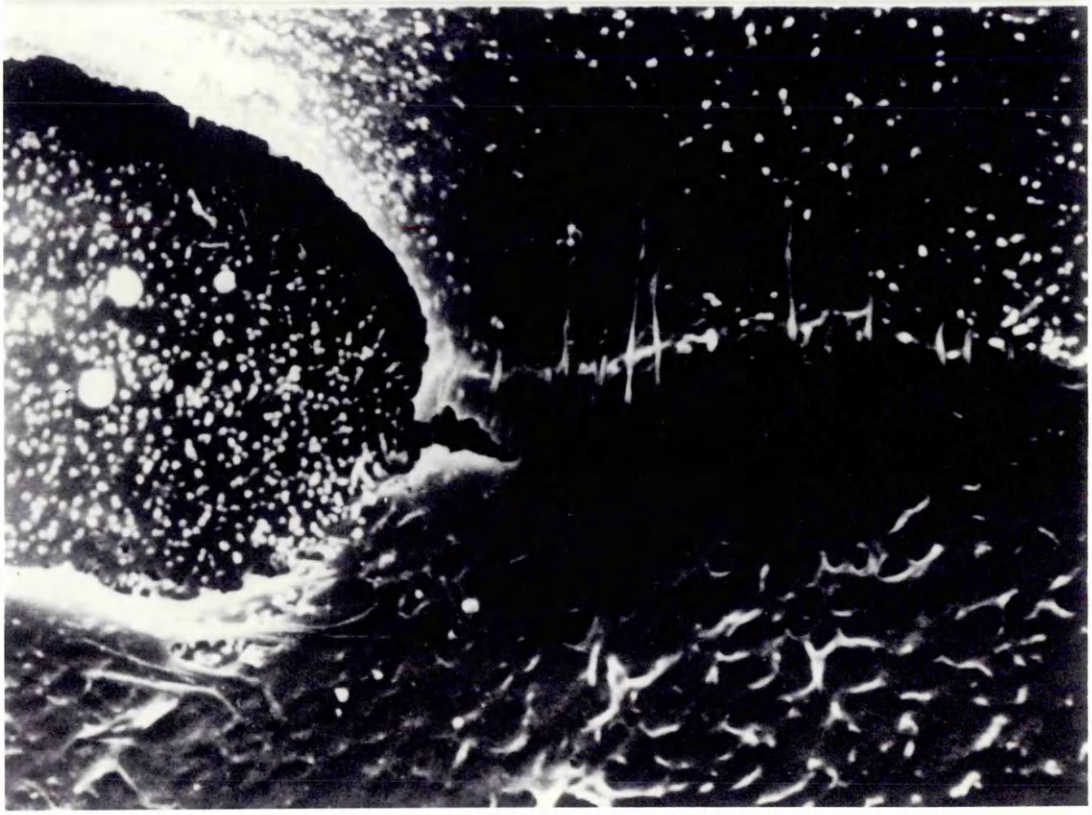
The heterogeneity of the surface morphology of mammary epithelial cells in primary culture is demonstrated. Many of the epithelial cells have a homogeneous microvillar distribution although the number of microvilli are variable. Some cells, such as the cell at the centre right, have microvilli present in specific regions of the cells.

B. Scanning electron micrograph of primary breast culture (Mag. x8320).

High magnification view of indentation in Fig. 5.2A. Cells between the superficial layer have short, stumpy microvilli and blebs on the cell surface. Intercellular bridges are present between the epithelial cells and there are ruffles on the cell which is at the forward right of the micrograph.

Cultures were prepared using the CPD technique on three differnt occasions.

A



B

while in the outer cell, there appear to be ruffles present on the cell surface.

An interesting feature in the CPD preparations was the presence of fillipodia which formed intercellular bridges. The significance of these structures is, however, unclear. Figs 5.2a and 5.2b also suggest that the epithelial cells may not always grow simply as monolayers as shown by the pit in these figures. This observation would require verification by sectioning of these cultures and further correlative studies by transmission electron microscopy (TEM).

It has previously been described that organoids of human breast epithelium attach to the feeder layer (see Fig.3.2) and then appear to migrate under the feeder layer (sect 4.23). The apparent edge of a breast colony, as determined by phase contrast miscroscopy, is illustrated in Fig 5.3a. There appears to be a distinct separation between the human breast epithelium and the mouse embryo fibroblast feeder layer. Examination of the edge of the organoid in Fig 5.3b provides further evidence to confirm the observations in sect. 4.23 that human mammary epithelial cells may grow underneath the feeder layer.

Examination of organoids, prepared by FD techniques reveals cells with differing surface morphologies. There appear to be at least 4 types of epithelial surface morphologies (see Figs. 5.3b and 5.4a):-

i) flattened cells with a relatively smooth homogeneous surface.
ii) cells with a homogeneous distribution of microvilli.
iii) cells with a heterogeneous microvillar distribution.
iv) rounded cells with a dense network of microvilli.

The blebs observed in the FD preparations probably represent fixation artefacts. They may be eliminated by fixation in glutaraldehyde alone (L. Tetley, personal comm.) although this would not allow adequate preservation of the surface morphology of the epithelial cells.

Fig. 5.3 Scanning electron microscopy of primary human breast cultures.

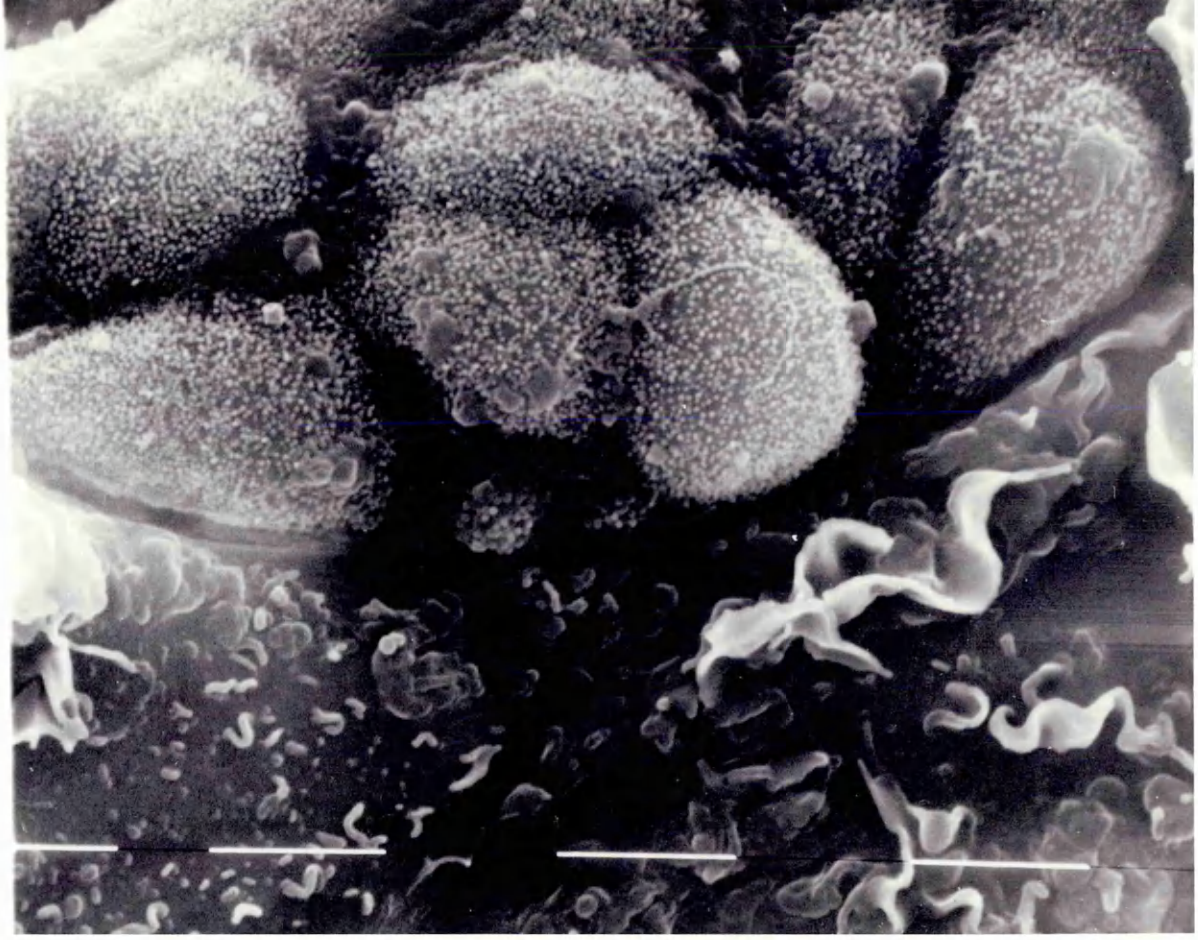
Breast cells were established in primary culture as in Sect. 2.72 on 10mm glass coverslips and maintained for two weeks. Fixation and processing was carried out as described in Sect. 2.64 and dehydration by freeze drying on six different occasions.

A. High magnification scanning electron micrograph of a primary breast culture (Mag. x4160).

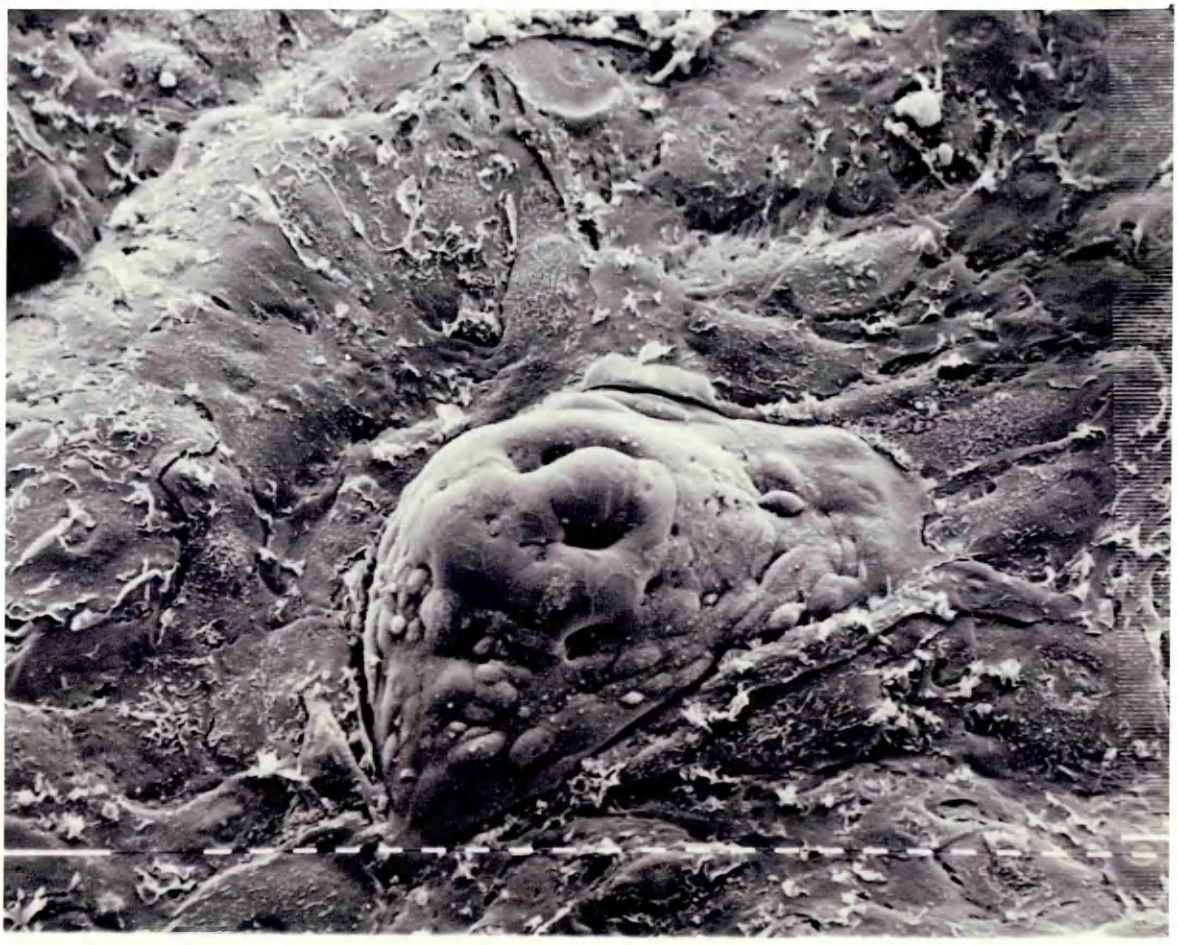
Primary cultures were established from an ER o/o biopsy, MTII, and grown in the presence of 0.02% (v/v) ethanol as control cultures. Short, stumpy microvilli are present on the epithelial cells while there are ruffles on the mouse embryo fibroblast cells.

B. Scanning electron micrograph of primary breast culture (Mag. x520).

Primary cultures were established from an ER o/o biopsy MTII. The outgrowth of cells from the organoid was found to migrate underneath the feeder layer.



A



B

Fig. 5.4 Scanning electron microscopy of primary human breast cultures.

Primary cultures were established from an ER +/+ biopsy, BIM4, as described in Sect. 2.72 on 10mm glass coverslips and grown for two weeks prior to processing (Sect. 2.64). Cultures were dehydrated by freeze drying.

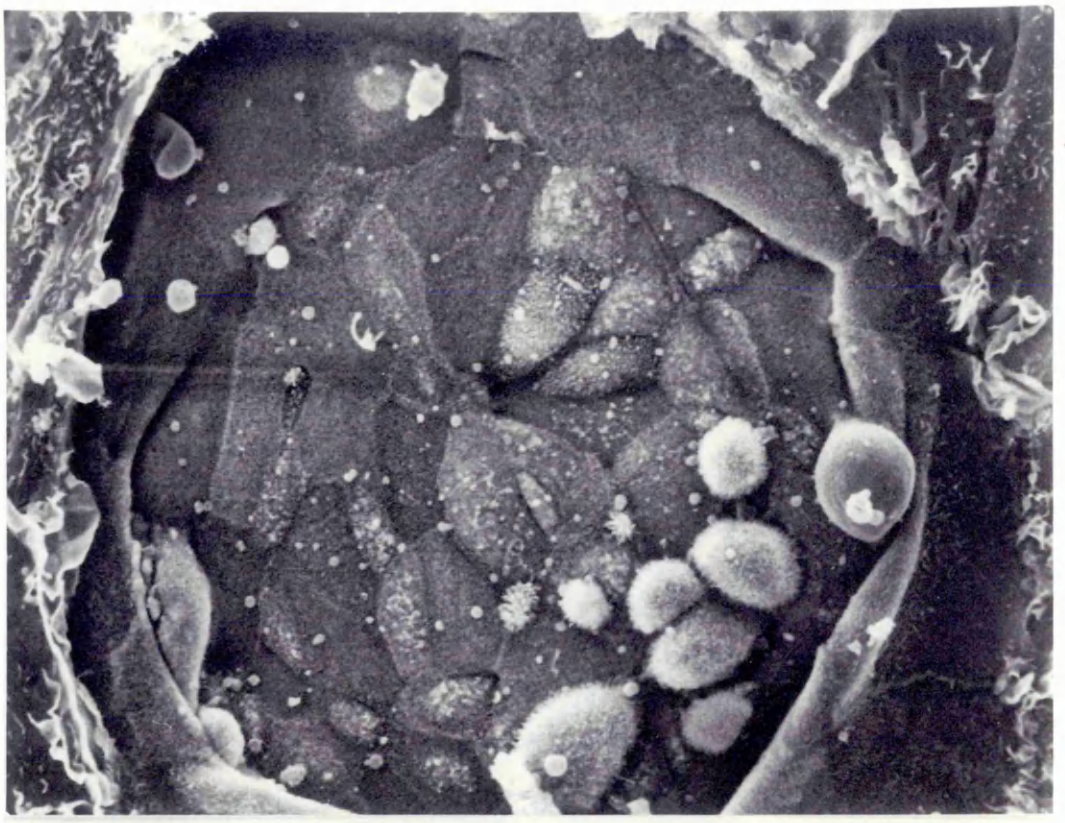
A. Scanning electron micrograph of primary breast culture (Mag. x2080)

The differing surface morphology of the primary culture was observed. Mammary epithelial cells were also found to grow underneath the feeder layer.

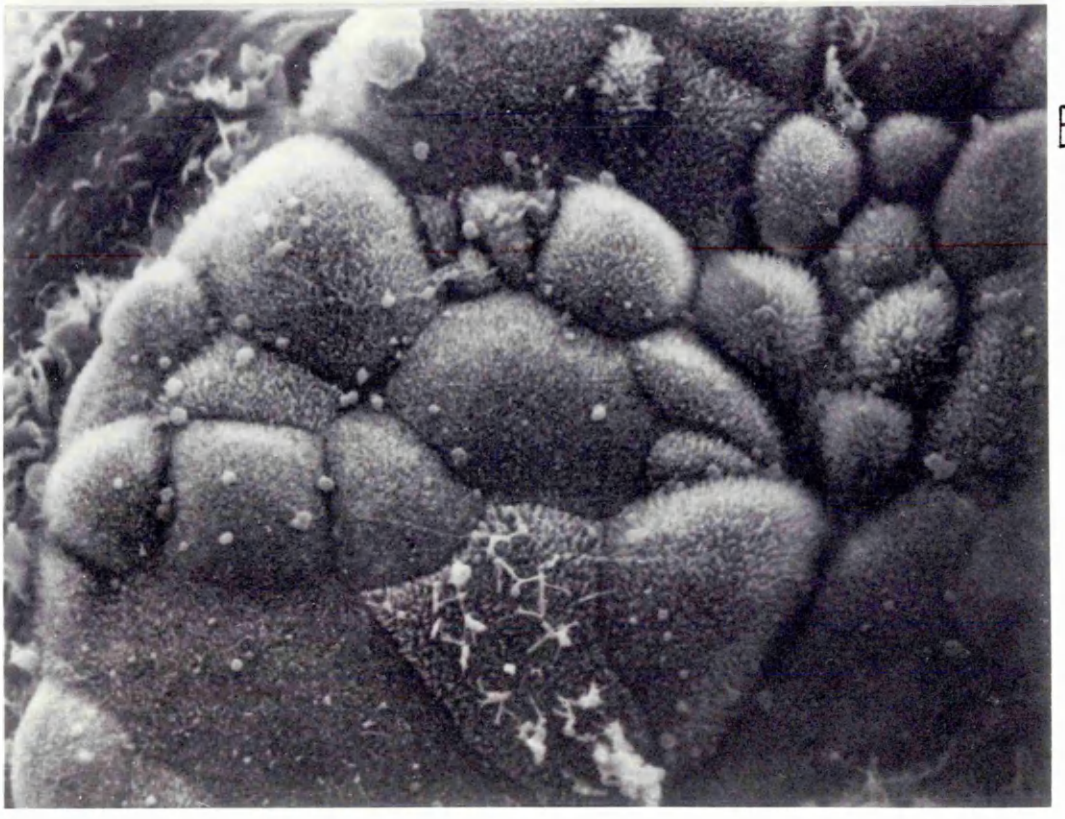
B. Scanning electron micrograph of primary breast culture (Mag. x 2080)

Changes in the surface morphology of primary cultures derived from ER +/+ biopsies were observed in response to oestradiol (10^{-9}M). The epithelial cells exhibited a greater degree of roundedness and showed increases in the number and length of microvilli. Blebs were also observed, although they are probably artefacts induced by the fixation procedure.

Experiments A and B were conducted on three and two occasions respectively.



A



B

5.23 Steroid effects on surface morphology.

The effect of oestradiol on the surface morphology of primary cultures derived from the same biopsy sample has been compared. Cells from ER positive biopsies grown in the presence of oestradiol were more rounded and had greater numbers of microvilli which were also longer (see Fig.5.4b) than those of identical cells grown in the absence of oestradiol (Fig. 5.5a).

In various primary cultures derived from ER positive tumours, analysis of the surface morphology in the presence of oestradiol shows the presence of epithelial cells with a homogeneous and dense distribution or microvilli (see Fig. 5.5b). Other categories of epithelial cells were not observed.

Cultures derived from ER negative tumours revealed the presence of an epithelial cell population in which individual cells had localised regions of very high surface morphology whilst the rest of the cell surface had a fairly homogeneous microvillar distribution (Fig. 5.6a). Comparison of the surface morphology of oestradiol-treated (Fig. 5.6a) and control organoid cultures (Fig. 5.6b) showed that there was little difference in microvillar organisation. The microvilli in these colonies whether E_2-treated or not, were short and stumpy as shown at higher magnification in Fig. 5.7. Differences were noted in the microvillar organisation and distribution when oestrogen-treated ER-positive and ER-negative cultures were compared (Figs. 5.5b and 5.6a respectively). The microvilli in the ER-positive derived cultures were longer and present at greater density than the microvilli in the ER-negative derived cultures.

In the presence of cortisol (10^{-7}M), two different populations of epithelial cells were observed. Some epithelial cells were found to have a homogenous distribution of small, stumpy microvilli while the other population has localised regions of high surface morphology (Fig. 5.8). Comparison of the

Fig. 5.5 Scanning electron microscopy of primary human breast cultures.

Breast cells were established in primary culture as in Sect. 2.72 on 10mm cover-slips and grown for two weeks. The cultures were subsequently processed according to the schedule in Sect. 2.64 and dehydrated by freeze drying. Blebs on the cell surface probably represent artefacts of fixation.

A. Scanning electron micrograph of a primary breast culture (Mag. x2080)

Control primary cultures from an ER +/+ biopsy, BIM4, were grown in the presence of 0.02% (v/v) ethanol. Cells at the periphery of the epithelial colony are relatively flat.

B. High magnification scanning electron micrograph of a primary breast culture (Mag. x8320).

Primary cultures from an ER +/+ biopsy were grown in the presence of 10^{-9}M oestradiol. The cells from the centre of the colony showed only one category of rounded epithelial cells with a homogeneous and dense distribution of long microvilli.

Experiments A and B were conducted on three and two occasions respectively.

A

B

Fig. 5.6 Scanning electron microscopy of primary human breast cultures

Primary cultures were established from an ER o/o biopsy as in Sect. 2.72 on 10mm glass coverslips and grown for two weeks in culture. Cultures were fixed and processed according to the methodology in Sect. 2.64 and dehydrated by freeze drying.

A. High magnification scanning electron micrograph of a primary breast culture (Mag. x4160).

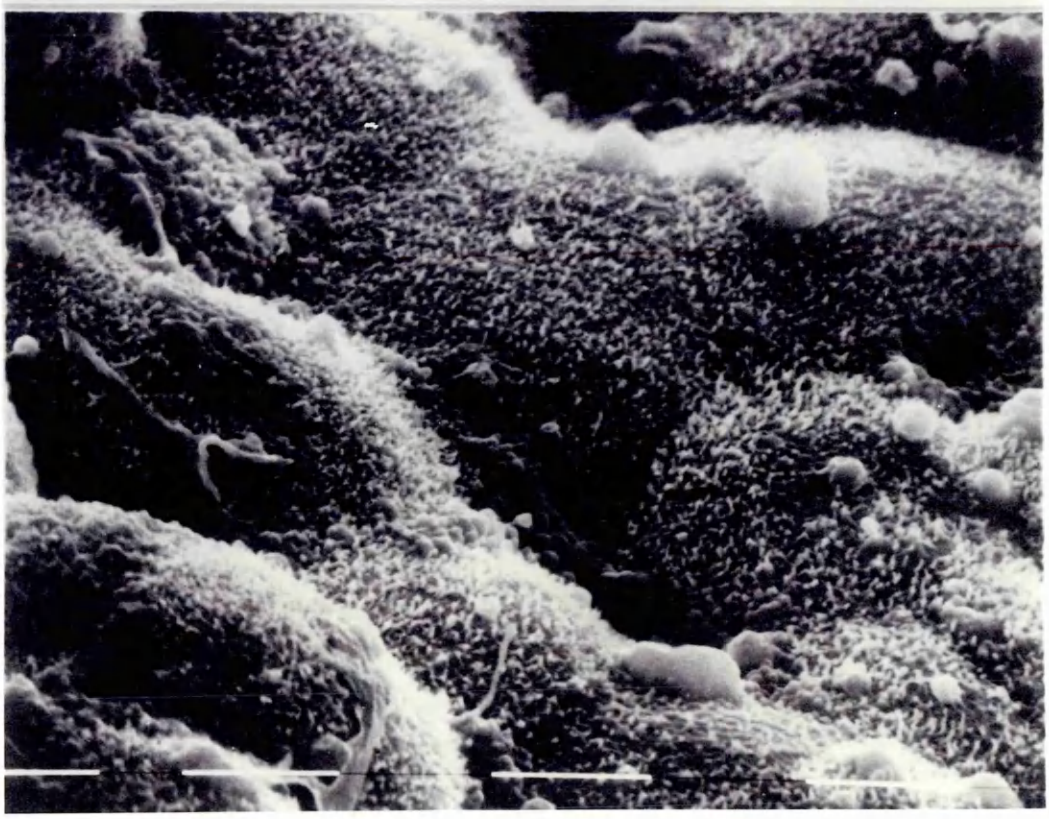
Primary cultures from an ER o/o biopsy were grown in the presence of 10^{-9}M oestradiol. The epithelial cells exhibited a heterogeneous surface morphology. The cell at the centre right had localised regions of very high surface morphology, while the rest of the cell surface had a fairly homogeneous distribution of short, stumpy microvilli.

B. High magnification scanning electron micrograph of a primary breast culture (Mag. x 4160).

Control primary cultures, derived from an ER o/o biopsy were grown in the presence of 0.02% (v/v) ethanol. The fine detail reveals a homogeneous distribution of small microvilli.

Experiments A and B were conducted on two and three occasions respectively.

A



B

Fig. 5.7 Scanning electron microscopy of a primary breast culture.

Primary cultures from an ER o/o biopsy were established as in Sect. 2.72 on 10mm glass coverslips. Cultures were grown for two weeks in the presence of 10^{-9}M oestradiol, processed for SEM and dehydrated by freeze drying (Sect. 2.64).

The high magnification scanning electron micrograph of the primary culture (Mag. x8320) shows the fine detail of the surface morphology. A homogeneous distribution of short, stumpy microvilli was observed.

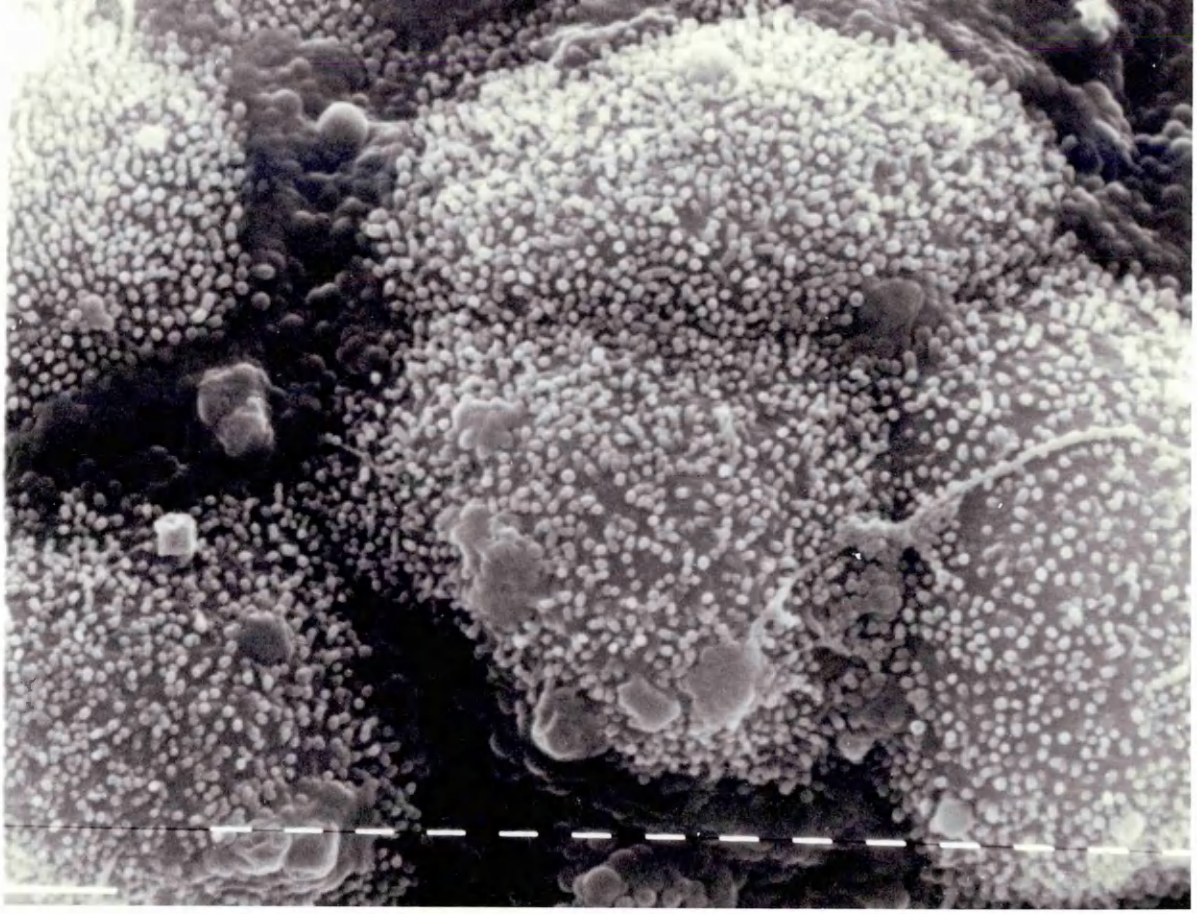
This experiment was performed on two occasions.

Fig. 5.8 Scanning electron microscopy of a primary breast culture.

Primary cultures from an ER +/+ biopsy, BIM4, were established on 10mm coverslips as described in Sect. 2.72. Cultures were grown in the presence of cortisol (10^{-7}M), processed for SEM and dehydrated by freeze drying as in Sect. 2.64.

Different types of surface morphology were observed in the scanning electron micrograph (Mag. x2080). The epithelial cells showed an intermediate degree of roundedness between the control and oestradiol treated cultures.

This experiment was performed on one occasion in duplicate.



microvilli of oestradiol and cortisol treated cultures from the same biopsy have shown that oestradiol treated cultures have more rounded cells with an increased density and length of microvilli. (See Figs. 5.4b and 5.8).

5.3 Discussion

The multiple surface morphologies of primary human mammary epithelial cell cultures have been described. The fine detail of the surface morphology has been found to be influenced by both fixation and dehydration procedures. Using CPD techniques, extensive cracking of the cultures occurred through shrinkage, but, allowed the identification of two epithelial cell types. Additionally, the presence of fillipodia at cell-cell junctions was observed, although their significance is unclear. The preservation of surface detail was improved by freeze drying dehydration procedures although the problem of thermal cracking was encountered. Greater differences in the surface morphology were apparent with four different epithelial cell types distinguished, confirming that there was a distinct improvement in the maintenance of surface morphology relative to CPD techniques.

Oestradiol was found to alter the surface morphology of primary cultures from ER-positive tumours but not the ER-negative tumours. The modification in surface morphology by oestradiol (10^{-9}M) resulted in the presence of a single population of very rounded epithelial cells with a dense and homogeneous microvillar distribution. The effect of cortisol (10^{-7}M) on surface morphology resulted in the identification of two epithelial populations with an intermediate degree of roundedness between the control and oestradiol-treated cell cultures.

6 General Discussions.

Specific discussions are presented at the end of chapters 3, 4 and 5. The chief conclusions are reiterated in this chapter and considered in relation to current concepts on oestrogen regulation of epithelial cell proliferation.

Feeder Layers.

Primary cultures of human mammary epithelial cells were established in order to minimise the selection of variant populations of cells within the original tumour cell population. The data obtained during these studies have highlighted the heterogeneous nature of the primary breast tumours. However, the data described in this thesis show that both ER positive and ER negative cells can be established in primary culture.

Primary human breast cultures have routinely been established on feeder layers of mitomycin - C treated mouse embryo fibroblasts following enzymatic disaggregation of human breast tumours. The most successful of the feeder layers proved to be the NIH - 3T3 and STO mouse embryo fibroblast cell lines on which approximately 80% of the breast tumour biopsies were successfully established in primary culture. The three-fold difference in the ability of the NIH-3T3 feeder layer over the Swiss-3T3 feeder layer, to select for the growth of breast epithelial cells remains unclear. It may, perhaps, be related to the provision of extracellular matrix components which promote both attachment and subsequent growth.

In terms of the mode of growth of the primary cultures, they are all derived from organoids which are small clumps of epithelial cells that attach and spread out on the feeder layer. Primary cultures grew either on (Fig. 4.3a) or underneath the feeder layers (Fig. 4.2b). In the latter case, the organoids plated onto the feeder layer appeared to migrate underneath

the feeder layer as shown by the immunofluorescence (see chapter 4) and autoradiographic studies (see chapter 3). The use of autoradiography to assess hormone dependence was abandoned after preliminary studies revealed the extensive variation in the (^{3}H) thymidine labelling patterns within single cultures.

Responses to hormones.

Selection of both the more differentiated premenopausal ER +/+ and the less well differentiated postmenopausal ER o/o groups occurred on the NIH - 3T3 feeder layer. A mitogenic response to cortisol and oestradiol alone was observed for the ER +/+ categories, while the ER o/o groups generally showed little response. The most significant increases in MTCA and MTCN occurred in the well differentiated ER +/+ groups in response to the cortisol/oestradiol combination. A synergistic response to the hormone combination was observed in the poorly differentiated ER o/o groups in terms of colony area but the effects on colony number were variable. There was no degree of overlap in the responses of the ER +/+ and o/o groups to cortisol alone or the cortisol/oestradiol combination with respect to either MTCA or MTCN. The response of the ER +/+ group to the oestradiol/cortisol combination was twice that of the ER o/o group, suggesting that half of the response is ER independent. This may occur through cortisol-priming of an oestrogen sensitive autocrine mechanism.

Growth on the STO feeder layer suggested that the premenopausal ER +/+ and premenopausal ER o/o groups were selected for on this feeder layer. Cortisol and oestradiol alone did not generally appear to exert a direct mitogenic action on cell proliferation. Primary cultures appeared to respond to cortisol and oestradiol in combination, independently of menopausal and, perhaps, receptor status as there was a greater response in the ER o/o group than the ER +/+ group. It was interesting to note that the postmenopausal ER o/o and premenopausal ER +/+ groups showed the greatest response to cortisol and oestradiol in combination. These

results, once again, suggest that some of the mitogenic response may occur independent of the ER.

Cultures grown on the STO feeder layer showed a direct mitogenic response to EGF in terms of both colony area and plating efficiency. Cortisol, in combination with EGF elicited a synergistic mitogenic effect, but, only an additive effect on plating efficiency.

Prolactin was found to significantly stimulate the growth of primary cultures while there was an accompanying decrease in colony number. The steroids cortisol and oestradiol, either alone or in combination, antagonised the mitogenic action of prolactin and also reduced the plating efficiency.

Further evidence for the involvement of an additional mechanism(s) in growth regulation of breast epithelium (apart from an endocrine mechanism) has come from the experiments with conditioned medium from human breast stromal fibroblasts. The results suggest the existence of a paracrine-type mechanism in the modulation of mammary epithelial cell growth.

In summary, external signals such as hormones and growth factors have exerted differential effects on both the plating efficiency and growth of the primary breast cultures. The mitogenic effects, observed in response to hormones and growth factors, have been of a relatively small magnitude with regard to the *in vivo* effects. The possible existence of non-endocrine growth regulatory mechanisms such as autocrine and paracrine mechanisms has been suggested. The data presented, confirm that there is a complex interplay of hormonal and growth factor regulatory mechanisms in the growth of mammary epithelium.

Cell Identification.

Characterisation of the human mammary epithelial nature

nature of the primary cultures was performed by immunofluorescence studies using a panel of antibodies. The mammary origin was confirmed by intense staining of the primary cultures with the HMFG - 2 monoclonal antibody. This has previously been reported to show a stronger reaction with both ductal carcinomas and cell lines derived either from them or their metastatic lesions (Burchell et al, 1983). The heterogeneity of staining with the M8 monoclonal antibody against the epithelial membrane antigen, confirms the observations of others (Edwards & Brooks, 1984). This heterogeneity has been attributed to either random antigen expression or different physiological states within the mammary epithelium.

The epithelial nature of the primary breast cultures was confirmed by specific staining with the anti-desmoplakin antibody in addition to the studies with the keratin antibodies. The staining with the wide-spectrum antibody alone was not adequate to confirm the true epithelial nature of the primary cultures since the basal myoepithelial cells also stain positively with the antibody. However, unequivocal proof of the glandular epithelial nature of these cultures was obtained by the specific staining obtained with the LE61 (anti-keratin 18) antiserum which stains glandular epithelium but not the basal cells.

Differences were also observed in the intensities of the fluorescence signal obtained for the LE61 staining. Localised regions of breast colonies, or less commonly single cells, exhibited intense staining. The variations in the fluorescence signals are unclear but may reflect the heterogeneous nature of the primary human mammary epithelial cells.

Vimentin, the intermediate filament type associated with mesenchyme-derived cells, was co-expressed in the primary breast cultures. This observation is in agreement with other

reports of vimentin co-expression in epithelial cultures (Franke *et al*, 1979; Ramaekers *et al*, 1983; Dairkee *et al*, 1984). It is particularly interesting since vimentin is not expressed in solid epithelial tumours *in vivo*, while its expression appears to be induced in culture. Vimentin expression *in vivo* has not been examined during the course of this study, but it is currently regarded that vimentin is co-expressed *in vitro* due to the release of the epithelium from the *in vivo* three-dimensional constraints of intact tissue.

The effects of various steroids and retinoic acid, on the expression of keratin 18, a marker of simple epithelium, were assessed by further immunocytochemical studies. Primary cultures showed little response to steroids, with the exception of the cortisol/tamoxifen combination which resulted in a significant change in the morphology of the epithelial cells. However, retinoic acid appeared to elicit a phenotypic alteration in the pattern of keratin 18 expression which seemed to have a more diffuse and linear arrangement. These observations with retinoic acid are similar to previous findings with primary cultures of human endometrial cells (Field, 1985). The apparent changes in the expression of keratin 18 need not necessarily occur through a reduction in the expression of the keratin 18, but may perhaps, represent the masking of antigenic determinants which are detected by the LE61 antiserum. These observations are particularly interesting since it is recognised that vitamin A has an important role in the regulation of epithelial growth and differentiation (Zile & Cullum, 1983). Moreover in some cancers, vitamin A has been attributed a protective role (Newberne & Rogers, 1981). If these observations on the apparent change in cellular phenotype are confirmed by biochemical and immunological techniques, these results would suggest that differentiation may, perhaps, induce a change in the keratin network. A correlation has also been proposed between changes in cytoarchitecture and oncogenic

transformation (see Sect. 1.611). These preliminary results, therefore, suggest that the possibility exists to manipulate the state of differentiation of tumour cells and, perhaps, their malignant properties.

Ultrastructure.

The surface morphology of primary human mammary cultures was examined in order to determine the epithelial nature of the breast colonies and to assess the response to hormones. The presence of microvilli on cultures derived from both ER-positive and ER-negative biopsies confirmed their epithelial nature. With regard to the responses to hormones, oestradiol was found to increase the degree of roundedness of the epithelial cells as well as the number and length of microvilli in cultures derived from ER positive biopsies. The morphology of cultures from ER negative biopsies was, however, unaffected by the addition of oestradiol. These observations on the changes in surface morphology in response to oestradiol are in agreement with the findings of Chambon et al, (1984) who found both an increased number and length of microvilli of some of the oestradiol-treated organoids. These authors also observed the presence of blebs or knobs in response to oestradiol. In the studies reported in this thesis, blebs have also been observed although they occurred regardless of the type of hormone treatment. These blebs probably represent artefacts and may be avoided by a modification of the post-fixation technique (L.Tetley, pers. comm.). Increased length and number of microvilli on breast epithelial cells in response to steroids supports the previous findings from my laboratory on the effects of oestradiol and medroxyprogesterone acetate on human endometrial carcinoma primary cultures (Field, 1985).

It is particularly interesting to note that cultures derived from ER positive tumours retain the ability to respond to oestradiol in ultrastructural terms. Similar cultures

established in exactly the same manner, however, fail to show a mitogenic response to oestradiol. These effects of oestradiol on surface morphology demonstrate that these primary cultures retain a functional state of differentiation. These differential effects of oestradiol on cellular proliferation and differentiation are in agreement with the findings of Sonnenschein and Soto, (1980), and support the view that breast epithelial cells respond directly to oestradiol in receptor-mediated modification of various parameters. However, the mitogenic response to oestradiol, seen *in vivo*, is apparently mediated through an indirect mechanism such as the stromal growth factors suggested in this thesis.

Further experiments.

Some of the preliminary data presented in the preceding chapters warrants further investigation. In particular, the growth regulation experiments with the various hormones, growth factors and conditioned medium which have suggested indirect mechanisms for oestrogen action on cellular proliferation should be extended. The paracrine-type mechanism, indicated by the conditioned media experiments, should be investigated further. The ultimate aim would be to purify the undefined mitogenic "factor(s)" present in the conditioned media from human breast stromal fibroblasts stimulated with oestradiol. This may necessitate growing the cells in serum-free medium in order to remove interfering serum proteins for the subsequent purification. Alternatively, the breast fibroblasts may be grown in the defined medium for human diploid fibroblasts, MCDB 104, and the mitogenic activity (if any) of this conditioned medium, determined and subsequently purified. It is indeed possible that the undefined mitogenic factor(s) may be present in higher amounts within the breast stromal cells and it may prove more fruitful to isolate the factor(s) from the cells themselves. The purification and subsequent sequence analysis of the mitogenic activity should enable a comparison with other characterised growth factors. The purification of the mitogenic activity would also enable an assessment of the relative stimulation of breast stromal fibroblasts and breast epithelial cells and improve our understanding of tissue-specific growth factor activity.

Similarly, the autocrine-type mechanism indicated by the effects of the cortisol/oestradiol combination on primary breast cultures merits further investigation. A similar approach to the one outlined above would probably be necessary. This may be aided by the growth of the primary breast cultures in the defined medium MCDB 170 which is now

available for the clonal growth of normal human mammary epithelial cells. However, this medium may have the drawback of a greater degree of selection although it would dispense with the requirement for serum and feeder layers. This would also simplify the interpretation of the responses to hormones and growth factors and permit the construction of dose-response curves for hormones, growth factors and also various drugs.

The present primary culture system has the potential to be developed into an assay for hormone dependence based on the detection of ER by immunocytochemical techniques. This would permit the responses to external signals to be related to ER levels present in the primary cultures and thereby provide an assessment of the degree of hormone dependence in the cultures and, perhaps, the parent tumours. The heterogeneity of the cultures may indeed provide a more accurate representation of the hormone dependence and, therefore enable patients to be stratified in terms of treatment on the basis of the hormonal stimulation experiments.

The preliminary data from the SEM and immunofluorescence studies has suggested that a functional state of differentiation is retained and that the differentiated state (and malignant properties) of the breast cells may be manipulated. The apparent changes in keratin 18 require verification by biochemical and/or immunological techniques. Other accompanying changes in the cytoskeleton should be considered e.g. actin expression, since they may be related to oncogenesis and growth factor activity. Additionally, the integrity of the cell-cell communication should be investigated by immunocytochemical techniques (using the anti-desmoplakin antisera) to assess changes induced by external signals and any modification in cell-cell contact related to the receptor status.

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