EGF AND PDGF RECEPTORS IN ENDOMETRIAL CANCER

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ABSTRACT

Despite its relatively good long term survival compared to other cancers in the female, endometrial carcinoma still kills 20% of those women who develop the disease worldwide. Abnormalities in growth factor receptors have been shown to be important in both the prognosis and probably the malignant transformation of the two other oestrogen-dependent cancers, breast and ovary, which suggests that similar defects may occur in endometrial cancer.

In this thesis, two of these growth factor receptors, those for the epidermal and platelet-derived growth factors, are studied in endometrial cancer at the levels of the gene and expression of the proteins on the cell surface, in an attempt to detect abnormalities which might be implicated in carcinogenesis.

No amplification or rearrangement of the EGFR gene was detected on 13 tumour samples initially, or of the EGF, PDGF α- and β-subunit receptor genes subsequently on a mixture of tumour samples and cell from endometrial cancer cell lines.

The size of the EGF and PDGF receptor proteins and the activation of their tyrosine kinase domains was assessed in an oestrogen responsive (Ishikawa) and an oestrogen independent (HEC-1-A) endometrial cancer cell line. Functioning receptors for EGF were demonstrated in both cell lines but no PDGF receptors were detected. EGF-binding studies were carried out and appropriate affinity constants and receptor numbers obtained in both cell lines.

The growth of Ishikawa and HEC-1-A cells in culture was studied but because of the rapid growth of both lines in the absence of serum no meaningful mitogenic effects were shown by any growth factor or steroid.

Phorbol ester and TGF-β inhibited the growth of HEC-1-A and Ishikawa cells, respectively. Of particular interest was that, despite the reported sensitivity of Ishikawa cells to oestrogen, no consistent stimulation was observed.

The growth of both cell lines in the absence of serum suggested that the cells might be secreting growth factors and conditioned medium was obtained from long term, large scale cultures. The medium was concentrated and passed through a size exclusion column and the fractions assayed for DNA synthesis and competition for EGF-binding to Swiss 3T3 cells. No consistent effect on either of these assays was shown and no further investigation was undertaken.

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LIST OF ABBREVIATIONS

A_{280}	absorbance at 280 nm
αTPA	alpha form of TPA
APC	adenomatous polyposis coli gene
APS	ammonium persulphate
bFGF	basic FGF
BES	N,N-bis(2-Hydroxyethyl)-2-aminoethanesulfonic acid
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CCM	concentrated, conditioned medium
cDNA	complementary DNA
cm	centimetre
CM	conditioned medium
cpm	counts per minute
CSF	colony stimulating factor
CSF-1	colony stimulating factor-1
CSF-1R	colony stimulating factor-1 receptor
DAG	1,2-diacylglycerol
dATP	deoxyadenosine triphosphate
DC	direct current; dextran:charcoal
DCC	deleted in colon cancer gene
DC-FCS	dextran:charcoal-stripped FCS
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
EDTA	ethylenediamine tetra-acetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ER	oestrogen receptor
FAP	familial adenomatous polyposis
FCS	foetal calf serum
FGF	fibroblast growth factor

FIGO	International Federation of Gynaecologists and Obstetricians
FPLC™	fast protein liquid chromatography
g	gram
G1,2,3	grade 1,2,3; first growth phase of cell cycle
GAP	GTPase activating protein
GFR	growth factor receptor
GDP	guanosine diphosphate
GTP	guanosine triphosphate
h	hour
HCl	hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ICI 164,384	N-n-butyl-11-3,17β-dihydroxyoestra-1,3,5(10)-trien-7α-yl-N-methylundecamide
^{125}I-EGF	^{125}I-labelled EGF
IGF-1	insulin-like growth factor-1
IGF-1R	insulin-like growth factor-1 receptor
IL-3	interleukin-3
IP_3	1,4,5-triphosphate
kb	kilobase
kD	kilodalton
l	litre
LICR	Ludwig Institute for Cancer Research
LOH	loss of heterozygosity
M	mole, molar
MCC	mutated in colon cancer gene
µg	microgram
µl	microlitre
mg	milligram
min	minute
ml	millilitre
mM	millimolar
mRNA	messenger RNA
MW	molecular weight
ng	nanogram
nM	nanomolar
NRK	normal rat kidney

$NR6^+$	subclone of Swiss 3T3 cells with transfected EGFR
OCs	oral contraceptives
OD	optical density
4-OHtam	trans 4-hydroxytamoxifen
O-2A	oligodendrocyte-type 2 astrocyte
p	short arm of chromosome
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PDGF-αR	PDGF alpha receptor subunit
PDGF-βR	PDGF beta receptor subunit
PDGFR	PDGF receptor
pg	picogram
PI	phosphatidylinositide
PI3-K	phosphatidylinositol 3'-kinase
PKC	protein kinase C
PLC-γ	phospholipase C-γ
pM	picomolar
PMSF	phenylmethylsulfonyl fluoride
PR	progesterone receptor
PTK	protein-tyrosine kinase
q	long arm of chromosome
R5020	promegestone
RB	retinoblastoma (gene/protein)
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
RR	relative risk
RU486	17β-hydroxy-11β-(4-dimethylamino-phenyl)-17α-(1-propynyl)-estra-4,9-dien-3-one
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	standard error of mean
SSC	standard saline citrate
SSV	simian sarcoma virus
TAE	tris-acetate EDTA
TE	tris-EDTA

TEMED	N,N,N',N'-tetramethylethylenediamine
TGF-α	transforming growth factor-α
TGF-β	transforming growth factor-β
tris	tris(hydroxymethyl) amino methane
TPA	phorbol 12-tetradecanoate 13-acetate
UV	ultraviolet
v-*onc*	viral oncogene
v/v	volume for volume
WT1	Wilms tumour gene
w/v	weight for volume

1 INTRODUCTION

The hypothesis underlying this thesis is that abnormalities in the structure or number of receptors for epidermal growth factor (EGF) and/or platelet-derived growth factor (PDGF) are present in endometrial cancers and may be implicated in tumour development. These abnormalities could occur either at the level of the gene or at any subsequent stage culminating in the production of receptor protein on the cell surface.

An objective of this work was to gain practical experience in a wide range of scientific techniques in a limited period. As science impinges on clinical medicine, e.g. the use of growth factors in wound healing and the stimulation of leukocyte proliferation, it becomes increasingly important for clinicans to understand the language, potential and limitations of laboratory-based research.

In this introduction endometrial cancer with particular emphasis on the factors potentially relevant to the development of the cancer will be described. The structure and function of EGF and PDGF and their receptors will be reviewed and the evidence that alterations in growth factors and their receptors are implicated in the development of a variety of malignancies outlined. Examples of tumours in which these alterations have been detected will be discussed. This discussion provides the rationale for the work carried out for the thesis, consisting of studies of the PDGF, EGF and their respective receptors in endometrial tumours and endometrial cancer cell lines.

1.1 Endometrial Carcinoma

Carcinoma of the endometrium is the most common malignancy of the genital tract in Western women and the fifth leading cancer in women world

wide. Despite the fact that endometrial cancer usually presents early in its development with abnormal vaginal bleeding, the 5-year survival remains at only 79-84% in Western cultures (Bonett *et al.*, 1990).

1.1.a Epidemiology

The descriptive and analytical epidemiology of endometrial cancer have been recently reviewed in detail (Parazzini *et al.*, 1991; Mack *et al.*, 1992) and the information presented in this section is based on these reviews. Worldwide the incidence of endometrial cancer varies considerably ranging from Western countries where it accounts for 8-10% of all cancers in women to the developing countries and Japan where the incidence is only 2-4%, with the lowest incidence rates seen in India and southern Asia (1%). However, the low figures in developing countries may be complicated by inadequacies in the registration systems in these countries. Despite this factor, endometrial cancer appears to be associated with Western society as, within individual countries, it is more common in urban than rural populations. Race also appears to be important as the incidence of endometrial cancer in white women is about double that of black women in most areas surveyed in the U.S.A..

Generally throughout the world the incidence of endometrial cancer has increased over the past 20 years in line with the increase in life expectancy in women. In the United States, however, the number of new cases rose dramatically through the 1960's, peaked in the early 1970's and slowly declined in the 1980's (Sutton *et al.*, 1990). Although errors in histological classification in the direction of over-diagnosis of cancer may have accounted for part of the increase, the reason commonly given for this discrepancy is the widespread use of exogenous oestrogens in the US over this period (Weiss *et al.*, 1976) as the mortality from endometrial cancer remained relatively stable. As the risks of unopposed oestrogen in the menopause became evident, their use declined in North America but

increased in other countries where the incidence of endometrial cancer has continued to rise e.g. Denmark, Sweden.

The risk factors associated with an increase in endometrial cancer have been widely documented but not usually subjected to rigorous statistical scrutiny. In particular, many studies have not adequately controlled for the impact of confounding variables such as obesity and progesterone deficiency. Obesity is one of the most important risk factors both before and after menopause with a relative risk (RR) of between 2 and 10 compared to women of normal weight and is stronger in women who have not used oestrogen replacement. There is also an indication that obesity in early life (second or third decade) may also increase the subsequent risk of endometrial cancer.

Late onset of menarche leads to about a 50% reduction in risk although this factor is complicated by weight as obese girls also have an earlier menarche. Similarly, a prolongation of menstruation due to a late menopause increases the RR between 1.5 and 2.5. The delivery of a child significantly reduces the risk of endometrial cancer with some, but not all, studies showing a decrease in incidence with increasing parity. The information is less clear on the effect of incomplete pregnancies (abortions) or age at first or last pregnancy.

There is an increased risk of endometrial cancer associated with oestrogen replacement therapy which rises with the dose and duration of use and this risk persists several years after the treatment stops. The addition of an adequate dose of cyclical progesterone for at least 10 days appears to reduce the risk of endometrial carcinoma to that of the normal female population (Persson *et al.*, 1989).

Interestingly, the antioestrogen, tamoxifen, has also been reported to increase the risk of endometrial cancer (Fornander *et al.*, 1989). In this Swedish study of 1846 women randomised to adjuvant tamoxifen in the treatment of early breast cancer the frequency of uterine cancers in tamoxifen users was 6.4 times that of control subjects and more marked in

the group taking tamoxifen for 5 years than those on tamoxifen for 2 years. This result is consistent with experimental evidence of the partial oestrogen agonist effect of tamoxifen on endometrial cancer cells *in vitro* (Anzai *et al.*, 1989). The results of the Scottish Cancer Trial of adjuvant tamoxifen in breast cancer, however, have not confirmed this effect of tamoxifen although a lower dose was used than in the Swedish study (Stewart and Knight, 1989).

Although the numbers of women reaching the menopause who have been on oral contraceptives (OCs) for significant periods of time is still small the accumulated data suggests that OCs reduce the risk of endometrial cancer by about 50% and that this reduction in risk persists for at least 10-15 years after stopping the use of the OC.

Endometrial cancer is a disease of white Western women and so is associated with increased dietary intake of protein, fat and calories. The relationship between diet and endometrial cancer is difficult to unravel because of the complicating effects of other variables, in particular, obesity which is also related to diet. Similarly, a history of diabetes or hypertension is reported to increase the frequency of endometrial cancer even when weight and socioeconomic status are taken into consideration. However there are few studies in which these variables are adequately controlled.

Smoking also appears to protect against endometrial cancer after the menopause. This may be due to a number of factors including the earlier onset of the menopause in smokers, reduced oestrogen levels because of the induction of liver enzymes and leaner body mass in smokers compared to non-smokers.

There also appear to be fundamental differences between endometrial carcinomas developing in women taking, and those not taking, oestrogens. Ninety-five percent of women with endometrial cancer taking oestrogen have Stage I disease at presentation compared to 74% of those not taking oestrogens (Studd, 1976). While it has been suggested that this reflects increased surveillance of women taking oestrogens, other findings suggest

that there are biological differences between the cancers induced by exogenous oestrogens and those occurring spontaneously. Patients with carcinoma not taking oestrogens are of lower parity, have a later menopause and are more likely to be obese, hypertensive and diabetic than women developing endometrial cancer on oestrogens (Silverberg *et al.*, 1980). Other workers have shown similar findings in that women with endometrial cancer not taking oestrogens are older, weigh less and their cancers have a poorer prognosis. Their tumours are more likely to be poorly differentiated, myometrial invasion is more common and, if present, more deeply invasive and lymph nodes and pelvic washings are more often positive (LaVecchia *et al.*, 1982).

1.1.b Pathogenesis

The most readily identifiable cause of endometrial cancer is the prolonged exposure of the endometrium to unopposed oestrogen. Epithelial cells of the endometrium divide in response to oestrogen, but the simultaneous presence of progesterone reduces or even inhibits mitosis (reviewed in Lane *et al.*, 1988). In the absence, or deficiency of, progesterone, proliferation can proceed unchecked and ultimately lead to cancer of the endometrium. All the risk factors described in Section 1.1.a which are associated with an increased chance of a woman of developing endometrial cancer can be explained by this model.

The association of unopposed oestrogen and endometrial cancer is seen in pharmacological, pathological and physiological settings. Case control studies in the 1970's established a 4- to 8-fold increase in the risk of endometrial cancer for women on oestrogens alone and further studies have confirmed this (reviewed in Mack *et al.*, 1992). For example, it was demonstrated that users of a sequential birth control pill, Oracon, had a risk of endometrial cancer 7.3 times greater than that of women not using hormonal contraception (Weiss and Sayvetz, 1980). Oracon contained a very

high dose of daily oestrogen - 100μg of ethinyl oestradiol - and a weak progestin, dimethisterone 25mg daily for only 5 days of each month.

Conversely, oral contraceptives (OCs) have a strong protective effect on the development of endometrial cancer (reviewed in Mack *et al.*, 1992). The use of OCs involves daily doses of oestrogen and progestin for 21 days followed by 7 days when no hormones are taken. The risk of endometrial cancer is presumably reduced because endogenous oestrogen levels are very low during the 7 hormone-free days as a result of the inhibition of ovulation which is one of the major contraceptive actions of OCs. In the proliferative phase of the menstrual cycle, oestrogen levels rise progressively in the absence of progesterone which is not produced until the time of ovulation mid cycle.

In a similar way, increasing parity progressively reduces the risk of endometrial cancer because, although oestrogen levels are very high in pregnancy, progesterone levels are also high. In contrast, anovulation from whatever cause leads to an absence of progesterone and often increased oestrogen production and therefore, any condition resulting in anovulation also increases the risk of endometrial cancer. Obesity and polycystic ovarian syndrome are both associated with chronic anovulation and an increase in endometrial cancer.

After the menopause endogenous levels of progesterone are usually very low and thus, any condition leading to an increase in oestrogen is also associated with an increase in endometrial cancer. Increases in circulating oestrogens may be due to the administration of unopposed oestrogen to treat menopausal symptoms, the abnormal production of oestrogen from oestrogen-secreting tumours, generally of the ovary, and in obese post-menopausal women. Aromatization of androstenedione to oestrone in adipose tissue is the major pathway of oestrogen production in postmenopausal women and raised plasma oestrone levels are found in obese older women, in those with endometrial hyperplasia and in postmenopausal women with endometrial cancer (MacDonald and Siiteri, 1974).

The risk factors outlined above only account for 30-60% of cases of endometrial cancer, however, and the interaction between the various factors is not well defined. 75% of endometrial carcinomas occur in post-menopausal women, most of whom are not on oestrogen replacement therapy and many of whom are not obese. As with cancers of other organs, other mechanisms must be implicated in the development of endometrial cancer. This is consistent with the multistep model of tumourigenesis (Foulds, 1958; Nowell, 1976) which proposes that two or more steps are required for the induction of cancer; namely initiation, promotion and progression. It is still not clear at which of these steps oestrogen is acting in the development of endometrial cancer.

1.1.c Histopathology

Although the endometrium is composed of both epithelial and stromal cells endometrial sarcomas account for less than one percent of endometrial malignancies (Sutton *et al.*, 1990). Histologically, most endometrial carcinomas are adenocarcinomas with or without benign squamous elements (previously described as adenoacanthoma) and can be subdivided on a histological basis as follows:

Adenocarcinoma	65%
Adenocarcinoma with squamous differentiation	19%
Adenosquamous carcinoma	13.8%
Clear cell carcinoma	1.2%
Secretory carcinoma	1.0%

(Reagan and Fu, 1981)

The degree of histologic differentiation of endometrial cancers is an important prognostic variable and is classified by the International Federation of Gynecologists and Obstetricians (FIGO) into well

differentiated (G1), moderately differentiated with partly solid areas (20-50% solid, G2), and poorly differentiated with predominantly or entirely solid areas (>50% solid, G3). The effect of degree of differentiation on prognosis is shown in the 5-year survival figures for Stage I endometrial cancer:

Grade 1	93 - 98%
Grade 2	76 - 95%
Grade 3	62 - 79%

(Huang *et al.*, 1992)

Endometrial cancer spreads initially through the myometrium, into the cervix and subsequently to the peritoneal cavity and the pelvic lymphatics. This pattern of spread is reflected in the FIGO staging system (see 1.1.d) which was revised in 1989 from a clinical to surgico-pathological system because of the importance of the surgical and, particularly, the histologic findings in terms of further management and prognosis.

The depth of myometrial invasion is a generally accepted prognostic factor and correlated with the grade of the tumour, i.e. the poorer the tumour is differentiated the more deeply it is likely to have penetrated the endometrium (Creasman *et al.*, 1987). In a survey of 621 Stage I (clinically staged) endometrial cancers myometrial invasion occurred as follows:

Endometrium only	14%
Inner 1/3	45%
Middle 1/3	19%
Outer 1/3	22%

(Creasman *et al.*, 1987)

In this study, both tumour grade and depth of myometrial invasion were related to the presence of pelvic and para-aortic nodal metastasis, the

most sinister marker of advanced disease. In addition, adnexal involvement by tumour, other spread outside the uterus and positive peritoneal cytology were all associated with nodal metastasis, consistent with the FIGO staging system.

1.1.d FIGO staging

The staging system for endometrial cancer was modified in 1989 on the basis of the FIGO cancer committee report because of inadequacies in the clinical staging of endometrial cancer. The current FIGO classification is independent of tumour grade and summarized below:

STAGE	Ia	Tumour limited to endometrium
	Ib	Invasion to < 1/2 myometrium
	Ic	Invasion to > 1/2 myometrium
	IIa	Endocervical glandular invovement only
	IIb	Cervical stromal invasion
	IIIa	Tumour invades serosa and/or adnexae and/or positive peritoneal cytology
	IIIb	Vaginal metastases
	IIIc	Metastases to pelvic and/or para-aortic lymph nodes
	IVa	Tumour invasion of bladder and/or bowel mucosa
	IVb	Distant metastases including intra-abdominal and/or inguinal lymph nodes

1.1.e Investigative pathology

Until recently the experimental biology of endometrial cancer was limited to the investigation of the steroid hormone receptors and their relationship to prognosis and other prognostic variables. Over the past 5 - 10 years there

has been an enormous increase in the use of laboratory-based techniques to study endometrial cancer and its biological behaviour. The results of these investigations have been reviewed recently (Gurpide, 1991), and include studies of oncogenes, growth factors and their receptors, and ploidy status in addition to the expanding body of information on the steroid receptors. The establishment of an endometrial cancer cell line, Ishikawa (Nishida *et al.*, 1985), with functional oestrogen receptors has enabled the interaction of hormonal, biochemical and biological events to be studied in more detail.

Steroid receptors

Because of the relationship between endometrial cancer and oestrogen, considerable attention has been focussed on the presence of both oestrogen (ER) and progesterone receptors (PR) in endometrial cancers. Most authors have found a correlation between positivity of both ER and PR and increased survival independent of tumour grade and degree of myometrial invasion (reviewed in Huang *et al.*, 1992). Overall, ER are identified in 61-100% and PR in 49-88% of endometrial cancers (reviewed in Kauppila, 1989). Receptor positivity is lost as tumours become less differentiated (Budwit-Novotny *et al.*, 1986) but this may simply reflect that oestrogen and progesterone receptors are markers of biochemical differentiation rather than central to tumour development or inhibition (Gurpide, 1991). When the data are collected, in early endometrial carcinoma the ER and PR concentrations are useful in predicting outcome, with PR being more predictive than ER (Kauppila, 1989; Kleine *et al.*, 1990). Combining the 2 measurements does not appear to improve the predictive value.

The antagonistic effects of progesterone on oestrogen-stimulated growth of endometrium and the requirement for the presence of oestrogen to stimulate the production of progesterone receptors led to studies on the relationship between receptor status and the effect of progestin therapy in advanced endometrial cancers. Pooling the data from a number of studies suggests that the presence of ER and/or PR is associated with a response to

progestin therapy, again with PR being more discriminatory than ER (Kauppila, 1989). This allows better selection of those patients for progestin treatment although, in practise, all patients with advanced disease tend to be treated regardless of receptor status as the treatment has few side effects and 10% of receptor negative patients will respond to progestins (Kauppila, 1989).

In early stage disease progestin treatment appears to have little or no effect irrespective of receptor status. Several randomized trials of progestin therapy in high risk, early stage endometrial cancer have failed to show any benefit (MacDonald *et al.*, 1988; COSA, 1990). This may be a statistical problem because of the relatively low recurrence rate in endometrial cancer and hence, the large numbers of patients required to show a significant effect of alternative treatments.

Ploidy status

DNA ploidy patterns as determined by flow cytometry or conventional cytogenetic analysis appear to correlate with histologic grade but not other recognized prognostic factors. Aneuploidy was found in 24-31% of endometrial cancers (Stendahl *et al.*, 1989; Iversen and Laerum, 1985) and patients with aneuploid tumours had a higher recurrence rate and shorter disease-free interval when compared to those with diploid tumours (Iversen, 1986). In the aneuploid group death rates were also higher, the median survival was shorter and ploidy was a better prognostic variable for survival than tumour grade. In 5 out of 8 patients with metastatic disease the secondary deposits were aneuploid suggesting that aneuploid tumours are more likely to metastasize.

In the latter study (Iversen, 1986) the fraction of cells with S-phase DNA content (i.e. the cells actively proliferating) was measured and averaged 22.7% and 9.7% in aneuploid and diploid tumours, respectively. Another analysis using flow cytometry showed a good correlation between aneuploid tumours or those with a S-phase rate >15% and early death from

disease in endometrial cancer (Stendahl *et al.*, 1989). The fraction of cells actively proliferating in tumours can be determined using flow cytometry to look at an antibody, Ki67, that stains nuclear antigen present only in proliferating cells. Using this technique the population of proliferating cells was reported to be significantly higher in endometrial carcinomas (27%) compared to that in normal endometrium (14.5%) and was elevated in tumours with other poor prognostic factors (Yabushita *et al.*, 1992).

Oncogenes, growth factors and their receptors

The presence and significance of this heterogeneous group of proteins in endometrial cancer is discussed in Section 1.8.

1.1.f Treatment

Although all are in agreement that surgery is the preferred therapy for early stage endometrial cancer, there remains considerable debate over the optimum use of radiotherapy in conjunction with the surgery. Historically, both pre- and post-operative radiotherapy have been used and both still have their advocates. However, in the management of early stage disease most centres are now moving to selective post-operative radiotherapy based on the surgical and pathologic findings.

An appropriate rationale for treatment is outlined by Creasman and Weed (1992). Initial surgery should involve total abdominal hysterectomy and bilateral salpingo-oophorectomy and, particularly in the G2 and 3 lesions, pelvic and para-aortic lymphadenectomy. At surgery peritoneal washings should be obtained on entering the peritoneal cavity and sent for cytology. Some centres would not perform para-aortic lymph node sampling routinely because of the increased technical difficulty and the small chance of the para-aortic nodes containing tumour in the absence of pelvic node metastasis.

Following surgery the histology is reviewed and further management

planned on the basis of these findings. Most patients will require no further treatment but if poor prognostic factors such as deep myometrial penetration, positive peritoneal cytology or pevic lymph nodes are present then radiotherapy is given. In this way, most patients will avoid unnecesary radiation and the radiotherapy in those who require it can be tailored to meet their individual needs. Postoperative radiotherapy typically involves the use of an intravaginal source to reduce local recurrence and external beam to the pelvis if there has been deep myometrial invasion or lymph node involvement.

Stage II disease has conventionally been treated with pre-operative irradiation followed by surgery. However, the inability of clinical staging to reliably detect cervical involvement has led more recently to a similar management to that of Stage I disease described above. Some centres would do an extended or radical hysterectomy for proven Stage II disease. The use of progestins in Stages I and II has not been shown to be beneficial.

An individualized approach to the management of late Stage and recurrent endometrial cancer is essential (reviewed in Cohen, 1992). Disease that is surgically resectable should be removed without jeopardizing the patient unduly although the evidence that debulking has any benefit in terms of survival or disease-free interval is lacking. Further treatment largely reflects the bias of various centres but radiotherapy is generally used for local spread or recurrence and chemotherapy has been increasingly employed for disease that is more widespread. Chemotherapeutic regimes in current use for endometrial cancer usually include a combination of cyclophosphamide, cisplatinum and adriamycin.

1.2 Carcinogenesis

Studies of chemical carcinogenesis and epidemiological studies indicate that most human cancers are caused by environmental carcinogens or mutagens

that are presumed to damage DNA (Ames, 1979). However, the exact nature of the damage that causes cancer is incompletely understood and a long latent period is needed for the induction of cancer after the initial damage to DNA has taken place. There is evidence for mounting genetic instability within cells that are progressing toward a malignant phenotype, as if early events in tumourigenesis confer a predisposition to mutation on the cells (reviewed in Meuth, 1990). The genetic abnormalities result in the production of structurally abnormal proteins or abnormal quantities of normal proteins, either too little or too much, which lead to disturbances in cell functioning.

1.2.a Genetic abnormalities in cancer

Four mechanisms resulting in genetic abnormalities have been identified in human cancers:

1) Loss or gain of a haploid or diploid set of chromosomes. This leads to severe aneuploidy that characterizes many cancer cells and is associated with clinically more aggressive tumours (see Section 1.1.e).

2) Gene rearrangement or deletion. Rearrangement or loss of variable amounts of DNA within chromosomes may result in changes that may be submicroscopic or visible on cytogenetic analysis (see Section 1.5.b).

3) Gene amplification. Specific genes may be amplified a few-fold to several hundred-fold and appear on chromosomes as homogeneously staining regions or independently as double minute chromosomes (see Section 1.6.a).

4) Point mutation. Alteration of a single nucleotide at a critical site has been shown to result in the activation of oncogenes (see Section 1.5.b).

It appears in the human cancers, at least in those that the genetic abnormalities have been most extensively studied, that multiple genetic lesions are necessary to induce cancer.

1.2.b Oncogenes

Retroviral oncogenes:

The rapidly transforming retroviruses cause cancer in a variety of vertebrate species, including primates. The molecular characterization of the genome of the retroviruses led to the identification of DNA sequences that were directly responsible for their potential to cause cancer. These genes were subsequently called oncogenes and given specific names e.g., v(viral)-*sis*, v-*myc*, v-*ras*, v-*erb*, v-*src*. Using the DNA sequences of the viral oncogenes to generate cDNA probes, cellular homologues of the viral oncogenes were detected in normal vertebrate DNA and subsequently termed proto-oncogenes (reviewed in Bishop, 1983). It is likely that viral oncogenes arose from the mutation of normal cellular genes (the proto-oncogenes) the virus incorporated on its passage through the host cell, a process known as transduction.

It is now clear that proto-oncogenes encode proteins with various functions involved in cell growth and/or differentiation such as growth factors, growth factor receptors, or intracellular components of signal transduction pathways. The notion of mutant forms of normal cellular proteins causing a functional disturbance within cells containing transforming genes was supported by the discovery that the protein encoded by the retroviral oncogene v-*sis* was a homologue of the B chain of the growth factor, PDGF (Waterfield *et al.*, 1983; Doolittle *et al.*, 1983).

Oncogenic forms of cellular proto-oncogenes homologous with the viral oncogenes were first isolated by the transfection of DNA from human cancer cells into mouse NIH3T3 cells (Shih *et al.*, 1981, Krontiris & Cooper, 1981). This process resulted in malignant transformation of the 3T3 cells and this technique was used subsequently to detect many of the known oncogenes. This system has limitations, however, its most serious being that it relies on a single mesenchymally-derived cell line which may not be susceptible to transformation by oncogenes from other cell types.

Cooperation between oncogenes

The necessity for activation of more than one oncogene to induce cancer was initially described *in vitro* with two viral oncogenes - the middle T and large T genes of polyoma virus (Rassoulzadegan *et al.*, 1982). When introduced into rat embryo fibroblasts neither is able to fully transform the cells alone but when both oncogenes are present in the cells a fully transformed phenotype is elicited. This is consistent with the multistep model of carcinogenesis in which there is successive activation of different oncogenes, each of which confers on the cell one of the phenotypes which in aggregate constitute a fully transformed cell.

This cooperation between oncogenes is most clearly demonstrated *in vitro* by the cellular oncogenes c-*ras* and c-*myc*, where cointroduction of both oncogenes into rat embryo fibroblasts is necessary to induce complete malignant transformation (Land *et al.*, 1983). A number of oncogenes can be divided into two functional categories based on their abilities to complement either c-*ras* or c-*myc* in transformation assays in the mouse NIH3T3 cell system (reviewed in Weinberg, 1985). Interestingly, the "*ras*" group of oncogenes encode cytoplasmic proteins, some of which have been shown to be involved in intracellular signalling, while the "*myc*" group encode nuclear proteins regulating nuclear transcription. Expression of the cytoplasmic oncoproteins alone leads to altered cell morphology and growth factor or anchorage requirements but does not immortalize the cells, while the converse is usually true when the nuclear oncoproteins are expressed by themselves (reviewed in Hunter, 1991). Although this division may be somewhat simplistic, it emphasizes the point that multiple genetic lesions are necessary for complete transformation.

1.2.c Tumour suppressor genes

This explanation for oncogenesis assumes that activated oncogenes act in a dominant manner, i.e. expression of the oncoprotein overrides the normal,

physiological function of the wild-type protein. However, in studies of somatic cell hybrids, the fusion of malignant cells with normal cells results in nontumourigenic hybrid cells (Stanbridge, 1976). This phenomenon of tumour suppression suggests that a gene or genes from the normal cells replaces the defective function in the tumour cells and renders them responsive to normal growth regulators. The concept of a loss of genetic material being a critical event in the genesis of cancer is supported by the specific chromosome deletions observed in certain human malignancies (Ponder, 1988). This evidence suggests that there is a class of genetic elements which must be inactivated in order for a cell to become malignant.

These elements have been termed tumour suppressor genes or antioncogenes and the first gene of this kind was detected in retinoblastomas (Friend *et al.*, 1986). Loss of function of this gene, RB, either through deletions, translocations or point mutations, leads to the production of a defective RB protein. RB protein is thought to play a regulatory role in the cell cycle (DeCaprio *et al.*, 1989) and retinoblastoma cells lack a functional RB protein. Approximately 25-30% of cases have a family history of the disease and in the familial forms affected individuals inherit a mutant, loss-of-function allele (reviewed in Cowell, 1991). Tumours in these patients are usually bilateral, multifocal and have an earlier age of onset than the sporadic cases. Although the abnormal RB allele is inherited as an autosomal dominant, a somatic event must inactivate the remaining normal allele in order for the disease to develop. In contrast, sporadic forms of the disease involve two somatic mutational events (commonly a deletion associated with an abnormality in expression from the remaining allele) in the same cell and the tumours therefore occur later in life and are usually single. Replacement of the normal RB protein into retinoblastoma cells induces profound changes in cellular characteristics and complete loss of their tumourogenic activity in nude mice (Huang *et al.*, 1988). Similar results were obtained in human prostate carcinoma cells, DU145, which express an abnormal RB protein. Transfection of the normal RB gene into these cells led to a loss of

their ability to form tumours in nude mice although their excessive growth rate was unchanged (Bookstein *et al.*, 1990). These studies confirm that the presence of the normal or wild-type RB protein prevented tumour development.

Subsequently other antioncogenes were detected by studying genes at the sites of characteristic chromosome deletions in human cancer. The most productive of these to date has been colorectal cancer where the tumour suppressor genes p53 (Baker *et al.*, 1989), DCC (Deleted in Colon Cancer, Fearon *et al.*, 1990), MCC (Mutated in Colon Cancer, Kinzler et al., 1991a) and most recently, APC (Adenomatous Polyposis Coli, Kinzler *et al.*, 1991b; Nishisho *et al.*, 1991; Groden *et al.*, 1991; and Joslyn *et al.*, 1991) have been detected in this manner. The genes characterized to date have very different functions e.g. p53 which acts as a DNA binding protein (Baker *et al.*, 1989), DCC is probably a cell surface glycoprotein (Fearon *et al.*, 1990) while the functions of MCC and APC are not known. Interestingly, the MCC and APC genes are located very close to one another on Chromosome 5q21, a common site for deletions in colorectal cancer (reviewed in Bourne, 1991). While MCC deletions and point mutations are seen in sporadic cancers, germ-line abnormalities in the APC gene give rise to familial adenomatous polyposis (FAP), the inherited condition in which multiple colonic polyps develop at an early age and ultimately lead to colorectal cancer if not treated. Mutations in the APC gene are also seen in sporadic colon cancers but to date no germ-line mutations have been detected in the MCC gene in patients with FAP.

p53 was initially thought to be an oncogene as it transformed normal rat fibroblasts when co-transfected with activated Ha-*ras* (Parada *et al.*, 1984; Eliyahu *et al.*, 1984). Subsequently this was shown to be an effect of mutant p53 and the normal allele functions as a tumour suppressor gene. Mutations in the p53 gene have been demonstrated in a number of tumours including hepatomas and in over half of breast, lung, colon and bladder cancers (reviewed in Harris, 1991). Accumulating data on alterations in the

p53 gene suggest that it is the gene most commonly invoved in human carcinogenesis and its inactivation may be essential for the development of malignancy (Lane and Benchimol, 1990). It appears that p53 is a transcription factor which binds at a sequence-specific site on DNA and levels of p53 rise when damage to DNA occurs (reviewed in Lane, 1992). This causes an arrest of the cell cycle in G1 which switches off replication to allow time for repair of the DNA. Tumour cells in which the p53 is abnormal are therefore unable to stop replication leading to a progressive accumulation of genetic damage.

Inactivation of tumour suppressor genes can therefore occur either by loss-of-function (recessive) mutations that inactivate the protein or delete the gene (e.g. RB) or gain-of-function mutations that convert the protein to a dominant form that suppresses the activity of the wild type protein (e.g. p53). In the future it may be possible to reverse the effects of activated dominant oncogenes in a therapeutic fashion with the use of antisense oligodeoxynucleotides which bind to oncogenic mRNAs and inhibit their expression (reviewed in Calabretta, 1991).

In colorectal cancer the molecular events underlying the initiation and progression of the cancer are perhaps best understood (reviewed in Fearon and Vogelstein, 1990). Colorectal tumours arise in pre-existing benign tumours (adenomas) and progressively develop into frank carcinoma. This continuous process can be identified as a series of histologic stages. Tumours from each of these stages can be obtained for study purposes and this has facilitated the investigation of the molecular genetic abnormalities in colorectal carcinoma. In these tumours there is mutational activation of oncogenes and inactivation of tumour suppressor genes and it is the accumulation of these changes that results in cancer. The mutation of at least 4 or 5 genes is necessary to produce a malignant tumour while benign tumours contain fewer detectable changes. This is consistent with epidemiological studies on the frequency of human cancer with age which suggest that 5 or 6 independent steps are necessary for the development of

cancer (Peto *et al.*, 1975). Similar activation of multiple oncogenes and inactivation of antioncogenes has now been described in a number of human cancers including carcinomas of the breast and lung, astrocytomas and neuroblastomas (reviewed in Bishop, 1991).

1.3 Growth Factors

Polypeptide growth factors are small molecules secreted by cells which bind locally to specific high-affinity cell surface receptors. Ligand binding activates the cytoplasmic portion of the receptor initiating a series of intracellular events culminating in the regulation of growth and/or differentiation in the target cell (reviewed in Rozengurt, 1986). Growth factors act locally on adjacent cells (paracrine effect) or on the same cell that secreted them (autocrine effect). In most instances they are secreted into the extracellular milieu although in some cases they may remain attached to cell surface (Gordon *et al.*, 1987) or as an integral membrane protein (Pfeffer and Ullrich, 1985). Their function *in vivo* is assumed to be the regulation of growth and differentiation to maintain celular homeostasis.

Growth factors, although named for the biological activity or assay leading to their initial discovery (e.g. epidermal growth factor, transforming growth factor), appear to have different functions dependent upon the target cell type and presence of other growth factors (reviewed in Sporn and Roberts, 1988). For example, EGF was initially detected and purified using a bioassay of its proliferative effect on immature mouse keratinocytes which led to precocious opening of the eyelids in newborn mice (Cohen, 1962). In contrast, EGF inhibits proliferation of hair follicle cells and a number of cancer cell lines and, in an effect unrelated to proliferation, suppresses gastric acid secretion in humans (Gregory, 1975).

The presence of other growth factors may modify the action of a particular growth factor and this is exemplified by transforming growth

factor β (TGF-β) which stimulates fibroblast proliferation in the presence of PDGF but is inhibitory when EGF is present (Roberts *et al.*, 1985). The various isoforms of TBG-β are widely distributed throughout both fetal and adult tissues and either promote or inhibit proliferation, differentiation and chemotaxis in a cell-type specific manner (Barnard *et al.*, 1990). The actions of growth factors may also be concentration-dependent and this is demonstrated in fibroblasts where fibroblast growth factor in low concentrations elicits a mitogenic response but is chemotactic at higher concentrations (Linemeyer *et al.*, 1987).

A growth factor may also modify the action of another growth factor indirectly by affecting receptor numbers. PDGF acts on EGF receptors to reduce binding affinity and consequently kinase activity (see Section 1.4.e) and expression of the α-subtype of the PDGF receptor is decreased in Swiss 3T3 cells treated with TGF-β (Gronwald *et al.*, 1989).

A further role for growth factors has been demonstrated in the differentiation of specific cell lineages. In haemopoietic cells, high concentrations of interleukin-3 (IL-3) suppress the response to the colony stimulating factors (CSFs). However, pretreatment of the cells with a low concentration of IL-3 enables them to respond to the CSFs and develop along the granulocyte/macrophage lineage (Cross and Dexter, 1991). In a similar manner, the presence of PDGF allows rat oligodendrocyte type 2 astrocyte (O-2A) precursors to differentiate *in vitro* on the equivalent of day 21 of embryogenesis regardless of when the cells were removed from the optic nerve (Raff *et al.*, 1988). In the absence of PDGF the O-2A progenitors differentiate prematurely into oligodendrocytes while exposure to both PDGF and FGF leads to continued proliferation without differentiation (Bogler *et al.*, 1990).

1.3.a Epidermal growth factor

EGF is a single polypeptide chain of 53 amino acids with 3 internal

disulphide bonds and a molecular weight of 6045. It was initially isolated from extracts of mouse submaxillary glands after the serendipitous observation that these extracts induced precocious eyelid opening and incisor eruption in newborn mice (Cohen, 1962). EGF can be detected in most human body fluids and is present in a variety of tissues, including placenta, kidney, stomach, bone marrow and duodenum, but at very low concentrations (Kasselberg *et al.*, 1985). EGF is synthesized as 1217 amino acid transmembrane protein with subsequent proteolysis releasing the active factor (Gray *et al.*, 1983). The membrane-bound precursor may act as an immobilised growth factor capable of stimulating adjacent cells (Mroczkowski *et al.*, 1988) or as a receptor for an as yet unidentified ligand (Pfeffer and Ullrich, 1985). The latter authors arrived at that conclusion because of the sequence homology and similarity of other structural features between the EGF precursor and cell surface receptors for low density lipoproteins.

1.3.b Transforming growth factor-α (TGF-α)

A further ligand for the EGF receptor (EGFR) was discovered in the conditioned media of sarcoma virus-transformed cells (De Larco and Todaro, 1978) and, because of its ability to induce a transformed phenotype in previously normal fibroblasts, was named transforming growth factor. Subsequently TGF was found to consist of two mitogens (TGF-α and TGF-β) with divergent properties, one of which, TGF-α, binds to the EGFR. Like EGF, TGF-α is synthesized as a transmembrane precursor protein of 160 amino acids and subsequently cleaved to release the 50 amino acid polypeptide. Recent evidence demonstrates that the membrane bound precursors can bind to and activate EGFR on adjacent cells and may therefore function directly in cell-cell communication (Wong *et al.*, 1989; Brachman *et al.*, 1989).

TGF-α is expressed in normal tissues of the fetus and a number of

adult tissues including keratinocytes, activated macrophages, brain and pituitary. Its function in normal tissues is unknown but it has a mitogenic effect on keratinocytes in culture (Barrandon and Green, 1987) and is expressed at increased levels in proliferative keratinocytes from psoriatic skin (Elder *et al.*,1989). Messenger RNA for TGF-α is detected in a large number of human sarcomas and carcinomas and cell lines derived from these tumours (Derynck *et al.*, 1987), suggesting that TGF-α may play a role in malignant transformation and tumour formation *in vivo*.

1.3.c Platelet-derived growth factor

Structure

As its name implies, PDGF was initially isolated from platelets (Ross *et al.*, 1974) but was subsequently found to be an ubiquitous growth factor formed by normal and abnormal cells and the major mitogen present in serum (reviewed in Ross *et al.*, 1986). PDGF derived from platelets has a molecular weight of 30 kD and is composed of an A and a B chain of 16 and 14 kD, respectively, with an overall amino acid sequence homology of their precursors of 40% (Betsholtz *et al.*, 1986). PDGF can therefore be secreted in three isoforms - PDGF-AA, PDGF-BB and PDGF-AB. Subsequent analysis has shown that about 70% of the PDGF derived from platelets is secreted as the heterodimer AB, most of the rest as PDGF-BB and a little PDGF-AA while PDGF derived from other sources is usually secreted as homodimers of the A or B chain (reviewed in Ross, 1989). When the amino acid sequence of the B chain was determined it was found to have significant homology with the $p28^{sis}$ transforming protein from the simian sarcoma virus (Waterfield *et al.*, 1983), while the A chain was sequenced from an osteosarcoma cell line which secretes homodimers of the A chain (Heldin *et al.*, 1986). The genes for the A and B chains are located on different chromosomes, 7 (Betsholtz *et al.*, 1986) and 22 (Swan *et al.*, 1982) respectively, but can be expressed either independently or coordinately in

different cell types (Westermark *et al.*, 1986).

Biological activity

PDGF is mitogenic and chemotactic to a wide range of normal and transformed cells, principally of the mesenchymal lineage. In addition to platelets, PDGF is secreted by activated macrophages, endothelium, smooth muscle cells and in a number of fetal tissues (reviewed in Ross *et al.*, 1986).

Evidence that certain coagulation factors induce the release of PDGF from arterial endothelial cells (Gajdusek *et al.*, 1986) and the fact that platelet aggregation is the first step in the repair of vascular damage of any kind suggest that PDGF may have a physiological function in the clotting/repair process. Macrophages are central to the normal inflammatory process and activated macrophages transiently express the genes for PDGF-A and -B and secrete the proteins (Shimokado *et al.*, 1985) suggesting that PDGF has a role in the inflammatory response.

Neither endothelial cells nor macrophages respond to PDGF indicating that the secreted PDGF probably stimulates growth in the surrounding connective tissue in a paracrine manner. PDGF commonly acts in this fashion, however the reverse is true in fibroblasts and smooth muscle cells where interleukin-1 induces the expression of the PDGF-A chain and the PDGF-AA secreted is mitogenic to these cells (Ross, 1989).

The interaction between PDGF and other mitogens may be complex. Human mesangial cell lines cultured from renal glomeruli express both PDGF A and B chains, and a number of mitogens for these cells including EGF, TGF-α, basic fibroblast growth factor (bFGF), tumour necrosis factor type α and PDGF isoforms, all increase levels of PDGF A and B mRNA (Silver *et al.*, 1989). The mitogenic response of these mesangial cells to EGF is partially inhibited by anti-PDGF antibodies, suggesting that PDGF may be an effector molecule in the mitogenic response to these other growth factors.

PDGF also appears to have a function in the myometrium where the

expression of the PDGF-A chain, but not the B chain, increased in human uterine smooth muscle cells as the muscle hypertrophied during pregnancy (Mendoza *et al.*, 1990).

1.4 Growth factor receptors

Receptors for the majority of peptide growth factors characterized to date have a similar structure. They consist of an extracellular N-terminal ligand-binding domain and a C-terminal protein-tyrosine kinase (PTK) domain separated by a short hydrophobic transmembrane region. Structural similarities within the PTKs have allowed the family to be subdivided into subclasses (from Aaronson, 1991) as shown in Fig 1.1.
The tyrosine kinase domains of all subclasses are similar except for the PDGF and FGF receptor groups in which a hydrophilic region of varying length is inserted in the kinase domain (Hanks *et al.*, 1988).

The extracellular domains exhibit more variation between the subclasses. Members of the EGF receptor subclass are monomers and possess 2 highly conserved cysteine-rich repeat sequences. Insulin receptors have similar cysteine-rich sequences in the extracellular domain but the receptors are disulphide-linked $\alpha_2\beta_2$ structures. PDGF and FGF receptor subclasses are monomeric with 5 and 3 immunoglobulin repeats in their extracellular domains, respectively. The remaining groups of receptors are all recent discoveries and are less well characterized. All possess cysteine-rich sequences in the external domain except the receptors for the neurotrophic factors. They are monomers apart from the receptor for hepatocyte growth factor which has disulphide-linked subunits of 50 kD (α) and 145 kD (β) of which the α subunit is extracellular (Park *et al.*, 1987).

Although not described with all receptors, ligand binding induces receptor dimerization which brings the 2 kinase domains into apposition causing transactivation of the kinase domains of both receptors (reviewed in

Ullrich and Schlessinger, 1990). Activation of the kinase causes autophosphorylation of tyrosine residues on the receptor and phosphorylation of cytoplasmic substrates. Ligand-receptor complexes then aggregate in coated pits and are internalized in coated vesicles and transported to lysosomes for degradation (Carpenter and Cohen, 1979).

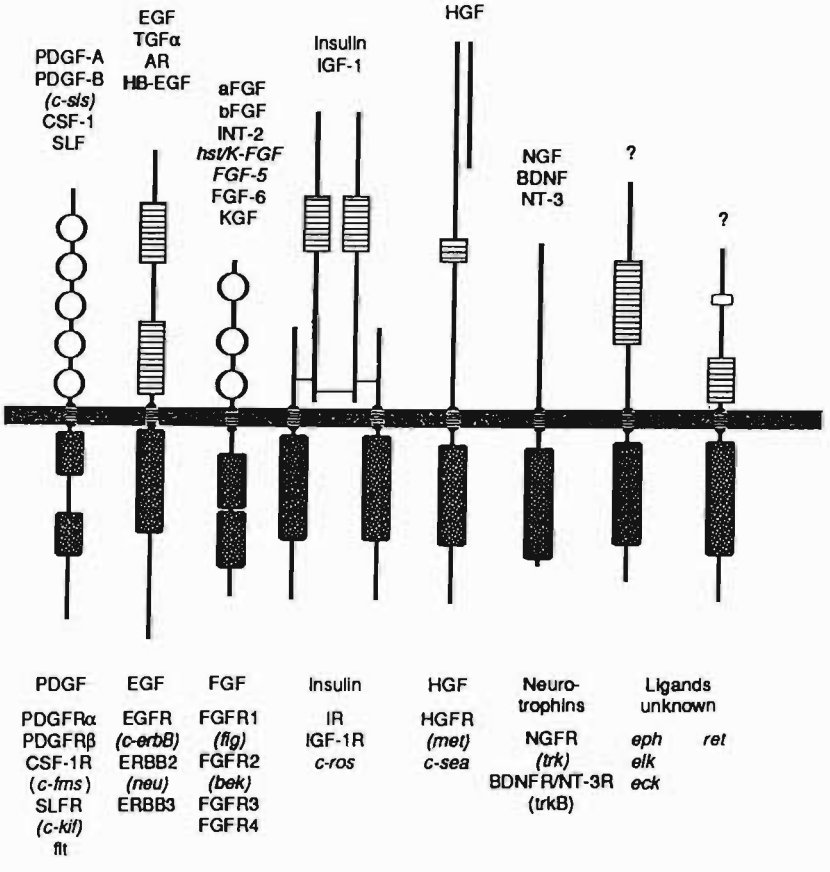


Fig 1.1 Schematic representation of protein-tyrosine kinase receptor subclasses. Each subclass is designated by a prototype ligand with other ligands listed above and other receptors in the family listed below. Open circles indicate immunoglobulin repeats. Hatched boxes signify cysteine-rich domains and dotted boxes indicate tyrosine kinase domains. (from Aaronson, 1991)

1.4.a Epidermal growth factor receptor

The structure and function of the EGFR have been defined in

considerable detail. As described above, the receptor comprises an extracellular ligand binding domain and a cytoplasmic tyrosine kinase domain linked by a short hydrophobic transmembrane region. The extracellular domain contains an amino terminal sequence termed domain I, and two cysteine-rich repeats, domains II and IV, separated by domain III (Ullrich and Schlessinger, 1990). Domain III has been proposed as the ligand binding domain (Lax *et al.*, 1989) and a 4 domain model has been proposed (see Fig 1.2) in which EGF or TGF-α bind to a cleft formed between Domains I and III while domains II and IV are in close contact with each other and the plasma membrane (Ullrich and Schlessinger, 1990).

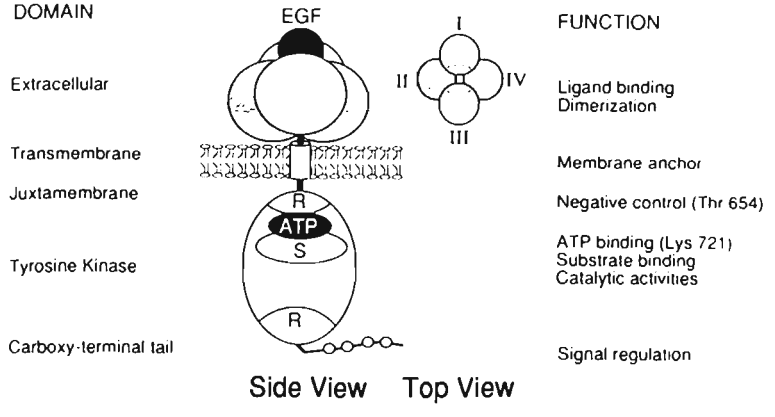


Fig 1.2 Proposed structure-function topology of the EGFR.
Subdomains II and IV (stippled) represent the cysteine-rich regions of the extracellular domain. Most of the structural determinants that define EGF binding affinity are proposed to be located in the cleft formed by subdomains I and III. The symbols S and R represent proposed interaction sites for substrates and regulatory factors.

(from Ullrich and Schlessinger, 1990)

The mechanism currently favoured for activation of the EGFR kinase is that ligand binding induces dimerization of receptors bringing two receptor kinase domains into apposition. These domains then phosphorylate

each other (Honegger *et al.*, 1989) and initiate the phosphorylation of a series of substrate proteins which are responsible for transduction of the activation signal.

Signal transduction by the EGFR

Elegant experiments using receptors composed of various chimeric combinations of the internal and external domains of the EGF and insulin receptors have provided convincing evidence that it is the cytoplasmic domains that determine the substrate specificity of the kinase region and therefore the ensuing mitogenic pathways (Riedel *et al.*, 1989).

A major intracellular signal transduction pathway activated by the EGFR involves tyrosine phosphorylation of GTPase-activating protein (GAP) which catalyses the conversion of the active GTP-bound forms of $p21^{ras}$ proteins to the inactive GDP-bound forms (Molloy *et al.*, 1989). The role of $p21^{ras}$ proteins in normal mitogenic signalling is unknown but the oncogenic forms of the protein are mitogenic and insensitive to the regulatory action of GAP (reviewed in Cantley *et al.*, 1991). In normal cells therefore, tyrosine phosphorylation of GAP by tyrosine kinase may modulate its interaction with the $p21^{ras}$ proteins and regulate the biological activity of these proteins. Recent evidence has confirmed that the addition of EGF to quiescent cells leads to a rapid increase in the amount of active GTP-bound $p21^{ras}$, presumably through activation of GAP (Satoh *et al.*, 1990). However, other pathways of $p21^{ras}$ activation do exist and in haemopoietic cells ligand-induced activation of a variety of interleukin and CSF receptors results in an increase in tyrosine kinase and $p21^{ras}$ activity without phosphorylation of GAP (Duronio *et al.*, 1992).

Other proteins which are phosphorylated by the EGFR kinase include the c-*raf* proto-oncogene product (Morrison *et al.*, 1988) which is involved in the signalling cascade (see Section 1.3.d) and lipocortin I (Sawyer & Cohen, 1985). Lipocortin I is a steroid-induced protein which inhibits phospholipase-A_2 (see below) and has anti-inflammatory activity. Evidence

suggests that the inhibition of phospholipase-A_2 is regulated by the phosphorylation of lipocortin by the EGFR tyrosine kinase (Pepinsky and Sinclair, 1986).

EGF promotes the activation of the protein-serine/threonine kinase, protein kinase C (PKC), at least in A431 vulval carcinoma cells which overexpress the EGFR, and PKC in turn causes an inhibition of high affinity EGF binding and a reduction in kinase activity (Whiteley and Glaser, 1986). PKC phosphorylates threonine 654 (T654) in the EGFR and it is likely that the phosphorylation of T654 is, at least partially, responsible for the negative effects of PKC on the EGFR. PKC may therefore have a role as a negative regulator of the EGFR although phorbol esters, tumour promoters acting through PKC activation, increase receptor synthesis over a period of hours (Bjorge and Kudlow, 1987). PDGF also activates PKC and has been shown to reduce EGF binding affinity, kinase activity and resultant mitogenic signalling (Decker and Harris, 1989), an effect known as receptor transmodulation.

In cells that overexpress the EGFR, EGF rapidly stimulates the formation of inositol 1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG) as a result of phosphorylation of phospholipase C-γ (PLC-γ, Margolis *et al.*, 1990) by the EGFR kinase. IP_3 and DAG are intracellular second messenger molecules and mediate hormone-stimulated increases in free intracellular Ca^{2+} (Berridge *et al.*, 1984) and PKC, (Nishizuka *et al.*, 1984) activity, respectively.

One of the outcomes of PLC-γ stimulation is the production of arachidonic acid, an important precursor in prostaglandin production. EGF has also been shown to activate phospholipase A_2, the rate limiting enzyme in the alternative pathway of arachidonic acid production (Margolis *et al.*, 1988), although the mechanism and significance of this action is not known.

In addition to stimulation of cellular proliferation, EGF also stimulates the transcription of a number of genes including c-*myc* and c-*fos* (Bravo *et al.*, 1985), prolactin (Murdoch *et al.*, 1985) and actin (Elder *et al.*,

1984), but the significance of this is unclear. EGF has also been shown to stimulate transcription of the mRNA for, and the synthesis of, its own receptor in a number of cell lines (Clark *et al.*, 1985; Kudlow *et al.*, 1986; Earp *et al.*, 1986), in a positive feedback mechanism.

1.4.b c-*erb*B-2

The ligand for the second member of this subclass of tyrosine kinase receptors, c-*erb*B-2, has only recently been characterized after its existence had been previously postulated (Yarden and Weinberg, 1989). It was found to be a 75 kD polypeptide which binds specifically to c-*erb*B-2, activates it and induces cellular proliferation in SKBR-3 breast cancer cells which over-express the receptor (Lupu *et al.*, 1992). The rat gene homologous with the human gene, *neu*, was initially isolated from rat neuroblastomas induced by ethylnitrosourea (Shih *et al.*, 1981) and encoded a 185 kD cell surface glycoprotein with close structural similarity to the EGFR (Schechter *et al.*, 1984). The human c-*erb*B-2 gene was defined using labelled cDNA probes made from highly conserved regions of the EGFR DNA sequence on Southern blots and was found to be similar but separate to the EGFR (King *et al.*, 1985) and mapped to a different chromosome (Schechter *et al.*, 1985). It has taken a further 7 years to confirm the assumption that c-*erb*B-2 was a PTK receptor.

The function of this receptor is unknown although the distribution of c-*erb*B-2 in adult rats suggests that it has a function in the growth of epithelial tissues (Kokai *et al.*, 1987). In immature rats it is expressed on a subset of neuronal cells in addition to most secretory epithelial cells.

EGF enhances the phosphorylation of c-*erb*B-2 and this transphosphorylation leads to increased tyrosine kinase activity in the c-*erb*B-2 (Stern and Kamps, 1988). It was initially confirmed that the c-*erb*B-2 receptor was a substrate for the EGFR tyrosine kinase (Connelly and Stern, 1990) and then discovered that the EGFR and c-*erb*B-2 form non-

covalently linked heterodimers on the addition of EGF (Qian *et al.*, 1992). Both of the receptors were heavily phosphorylated in the heterodimers suggesting that the dimer formation has functional significance. The mitogenic response cascade to c-*erb*B-2 is poorly understood so it remains to be seen what the implications of this association between 2 different receptors are, and what role the recently discovered ligand for c-*erb*B-2 has on activation of both the c-*erb*B-2 and EGF receptors.

1.4.c c-*erb*B-3

The third member of the *erb*B family, c-*erb*B-3, was initally detected using reduced stringency hybridization of v-*erb*B to normal human genomic DNA (Kraus *et al.*, 1989). Under these conditions 4 restriction fragments were detected: 2 identified as EGFR gene fragments, 1 as c-*erb*B-2 and a previously undescribed fragment which failed to hybridize when the stringency was increased. It was therefore called c-*erb*B-3, found to be expressed in a variety of tissues of epithelial origin and mapped to chromosome 12, distinct from either EGFR or c-*erb*B-2. The predicted amino acid sequence is similar to both EGFR and c-*erb*B-2 with 45% and 43% homology in the extracellular domain, respectively, and 60% and 62% in the kinase region. There is far less homology in the remainder of the C-terminal end and, in particular, the c-*erb*B-3 protein lacks the T654 site for PKC phosphorylation seen in the EGFR and is therefore unlikely to be regulated by PKC. The same gene was cloned and expressed independently with no significant differences detected (Plowman *et al.*, 1990).

1.4.d Platelet-derived growth factor receptor (PDGFR)

Two receptors for PDGF have been cloned and designated the α- and β-subunits. They associate and form stable dimers when 1 of the 3 isoforms of PDGF, AA, BB, or AB, binds. Binding studies have shown that the α-

subunit can bind to either PDGF-A or PDGF-B, while the β-subunit can only bind to PDGF-B (Seifert *et al.*, 1989). Therefore, PDGF-BB can bind to any of the 3 receptor subunit combinations (αα, ββ, αβ), PDGF-AA can only bind to αα receptors, and PDGF-AB binds to either αα or αβ receptors. PDGF receptors have been found on all cells which form connective tissue including fibroblasts, smooth muscle cells, glial cells and also on capillary endothelial cells but not on epithelial cells or arterial endothelial cells (reviewed in Ross, 1989).

The absolute and relative numbers of the receptor subunits vary on different cell types and determine the mitogenic sensitivity of these cells to the different PDGF isoforms. For example, human fibroblasts have about 20-fold more β-subunits than α-subunits while mouse 3T3 cells have approximately equal numbers. Consequently, PDGF-BB and PDGF-AB are about 2- to 5-fold more mitogenic than PDGF-AA for human fibroblasts while the 3 isoforms are equally effective in stimulating 3T3 cells (Seifert *et al.*, 1989). The reorganization of actin and membrane ruffling that occurs in human foreskin fibroblasts in response to PDGF was used in another study of PDGFR specificity (Hammacher *et al.*, 1989). The fibroblast response was only induced by PDGF-BB and PDGF-AB suggesting that it was mediated by the β-receptor. However at high concentrations of PDGF-AB the effect of PDGF-BB on actin reorganization was inhibited, which the authors suggest is due to the PDGF-AB binding to the β-receptor and preventing PDGF-BB from dimerizing, and hence activating, the β-receptors. This complex response to the different isoforms of PDGF illustrates how the response of cells could be fine tuned through various combinations of PDGF and receptor subtypes.

Structure of the PDGFR

The PDGF subclass of receptors has 5 immunoglobulin-like repeats in the extracellular domain and a kinase domain split by non-homologous sequences in the intracellular portion. The protein cores of both subunits

have molecular weights of approximately 120 kD and the β-subunit is rapidly glycosylated to a 160 kD precursor and subsequently matures to a 180 kD form at the cell surface (Claesson-Welsh *et al.*, 1988), while the glycosylated mature form of the α subunit is 170 kD (Claesson-Welsh *et al.*, 1989).

Both subunits have been cloned and sequenced and demonstrate 31% homology in their ligand binding domains and 75-85% homology in the two kinase domains (Yarden *et al.*, 1986; Matsui *et al.*, 1989). The gene for the β-subunit has been localized to chromosome 5 adjacent to the gene for c-*fms*, the receptor for colony stimulating factor 1 (CSF-1) (Yarden *et al.*, 1986). The gene for the α-subunit has also been localized to the same region as a proto-oncogene, c-*kit*, on chromosome 4 (Matsui *et al.*, 1989). The 4 molecules, PDGF-αR, PDGF-βR, c-*fms* and c-*kit*, are all members of the PDGF family of tyrosine protein kinases and, interestingly, it has been proposed that chromosomes 4 and 5 have a common ancestral origin (Comings, 1972).

Signal transduction by the PDGFR

The mechanism by which the signal initiated by ligand binding to the PDGFR culminates hours later in DNA replication and cell division has so far provided the most complete model for growth factor receptor signal transduction. Much of the work on PDGFR signalling has used the β-subunit so that the ensuing discussion on receptor function relates to the β-subunit. Whether the α-subunit functions in a similar way is not yet clear although recent work has suggested that there are significant differences in intracellular signalling functions between the 2 receptors (Yu *et al.*, 1991; Heidaran *et al.*, 1991). Many of the early experiments stimulating cells were done with PDGF AB which stimulates both receptor types so much of the older data requires reinterpretation.

Currently the most widely accepted model for receptor activation is that the disulphide-linked dimeric isoforms of PDGF induce dimerization of

the appropriate subunits (Heldin *et al.*, 1989) and subsequent activation of kinase regions in a similar manner to that described above for the EGFR (reviewed in Williams, 1989).

The early responses to PDGF binding and substrates for the kinase are similar to the EGFR: activation of the tyrosine kinase, alteration in cellular pH, hydrolysis of phosphatidylinositol (PI), increase in intracellular calcium, changes in the cytoskeleton, elevation of cellular cyclic adenosine monophosphate (cAMP), and internalization and degradation of the receptor (Williams, 1989). All of these actions except the latter two are dependent on activation of the kinase domain.

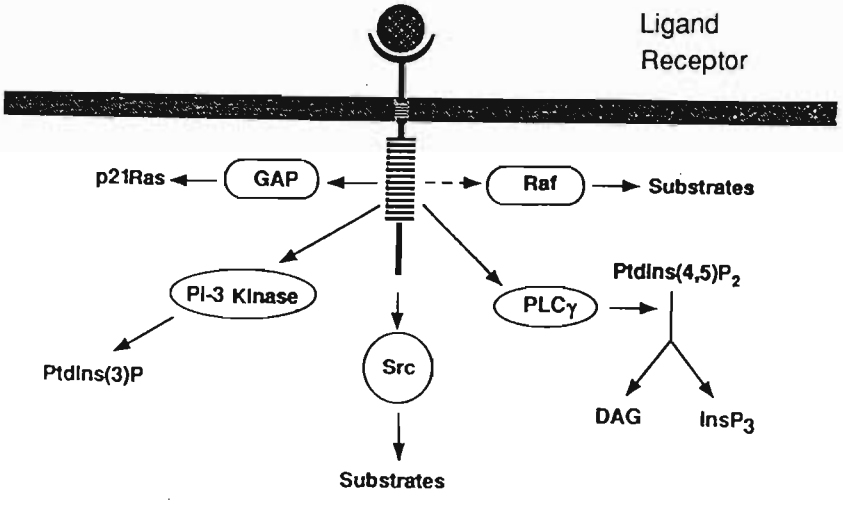


Fig 1.3 Substrates of receptor tyrosine kinases of which the PDGFR is the prototype. The substrates are described below in the text.

(from Aaronson, 1991)

As a result of kinase activation a number of proteins are phosphorylated on tyrosine residues (see Fig 1.3) including GAP (Molloy *et al.*, 1989), phosphatidylinositol 3'-kinase (PI3-K) (Kaplan *et al.*, 1987), PLC-γ (Meisenhelder *et al.*, 1989), the *src* family of non-receptor tyrosine kinases (Ralston and Bishop, 1985) and the product of the proto-oncogene c-*raf*, a serine/threonine kinase (Raf-1) (Morrison *et al.*, 1988). Following ligand binding, GAP physically associates with the β-receptor, PI3-K, PLC-

γ, and Raf-1 to form a signalling complex in which the receptor may function as a co-localizer for the other signalling molecules (Kaplan *et al.*, 1990).

Recently activation of both the PDGFR and the EGFR by their respective ligands has been found to cause phosphorylation of the product of the proto-oncogene *vav* (Bustelo *et al.*, 1992; Margolis *et al.*, 1992). This newly discovered proto-oncogene (Katzav *et al.*, 1989) is expressed in haemopoietic cells and is interesting because it has structural features usually found in transcription factors. It is therefore a new class of substrate for growth factor receptors and may provide a mechanism for direct signal transduction from cell surface receptors to the reulation of transcription.

The insert region in the kinase domain which is peculiar to the PDGF and FGF subclasses of tyrosine kinases also appears to be important for the intracellular substrate specificity of the kinase. Deletion of the insert interferes with some of the early responses to ligand binding and the mutant receptors fail to stimulate mitogenesis (Severinsson *et al.*, 1990). The insert region may contain the site at which GAP associates with the receptor and this may account for the failure of mutant receptors lacking the kinase insert to stimulate mitogensis but still elicit other responses to ligand binding such as PI hydrolysis and calcium flux changes (Kaplan *et al.*, 1990). Work on the PDGF-αR containing mutated kinase inserts showed that, unlike the PDGFR-βR, the association of the α-receptor with PI3-K is not necessary for PDGF-induced mitogenesis (Yu *et al.*, 1991) and the kinase insert region probably does not couple directly with the PI3-K (Heidaran *et al.*, 1991).

Interaction of PDGFR with other growth factors

As with the EGFR, levels of receptors and binding of PDGF can be modulated by other growth factors. Treatment of mouse 3T3 fibroblasts with TGF-β leads to a loss of α-subunits and hence a decreased mitogenic response to PDGF-AA (Gronwald *et al.*, 1989). These effects are thought to be a direct effect on receptor expression rather than down-regulation and

are associated with an increase in the number of β-subunits. In human foreskin fibroblasts TGF-β inhibits PDGF-stimulated DNA synthesis in a cell density-dependent manner, showing no inhibition at low density and increasing inhibition as cell density increases up to almost complete inhibition in dense cultures (Paulsson *et al.*, 1988). In rat arterial smooth muscle cells preincubation of the cells in insulin-like growth factor-1 causes a dose-dependent increase in PDGF binding although the mechanism underlying this is unknown (Pfeifle *et al.*, 1987).

1.5 Growth Factors as Transforming Proteins

It is possible to envisage the following ways in which growth factors could lead to disordered cell growth and hence cancer.

a) Abnormalities of growth factor production

b) Abnormalities of growth factor receptors

c) Disturbance of post-receptor signal transmission

d) Reduced production of, or sensitivity to, inhibitory factors.

1.5.a Abnormalities in growth factor production

TGF-α

TGFs were initially described because of the ability of supernatant from cells transformed by murine sarcoma virus to transform the phenotype, at least transiently, of normal rat kidney (NRK) fibroblasts (DeLarco and Todaro, 1978). Subsequently, TGF was found to have 2 components, α and β, with very different structures and receptors and different actions in different tissues and cell lines (see Section 1.3; reviewed in Derynck, 1988 [TGF-α]; Hsuan, 1989 [TGF-β]).

TGF-α expression has been demonstrated in many tumours including squamous, renal and mammary carcinomas. In oestrogen responsive breast

carcinoma cell lines oestrogen has been shown to induce the synthesis of TGF-α (Dickson *et al.*, 1987). TGF-α is secreted in various sizes by tumour cells reflecting incomplete cleavage of the membrane bound precursor form and variable glycosylation but the significance of this in terms of function or carcinogenesis is unknown (Linsley *et al.*, 1985).

The preceding evidence, and the role of TGF-α in malignant transformation of fibroblasts mentioned above, have led to the belief that it has a role in tumourigenesis *in vivo*. It is presumed that the transforming action of TGF-α is an autocrine and paracrine effect although direct evidence is lacking. Work on animal cell lines suggests that TGF-α secretion alone may not be sufficient for transformation (Finzi *et al.*, 1987), and the original studies on NRK cells described above demonstrated the need for both TGFs for complete transformation. Transformation of NRK cells by the *ras* or *sis* oncogenes, while inducing the expression of TGF-α, results in a more markedly transformed phenotype than that induced by TGF-α alone (reviewed in Derynck, 1988).

A further tumourigenic effect of TGF-α may be its action on blood vessels. Both EGF and TGF-α stimulate arterial blood flow in mice, but TGF-α is considerably more potent and also has angiogenic properties (Gan *et al.*, 1987). It is therefore possible that TGF-α, in addition to its paracrine effects on tumour cells, is also partly responsible for the increased vascularity upon which cancers are dependent (reviewed in Folkman and Klagsbrun, 1987).

EGF

In contrast to TGF-α, EGF is not commonly expressed by tumour cell lines and there are no clear examples to suggest that it might act in either an autocrine or paracrine manner to promote tumourigenesis. Although EGF and TGF-α have similar potencies in some assay systems, in others, such as the induction of new vessel formation (Schreiber *et al.*, 1986), TGF-α is

more potent than EGF. This supports the idea of TGF-α rather than EGF being the transforming ligand for the EGFR in tumours.

PDGF

PDGF and PDGF-like molecules have frequently been isolated from human tumour cell lines and the gene for the A-chain was cloned from a human clonal glioma cell line which secretes PDGF-AA homodimers (Betsholtz *et al.*, 1986). The transforming effect of simian sarcoma virus (SSV) on PDGF-responsive cell types appears to be mediated by $p28^{sis}$ (see Section 1.2.b), the viral protein homologous with human PDGF-B. SSV is an acutely transforming retrovirus which gives rise to glioblastomas and fibrosarcomas in infected monkeys. Work has suggested that, although ligand-receptor interaction is also necessary for SSV-transformation, the PDGF-like factor of SSV-transformed cells can activate its receptor inside the cell (reviewed in Heldin and Westermark, 1989). Although the autocrine stimulation that occurs in acutely SSV-transformed cells has provided the model for this form of subversion of a growth regulatory pathway, this alone is not sufficient to induce and maintain the malignant phenotype *in vivo*, consistent with the multistep model of carcinogenesis (Heldin and Westermark, 1989). This means that the autocrine stimulation that occurs as a result of SSV-transformation must be complemented by other genetic abnormalities in those monkeys infected with SSV who develop tumours.

In human tumour cell lines mRNA for one or both the A- and B-chains are frequently expressed and in those cell lines that possess receptors (e.g., sarcomas and gliomas) autocrine stimulation may occur, although formal evidence for this is lacking (Heldin & Westermark, 1989). The osteosarcoma cell line from which PDGF-AA was first purified to homogeneity also expresses mRNA for the B-chain but this is not secreted (Heldin *et al.*, 1986). It is possible that the B-chain is expressed at much lower levels, is not secreted efficiently, or remains membrane-associated (Ross *et al.*, 1986). Confusingly, in glioblastomas mRNA for both the B-

chain and the β-receptor are expressed in both tumour and adjacent normal endothelial cells indicating that, if autocrine stimulation is occurring, tumour tissue must contain other genetic alterations (Hermansson *et al.*, 1988). This emphasizes the point that in tumour cells in which a growth factor is acting in an autocrine manner, overexpression of the growth factor alone may not be sufficient for tumourigenesis.

PDGF is also produced by cells that do not possess appropriate receptors such as mammary carcinoma cell lines and it is postulated that in this situation PDGF may act in a paracrine manner on the surrounding stromal cells that are receptor positive (Bronzert *et al.*, 1987). This may contribute to the stromal response frequently seen around tumours although the importance of this response to tumourigenesis is not known.

1.5.b Abnormalities of growth factor receptors

It is conceivable that an increase in either the number or activity of growth factor receptors could lead to increased responsiveness of cells to growth factors. Indeed both mechanisms have been described in a wide variety of tissues and cell lines. Many squamous cell carcinomas overexpress the EGFR (Ozanne *et al.*, 1986) and this is most dramatically demonstrated by the human vulval carcinoma cell line A431 which has 2×10^6 rather than the normal 2×10^4 - 2×10^5 receptors per cell (reviewed in Carpenter, 1987). Elevated receptor expression can occur as a consequence of gene amplification, transcriptional or post-transcriptional mechanisms and all these mechanisms are operative in the A431 cell line. In addition to amplification of the normal EGFR gene, there is also a translocated and rearranged EGFR gene in A431 cells from which an aberrant truncated receptor is expressed and secreted (Waterfield *et al.*, 1983). This receptor lacks the intracellular and transmembrane domains and is able to bind EGF but probably has no physiological significance. The absolute numbers of EGFR per cell varies between different A431 sublines and those variants

that have the highest levels of EGFR have the greatest tumourigenic potential in nude mice (Santon *et al.*, 1986).

The gene product of the v-*erb*B oncogene from the avian erythroblastosis virus is also a truncated EGFR which was presumably transduced from the normal cellular chicken EGFR gene (Downward *et al.*, 1984). The oncogene product has a truncated external domain and a shorter intracellular c-terminus with loss of part of the autophosphorylation domain. This is thought to result in a receptor which is ligand-independent and constitutively activated. Deletion of the ligand binding domain of the normal EGFR gene is sufficient to induce constitutive renewal in erythroblasts and this effect is enhanced by a c-terminal truncation but the resultant molecules are less active than the v-*erb*B protein (Khazaie *et al.*, 1988). This suggests that there are other abnormalities of the v-*erb*B protein which are as yet undetected.

Perhaps the most striking example of a structural change resulting in a growth factor receptor acquiring transforming potential is the single amino acid substitution in the gene product of *neu*, the rat homologue of the human c-*erb*B-2 proto-oncogene. Rat *neu* was initially isolated from neuroblastomas induced in newborn rats after ethylnitrosourea was administered to their pregnant mothers (Shih *et al.*, 1981). A single point mutation results in the substitution of valine to glutamic acid at position 664 in the transmembrane region of the receptor encoded by the *neu* gene converting the normal gene into a potent oncogene (Bargmann *et al.*, 1986). It is thought that the substitution of glutamate for valine in the transmembrane region leads to abnormally elevated tyrosine kinase activity (Bargmann and Weinberg, 1988; Weiner *et al.*, 1989). In addition, it has been postulated that enhanced dimerization occurs between the altered *neu* molecules, facilitated by the larger size of of the glutamate residues and that the increased dimerization results in increased tyrosine kinase activity (Sternberg and Gullick, 1989; ibid., 1990). So far similar mutations have not been implicated in human tumours although amplification of the proto-

oncogene has considerable importance (see Section 1.6).

1.5.c Disturbance of post-receptor signal transduction

As described previously (Section 1.4), different receptors activate many of the same intracellular pathways and interference with the effector molecules in these pathways has potential for either increasing the sensitivity of the cell to growth factors or locking the cell into a continuous proliferation mode.

The oncogenes most widely detected in human cancers to date are various members of the *ras* family. Normal *ras* genes encode 21 kD GTP-binding proteins which are part of a post-receptor signal pathway (reviewed in Cantley *et al.*, 1991). The proteins produced from mutant *ras* genes have enhanced activity because of their resistance to hydrolysis of bound GTP which presumably leads to prolongation of the receptor signal. In an analogous manner, the large number of oncogenes coding for membrane-bound or cytoplasmic proteins with protein tyrosine kinase activity, e.g., *abl*, *src*, *raf*, *mil*, may also disturb signal transduction between the cell membrane and the nucleus. The best characterized of these, $pp60^{src}$, is a membrane bound protein tyrosine kinase which may act to recruit enzymes to critical locations in cell membranes (reviewed in Cantley *et al.*, 1991). The tyrosine 527 at the carboxy terminus which regulates tyrosine kinase activity is lacking in the oncoprotein $pp60^{v\text{-}src}$(Courtneidge, 1985) and mutation of this tyrosine to a phenylalanine increases the protein-tyrosine kinase activity and the transforming activity of $pp60^{c\text{-}src}$ (Cartwright *et al.*, 1987). This suggests that the absence of this tyrosine in $pp60^{v\text{-}src}$ results in constitutive activation of the kinase and forms the basis of its transforming ability.

Within the nucleus the implications of abnormalities in the genes that code for the DNA-binding proteins, e.g., *rel*, *myc*, *fos*, *jun*, *erbA*, are readily apparent. The gene products of *fos* and *jun* form part of the AP-1 nuclear transcription factor and contribute to its activity (reviewed in

Lewin, 1991). Oncogenic mutants of *fos* and *jun* appear to increase the transcription of target genes, whereas the oncogenic forms of *rel* and *erbA* appear to be dominant negative oncogenes, i.e., that the target genes for these transcription factors are tumour suppressor genes (antioncogenes) that are inactivated (their transcription is inhibited) by the oncoproteins (Lewin, 1991).

1.5.d Decreased inhibitory control

Information on the inhibitory controls of peptide growth factors is sparse and individual growth factors may have proliferative and inhibitory effects in different situations as described previously. A number of controls of the EGFR have been demonstrated. PKC-induced phosphorylation of T654 in the juxtamembrane portion of the EGFR results in an inhibition of of its kinase activity (Davis, 1988) and PKC also reduces high affinity EGF binding (Livneh *et al.*, 1987). This may be the mechanism responsible for the rapid reduction in receptor binding activity ("down regulation") that occurs after the addition of EGF to quiescent fibroblasts (Carpenter and Cohen, 1976). Transmodulation of the EGFR also occurs with activation of the PDGFR and this effect may again be mediated by PKC (Lin et al., 1986). PKC may therefore act as a negative feedback control of EGFR activity as activation of the EGFR kinase with ligand binding leads to an increase in PKC activity via phosphorylation of PLC-γ (Margolis *et al.*, 1990, see Section 1.4.a).

This negative regulation of the EGFR is complicated by the finding in a number of cell lines that EGF stimulates the mRNA transcription and synthesis of its own receptor (see Section 1.4.a). The short term inhibitory effects of PKC after EGFR kinase activation may therefore be modified in the medium term by an increase in receptor number, at least in some cells.

The carboxy terminal tail of the EGFR contains several autophosphorylation sites which may also function as negative regulators of

kinase activity. Deletions in this region result in increased sensitivity to exogenous EGF and increased oncogenic potential (reviewed in Ullrich and Schlessinger, 1990). Exogenous substrates appear to compete with the autophosphorylation sites for the receptor tyrosine kinase and it may be that deletion of these sites leads to increased access of cellular substrates to the kinase.

The intricacies of inhibitory controls on growth factor receptors will doubtless continue to be slowly unravelled and vary between different receptors and cell types. Using the EGFR as a model, it is clear how alterations in either the receptor or other molecules involved in the regulation of receptor function have the potential for decreased inhibitory control.

Tumour suppressor genes

The product of the tumour suppressor gene NF-1 has homology to the *ras* GTPase activating protein (GAP) catalytic domain and may exercise negative control over 1 or more *ras* proteins (Buchberg *et al.*, 1990). GAP increases the conversion of the active GTP-bound *ras* to the inactive GDP-bound form. In normal cells the NF-1 gene product functions as a negative regulator of *ras*, turning off the proliferation signal, but reduction in or absence of the product in transformed cells leads to a decrease or loss of regulation (reviewed in Marshall, 1991).

Other tumour suppressor genes, RB, p53 and the Wilms' tumour gene are thought to be DNA-binding proteins with roles either in transcription or DNA replication (reviewed in Marshall, 1991). As transcription factors these proteins may suppress the genes required for proliferation or, conversely, activate genes required for cell quiescence. Abnormalities in all three genes have been demonstrated in human malignancies with obvious consequences on the regulation of transcription (see Section 1.6). Human cancers often contain abnormalities of several tumour suppressor genes which presumably increases the number of deregulated genes (e.g., colon

carcinoma, reviewed in Fearon and Vogelstein, 1990).

TGF-β

TGF-β is inhibitory to a wide range of cells *in vitro* but its role as a growth inhibitor in a physiological setting is not clear. Its inhibitory effect on keratinocytes may be mediated by RB, acting to reduce the transcription of c-*myc* (Pietenpol *et al.*, 1990). Other work demonstrates that the gene product of RB can directly regulate TGF-β gene expression positively or negatively depending on the cell type (Kim *et al.*, 1991). Thus abnormalities in RB such as those described above could have a secondary deregulatory effect by reducing TGF-β inhibitory control.

Other mechanisms by which tumour cells have escaped from the direct inhibitory effects of TGF-β have been described (reviewed in Roberts *et al.*, 1988). The major proportion of TGF-β is released in a biologically inactive "latent" form which must be activated in order to bind with its receptor. This activation is possibly the principal physiological control mechanism of TGF-β activity and tumour cells which secrete latent TGF-β but fail to activate it have been reported (Wakefield *et al.*, 1987). This has a dual effect as not only are the tumour cells not inhibited by the TGF-β but the secreted factor is probably activated by, and stimulates, the fibroblasts and endothelial cells in the surrounding stroma in a paracrine manner. Other transformed cells, in contrast to their untransformed counterparts, have lost the ability to be inhibited by activated TGF-β, suggesting that transformation has interfered with the signal transduction pathway for TGF-β (Roberts *et al.*, 1988).

1.6 Proto-oncogenes in human cancers

A diverse array of proto-oncogene and tumour suppressor gene abnormalities have now been detected in an ever-expanding number of

human cancers (reviewed in Bishop, 1991). The development of adenocarcinoma of the endometrium in women with high levels of oestrogen in the absence of progesterone either through the administration of synthetic oestrogen to postmenopausal women or in pathological conditions which lead to raised levels of endogenous oestrogens has led to comparison with the other, more lethal, oestrogen-dependent cancers of breast and ovary. There is now a large body of data on these abnormalities in breast and ovarian carcinoma which are both hormone-dependent cancers similar in this respect to adenocarcinoma of the endometrium. It is therefore appropriate to look at these proto-oncogene and tumour suppressor gene abnormalities in these 2 cancers in more detail with particular emphasis on the implications for treatment and prognosis.

1.6.a Breast cancer

In a study of breast cancer, benign breast disease and cancer of the breast, non-specific tyrosine kinase activity was assessed using a phosphorylation assay on homogenized tissue samples (Hennipman *et al.*, 1989). Breast cancer tissue showed significantly greater tyrosine kinase activity than benign breast disease, which in turn was more active than normal tissue. Furthermore, it appeared that there was an association between high values of tyrosine kinase activity and biologically more aggressive tumours as determined by time to relapse. This assay of total tyrosine kinase activity in cancer is probably a measure of the increased metabolism in cancer cells and may prove to be useful in assessing tumour prognosis.

<u>EGFR</u>

In 1987 the first report was published implicating EGFR expression in the prognosis for breast cancer (Sainsbury *et al.*, 1987). The expression of EGFR in membrane preparations from primary breast cancers is inversely related to the oestrogen receptor content and is associated with a poorer

prognosis. Although the presence and number of axillary lymph node metastases remains the most important prognostic indicator, in patients with node-negative disease EGFR status is a more reliable prognostic marker than oestrogen receptor status, tumour size and grade in terms of relapse-free and overall survival (Nicholson *et al.*, 1991). An assessment of EGFR status in breast tumour biopsies or surgical specimens has become standard in many histopathology laboratories. This represents the first example of a growth factor or its receptor becoming a marker measured routinely and used for determining prognosis and further management.

c-*erb*B-2

One of the ways in which the gene for c-*erb*B-2 was initially identified was in a mammary carcinoma cell line (King *et al.*, 1985). In this cell line the gene is amplified 5- to 10-fold and this finding led to the investigation of primary human breast cancer for amplification of the c-*erb*B-2 gene. The gene was found to be amplified from 2 to greater than 20 times in 30% of breast cancers and was also found to be a significant predictor of both survival and time to relapse (Slamon *et al.*, 1987). Furthermore, in lymph node-positive disease, c-*erb*B-2 amplification was a better prognostic indicator than any other factor. Gene amplification correlates well with expression of the protein determined by immunohistochemistry or Western blotting (Venter *et al.*, 1987) suggesting that amplification of c-*erb*B-2 may be involved in the pathogenesis of some breast cancers.

Supporting evidence that overexpression of the c-*erb*B-2 gene alone can be sufficient for transformation has been provided in NIH3T3 cells transfected with the cDNA for c-*erb*B-2 (Di Fiore *et al.*, 1987a). In cells expressing low levels of c-*erb*B-2 protein the gene lacks detectable transforming activity. However, a 5- to 10-fold increase in expression of the protein results in malignant transformation which is independent of any ligand.

c-*erb*B-3

Markedly elevated levels of mRNA transcripts for the third member of the *erb*B family, c-*erb*B-3, are detected in some human breast cancer cell lines (Kraus *et al.*, 1989). No evidence of gene rearrangement or amplification is detectable in these lines using Southern blot analysis, but c-*erb*B-3 transcripts are more than a 100-fold higher than in control cell lines. Although this proto-oncogene has not been studied in primary tumours its overexpression in breast cancer cell lines suggests that it too may have prognostic implications.

c-*myc*

Amplification of the nuclear proto-oncogene c-*myc* is found in 20-30% of primary breast cancers (Escot *et al.*, 1986; Yokota *et al.*, 1986a). Although the c-*myc* proteins have yet to be shown to activate individual genes directly, they bind to DNA and probably have a role in the regulation of transcription (reviewed in Blackwood and Eisenman, 1991). Amplification of c-*myc* is seen in a wide variety of primary cancers and cancer cell lines (Nishimura and Sekiya, 1987) and the gene therefore appears to be a common site for deregulation of proliferative control. Amplification of c-*myc* is associated with aggressive tumours that are more likely to be disseminated at the time of diagnosis and higher levels of amplification are seen in the metastases than in the primary tumours from which they arise (Yokota *et al.*, 1986a).

An alternative means for increasing the expression of the c-*myc* gene is seen in some haematological malignancies where various mechanisms lead to increased levels of mRNA transcripts of c-*myc* (Bishop, 1991). Enhanced levels of c-*myc* mRNA are also seen in breast carcinomas both with, and without, associated amplification of the gene but the mechanism for this has not been elucidated (Escot *et al.*, 1986).

Fibroblast growth factors (FGF)

Two FGF-like proto-oncogenes, *int*2 and *hst*1, are both integration sites for the mouse mammary tumour virus, a retrovirus implicated in the development of mammary carcinomas in several strains of mice (reviewed in Nusse, 1988). The genes are closely linked in the human genome and have been localized to chromosome 11q13 (Adelaide *et al.*, 1988). This region is amplified in a number of solid human tumours including breast cancer and squamous carcinoma (Zhou *et al.*, 1988) and has therefore been implicated in tumourigenesis. In patients with node-negative disease *int*2/*hst*1 coamplification was found to be associated with a shorter disease-free interval compared to those without amplification (Borg *et al.*, 1991). There was no significant correlation with overall survival, however, and it is not clear whether the *int*2/*hst*1 genes are contributing to the transformation or whether another unidentified adjacent gene which is also amplified is responsible.

Cytogenetic abnormalities

Allele losses commonly occur on chromosomes 11p and 13q in sporadic breast cancers (Ponder, 1988). This raises the possibility that there are tumour suppressor genes on these chromosomes whose loss of function as a result of deletion permits tumour initiation or progression. Interestingly, of the tumour supressor genes characterized to date, the Wilms' tumour gene (WT1) and RB map to chromosomes 11p and 13q, respectively. Although WT1 abnormalities have not been described, inactivated RB alleles do occur in a small fraction of sporadic breast carcinomas (Horowitz *et al.*, 1990). More commonly, a loss of heterozygosity (LOH) is observed for the short arm of chromosome 17 which contains the site of the p53 gene. More than 60% of breast cancers show LOH for the 17p region and p53 mutations are seen in 30-50% of breast cancers (Prosser *et al.*, 1991; Osborne *et al.*, 1991). Expression of mutant p53 protein may be a marker of more aggressive carcinomas but the prognostic power is weak and therefore not

likely to be clinically significant (Ostrowski *et al.*, 1991).

1.6.b Ovarian cancer

c-*erb*B-2

In their second study of c-*erb*B-2 in a large number of primary breast cancers, Slamon and co-workers (1989) also screened DNA from non-small cell lung cancers, colon carcinomas and ovarian cancers. Of these, only the ovarian carcinomas showed significant amplification of the c-*erb*B-2 gene. They therefore looked at 120 primary epithelial ovarian cancers in more detail and detected amplification of the c-*erb*B-2 gene in 31 (26%) with 23 of the 31 showing 2- to 5-fold, and the remaining 8 showing greater than 5-fold amplification. All the tumours with amplified genes also demonstrated overexpression of the protein and a further 6 cases (12%) had increased levels of protein expression but no amplification of the gene. This emphasizes the point that, irrespective of the gene amplification or rearrangement, it is the amount of protein which is probably of ultimate importance in transformation. Like breast cancers, increasing c-*erb*B-2 amplification is associated with a progressively worse prognosis for ovarian cancer. The median survival for patients with single copy c-*erb*B-2 genes was 1879 days compared to 243 days for those women with greater than 5 copies.

A further study using immunohistochemical staining for c-*erb*B-2 protein in 72 invasive ovarian tumours showed no significant association between level of staining and either relapse-free or overall survival (Kacinski *et al.*, 1992). However, more of the patients whose tumours showed strong membrane staining for c-*erb*B-2 protein suffered relapses of disease at 3-4 years than did patients with weak or absent staining.

c-*myc*

In a study of c-*myc* in primary ovarian carcinomas no evidence of gene

rearrangement was detected but amplification was found in 5 of the 17 (29%) tumour samples (Baker *et al.*, 1990). Contrary to findings in other tumours, there was no relationship between c-*myc* amplification and other prognostic indicators or the response of the tumour to platinum-based chemotherapy. However, amplification was only present in late stage tumours, consistent with other data which suggests that c-*myc* amplification is a late event as described in many types of solid and haemopoietic tumours (Yokota *et al.*, 1986a).

c-*fms*/colony stimulating factor-1 (CSF-1)

Both the genes for c-*fms* and its ligand, CSF-1, are expressed in ovarian cancers as determined using Northern blot analysis (Baiocchi *et al.*, 1991). Neither gene was expressed in normal ovarian tissue but 16 of 18 (89%) tumours expressed c-*fms* and 14 of 18 (78%) CSF-1. In all but 2 cases tumours expressed both c-*fms* and CSF-1. Co-expression of both c-*fms* and CSF-1 was also found in metastases from 2 primary tumours which did not express either gene. Baiocchi *et al.* (1991) found no correlation with the clinical aggressiveness of the tumours but in another study using *in situ* hybridization and immunohistochemistry, expression of c-*fms* and CSF-1 was associated with clinically aggressive tumours (Kacinski *et al.*, 1988). Abnormalities in the c-*fms* gene product have been implicated in tumourigenesis (Roussel *et al.*, 1988) raising the possibility that co-expression of c-*fms* and CSF-1 contributes to ovarian carcinogenesis through increased ligand-receptor interaction.

Cytogenetic abnormalities

Lesions on chromosomes 3p and 6q have been described in ovarian cancer (reviewed in Trent *et al.*, 1989), but neither are associated with the tumour suppressor genes characterized to date. However, the regions on chromosome 3, 3p21 and 3p13, have been implicated as potential sites of tumour suppressor genes in lung and kidney carcinomas, respectively

(reviewed in Bishop, 1991). In addition, 2 proto-oncogenes, *ros* and *myb*, occupy the area on Chromosome 6 shown to be abnormal in ovarian cancer (reviewed in Trent *et al.*, 1989). There are few reports on the cytogenetic lesions of solid tumours and gynaecological tumours specifically so the significance of these associations is unknown.

More recent studies looking at loss of heterozygosity (LOH) in ovarian cancers have revealed additional allele losses on chromosomes 11p (Ehlen and Dubeau, 1990), 13q (Li *et al.*, 1991) and 17p (Russell *et al.*, 1990). The RB locus is situated at 13q14 and allelic loss at this locus was seen in 30% of informative cases (Li *et al.*, 1991) suggesting that abnormalities in RB may contribute to the development of some ovarian cancers.

p53

A recent report on 24 ovarian carcinomas using immunohistochemistry showed 11 of 16 (69%) serous carcinomas and 1 of 6 endometrioid carcinomas showed abnormal p53 expression (Eccles *et al.*, 1992). Southern blotting was used to look for LOH on chromosome 17p13 and found in 11 of the 12 tumours staining for abnormal p53. LOH was also detected in 4 other tumours not staining for mutant p53. In this study the authors suggest that p53 staining may have prognostic implications as the serous tumours were all late stage and poorly differentiated, while the other tumours in the series were all stage I except for one endometrioid carcinoma.

1.7 Growth factor receptors in normal endometrium

The study of growth factor receptors in the endometrium is in its infancy. The available data is sparse and the relevance to the cyclical changes in the endometrium is unclear. The following sections review what is known about the presence of growth factor receptors in normal endometrium.

1.7.a Epidermal growth factor receptor

The data on levels of EGFR in normal endometrium are confusing, partly because of the variety of methods used to obtain the information. The methods used, immunohistochemistry and binding studies on cell membrane preparations, have given contradictory results. The presence of EGFR in endometrium was initially confirmed in tissue homogenates (Hofmann *et al.*, 1984) but no reference to menstrual status was made in this study. Other studies using endometrial membranes have shown no variation with menstrual cycle (Sheets *et al.*, 1985) or a gradual increase during the proliferative phase and subsequent fall after ovulation (Taketani and Mizuno, 1988). EGF-binding to endometrial membranes prepared from immature female rats was increased 3-fold by pre-treatment with oestrogen suggesting hormonal dependence (Mukku and Stancel, 1985).

Histological studies are similarly difficult to interpret because of conflicting data. EGFR were detected in endometrial glands only in the proliferative phase (Damjanov e*t al.*, 1986) using the monoclonal antibody EGFR1 which recognizes the external domain (Waterfield *et al.*, 1982). However in 2 studies using a murine monoclonal antibody 528 that also recognizes the external domain of the EGFR (Berchuck *et al.*, 1989; Prentice *et al.*, 1992), EGFR were detected in all endometrial cell types without variation during the menstrual cycle. Similar findings were also reported using an autoradiographic technique (Chegini *et al.*, 1986).

In summary, from the above it is clear that, although EGFRs are present in the endometrium, the influence of the menstrual cycle on their distribution and quantity is undecided.

1.7.b c-*erb*B-2

Using an immunohistochemical approach, homogenous staining for c-*erb*B-2 can be demonstrated in normal human endometrial glands (Berchuck *et al.*,

1991). No staining is present in the stroma or myometrium and there appears to be no relationship to the menstrual phase.

1.7.c Platelet-derived growth factor receptor

In porcine uteri the β-type PDGFR is present in endometrium and myometrium (Terracio *et al.*, 1988) with higher levels of both mRNA transcripts and protein detected in the endometrium. Cellular localization using immunohistochemistry and RNA *in situ* hybridization demonstrates that the receptors are principally located on stromal cells adjacent to endometrial glands and in the walls of small blood vessels deeper in the endometrium. No reference to the oestral state of the animals is made in this study so the effect of oestrogen and progesterone is unknown.

1.7.d Insulin-like Growth Factor-1 Receptors (IGF-1R)

Membrane preparations from human endometrium possess IGF-1R and levels do not change during the menstrual cycle (Rutanen *et al.*, 1988). However, secretory endometrium synthesizes and secretes an IGF-1-binding protein which is a competitive inhibitor of IGF-1 binding to its receptors in this phase of the menstrual cycle (Rutanen *et al.*, 1988). This results in decreased availability of IGF-1 in this phase of the menstrual cycle but whether this has a physiological role in the regulation of endometrial growth is not known.

1.8 Proto-oncogenes in endometrial cancer

1.8.a Epidermal growth factor receptor

In a study of c-*erb*B proto-oncogenes in female genital tract carcinomas

using Southern blotting techniques, an additional band suggesting a gene rearrangement was detected in the 5' region of the EGFR gene of an adenocarcinoma of the endometrium (Zhang *et al.*, 1989). This was the only uterine malignancy examined in the study and the presence of a rearrangement in the external domain of the EGFR gene is comparable to the chicken v-*erb*B oncogene which gives rise to a c-terminal truncated, constitutively activated EGFR (Downward *et al.*, 1984; see Section 1.5.b). No sequence data were reported so it is not possible to make a closer comparison with v-*erb*B.

In an immunohistochemical study of 40 adenocarcinomas of the endometrium, 32.5% did not express the EGFR (Berchuck *et al.*, 1989). This is in contrast to normal endometrium (see preceeding section) but the failure to express EGFR was not related to any of the recognized prognostic indicators such as histological grade, myometrial invasion, steroid receptor status, presence of metastases or development of recurrent disease. From this report it appears that EGFR status has little to contribute to the clinical mangement of the disease but the work confirms that abnormalities in EGFR expression do occur in endometrial carcinoma.

1.8.b c-*erb*B-2

The data on c-*erb*B-2 in endometrial carcinoma are conflicting. In a study of the DNA from 16 endometrial adenocarcinomas, 11 tumours showed amplification of c-*erb*B-2 (Borst *et al.*, 1990). Although the selection of tumours showed some bias in that only 1 of 16 (6%) was well differentiated (c.f. 34% in an unselected large series of endometrial carcinomas, Morrow *et al.*, 1991), amplification was associated with advanced stage and moderate to poor tumour differentiation. Four of the 11 patients with c-*erb*B-2 amplification died of disease an average of 16 months after diagnosis and the other 7 patients had recurrent disease within 2 years. The 5 patients with tumours lacking amplification were disease-free an average of 31 months

after diagnosis.

In a larger study of 95 endometrial adenocarcinomas looking at c-*erb*B-2 expression with immunohistochemical techniques, only 9% of the tumours were found to have greater staining than that seen in normal endometrium (Berchuck *et al.*, 1991). Like normal endometrium staining was confined to the glands and was not observed in the stroma or myometrium. There was a significant relationship between heavy staining for c-*erb*B-2 and the presence of metastatic disease particularly intraperitoneal metastases. Increased expression of c-*erb*B-2 protein was also more common in papillary serous (25%) than in endometrioid (8%) or adenosquamous cancers (0%). This is consistent with the poorer overall prognosis of papillary serous carcinomas of the endometrium (Hendrickson *et al.*, 1982).

The discrepancies between the 2 studies of c-*erb*B-2 are currently inexplicable and further evidence is required to resolve these differences. The two methods of assessment may contribute to the differences observed, however amplification and expression of c-*erb*B-2 protein show a good correlation in breast cancer (see Section 1.6.a).

1.8.c c-*myc*

Amplification of c-*myc* was also investigated in the study of c-*erb*B-2 amplification described previously (Borst *et al.*, 1990). The findings were similar to those reported for c-*erb*B-2 with 10 tumour samples of 15 exhibiting c-*myc* amplification. Again amplification of c-*myc* correlated with advanced stage disease, poor differentiation and reduced survival. Four out of 6 patients died in whom there was amplification of both genes.

1.8.d CSF-1/c-*fms*

Using Northern blot analysis, CSF-1 and c-*fms* expression was detected in

5/9 and 6/9 endometrial carcinomas, respectively (Baiocchi *et al.*, 1991), with all but one tumour expressing both ligand and receptor. Normal endometrial samples did not express RNA for either protein and in 2 of the tumours expressing CSF-1 and c-*fms*, adjacent normal endometrium showed no expression. In this study there was no correlation between expression of either gene and tumour behaviour. This contrasts with data from a study using a combination of Northern blotting and immunohistochemistry in which the expression of c-*fms* was associated with clinically aggressive disease (Kacinski *et al.*, 1988). In this study, high levels of expression correlated strongly with clinicopathological features predictive of poor outcome (high histological grade, late clinical stage, deep myometrial penetration).

1.8.e p53

Using RFLP analysis on frozen tissue from endometrial cancers, loss of heterozygosity was detected most frequently on the short arm of chromosome 17 (Okamoto *et al.*, 1991). The p53 gene is located on the the short arm of chromosome 17 and mutations in this gene were detected in 3 of 24 tumours in this study. No relationship to tumour behaviour was discernible with these small numbers, however the presence of these mutations suggests that inactivation of the p53 gene is involved in the development of some endometrial cancers. This finding might be expected given the frequency of p53 gene abnormalities in many types of human cancers (see Section 1.2.c).

1.9 Summary

In the preceding discussion the normal structure and function of EGF and PDGF and their receptors have been reviewed. These proteins are part of a

large group of proto-oncogene products whose members are all involved in the transmission of regulatory signals from the cell surface to the nucleus. Subversion of the normal structure and function of these products both in transformed cell lines and in tumours has been described. Carcinogenesis results from the progressive accumulation of genetic abnormalities in proto-oncogenes and antioncogenes leading to a transformed phenotype.

Abnormalities in proto-oncogenes and antioncogenes have been detected in hormone-dependent human cancers (breast and ovary) and may therefore be involved in the development of these cancers. Extrapolating this data to endometrial cancer leads to the conclusion that such abnormalities may also be present although little has been published on the presence and/or normality of growth factor receptors in the endometrium and, more particularly, endometrial cancer. Work to date suggests that there may be relevant abnormalities in the c-*erb*B, c-*fms* and CSF-1 genes and their expression in endometrial cancer but the data is conflicting and the importance of these abnormalities requires confirmation.

EGFR expression has prognostic significance in breast cancer and is altered in endometrial cancer. Rearrangement of the external domain of the EGFR comparable to that seen with v-*erb*B has been reported in endometrial cancer (see above). Amplification and overexpression of c-*erb*B-2 is associated with clinically aggressive disease in breast, ovarian and possibly endometrial cancers.

Normal porcine endometrium contains PDGF β receptors but there are no reports in the literature on the presence or otherwise of either of the PDGF receptors in normal human endometrium or endometrial cancer.

The work performed for this thesis attempted to detect abnormalities in the EGF and PDGF receptors at both the genetic and protein levels in primary endometrial cancers and established endometrial cancer cell lines. Cell lines were chosen to facilitate studies of receptor expression and, as immortalized cells, were thought more likely to contain genetic lesions which could then be pursued further at the level of primary tumours.

2 METHODS

2.1 Cell Culture

2.1.a Routine cell culture

Maintenance

Cells stored in freezing vials in liquid nitrogen were thawed rapidly in a water bath at 37°C and plated out onto 10 cm tissue culture dishes (Nunc, Gibco-BRL, UK). Unless stated otherwise, cells were grown in Dulbecco's modified Eagle's medium (DMEM; 10 ml per dish) supplemented with 10% (v/v) foetal calf serum (FCS), 2 mM L-glutamine, penicillin (100 IU/ml) and streptomycin (100 µg/ml). The culture dishes were placed in an incubator which contained a humidified atmosphere of 95% air and 5% CO_2 at 37°C. The medium was changed every 2 days. All cell culture procedures were performed in a class II laminar flow cabinet (TUV GMBH, West Germany) with an external exhaust. The status of cell growth was assessed using an inverted phase contrast microscope (Nikon, UK).

Passaging of cultured cells

The cells were passaged when they approached confluency. Medium was aspirated off the cell layer and the cells were washed with 5 ml of warmed phosphate-buffered saline (PBS) containing trypsin (0.4%, w/v) and EDTA (0.02%, w/v). The solution was aspirated off immediately, replaced with a further 2 ml of the same solution and the dish was incubated at 37°C for 2-3 min until trypsinization was complete. The activity of the trypsin was then inhibited by the addition of 10 ml of DMEM/10% FCS. The cells were

uniformly suspended and then the cells and medium were aspirated into a pipette. The suspension was equally divided into 4-6 dishes depending on the cell line and the volume of medium on the cells was then made up to 10 ml.

Storage of cultured cells

Cells were periodically stored in liquid nitrogen to provide backup stocks. Cells in the exponential phase of growth were gently trypsinized as described above except that the trypsin solution was removed before the cells had detached. One ml of DMEM containing 20% FCS was added to the dish, the cells suspended using a pipette and transferred to a sterile universal container on ice. An equal volume of cold DMEM containing 20% FCS and 20% dimethylsulphoxide was then added dropwise to the cell suspension bringing the final concentrations of FCS and dimethylsulphoxide to 20% and 10%, respectively. The suspension was dispensed into cryotubes in 1 ml aliquots which were placed at -70°C overnight and transferred to a liquid nitrogen storage tank the following day.

Reagents used for cell culture

DMEM	Dulbecco's modified Eagle's medium containing 1000 mg/l glucose and L-glutamine 2 mM (Gibco-BRL, UK)
Penicillin/Streptomycin	100 x stock solution (Gibco-BRL, UK)
FCS	Foetal calf serum (Imperial Laboratories, UK). Aliquoted into 50 ml tubes and stored at -40°C.

Trypsin/EDTA	20 x stock solution (Gibco-BRL, UK). Aliquoted in 20 ml tubes and stored at -20°C. Diluted 1:4 with PBS for use.
PBS	Phosphate-buffered saline (Flow Laboratories, UK)

2.1.b Large scale cell culture

In order to obtain large volumes of conditioned medium a suspension culture system using Cytodex^{TM} 3 microcarriers (Pharmacia, UK) in a stirring flask (Techne, UK) was chosen. Cytodex 3 microcarriers consist of a thin layer of denatured collagen chemically coupled to a matrix of cross-linked dextran with each microcarrier having an average diameter of 175 µm. Each gram (dry weight) of microcarriers contained approximately 4 x 10^6 microcarriers with a total surface area of 4,600 cm^2 (Pharmacia, product data sheet #52-1611-00-04). The Techne stirring vessel is a glass culture vessel with a rounded and indented base and a suspended magnetic stirring rod. The vessel is placed on a low speed magnetic base stirring unit and the bulb at the end of the magnetic rod circles round the base, kept in place by the shape of the bottom of the vessel.

Preparation of culture vessels

Prior to culturing, the vessels were siliconized to prevent the microcarriers sticking to the glass. A small volume of siliconizing fluid (Sigmacote, Sigma, UK) was added to the clean Techne flask and used to wet all glass surfaces that might come into contact with the microcarriers. Excess fluid was drained from the vessel which was allowed to dry and then washed

thoroughly with distilled water. One application of siliconizing fluid was sufficient for several experiments.

Preparation of microcarriers

Dry microcarriers were added to a siliconized Techne flask and hydrated in PBS (Ca^{2+}- and Mg^{2+}-free) for at least 3 h at room temperature. The supernatant was decanted and the microcarriers were washed once with gentle agitation for a few min in PBS. The PBS was discarded and replaced with further PBS and the vessel and microcarriers sterilized by autoclaving.

Prior to use the sterilized microcarriers were allowed to settle and the supernatant was removed. The microcarriers were rinsed in warmed medium (37°C) to remove the PBS from between and within the microcarriers. The medium was removed and the cells for culture were then added in culture medium.

Maintenance of cultures

All culture procedures were performed in a class II laminar flow cabinet. 250 ml and 1 litre Techne flasks were used at different stages in these experiments. Initial culture volumes were 50 ml and 500 ml of DMEM plus 10% FCS, respectively. Cells were seeded at a density of ~10 cells per microcarrier with 0.3 g (for 250 ml flask) and 3 g (for 1 litre flask) of dry microcarriers prepared as in Section 2.1.b. The medium was equilibrated by incubating the flask containing medium in an atmosphere of 95% air: 5% CO_2 for 5 min. The cells were added and then allowed to attach to the microcarriers by incubating the Techne flask at 37°C for 2 h, stirring the culture at 20 rpm for 2 min every 30 min. Continuous stirring then commenced at 40 rpm. After allowing 2 days for the cells to become

firmly attached and to condition the medium, the volume of medium was doubled.

Half the medium was changed every 4 days or earlier if the culture was becoming acidic (as determined by the colour change in the medium). At this time a 1 ml aliquot was taken and cell numbers were counted (see Section 2.1.b), cell viability was assessed and the glucose concentration estimated with Glucostix (Miles Scientific, UK). After the medium had been changed, the vessel was gassed with CO_2 for 2 min to maintain the CO_2 concentration. When exponential growth was confirmed (day 6-9), half the culture medium was replaced with serum-free DMEM. This was repeated at each medium change to progressively reduce the serum concentration. When the serum concentration was 0.3% or less the conditioned medium was collected and frozen at -70°C. This was continued until the cultures became contaminated or cell numbers started falling (50-90 days).

Counting cell numbers

A 1 ml sample of evenly suspended culture was placed in an Eppendorf tube and the supernatant was removed after the microcarriers had settled. The microcarriers were washed with 1 ml of PBS (Ca^{2+}- and Mg^{2+}-free), the supernatant was removed when the microcarriers had settled and 1 ml of trypsin (0.125%, w/v) and EDTA (0.01%, w/v) in PBS (Ca^{2+}- and Mg^{2+}-free) was added. The tube was incubated at 37°C for 15 min with occasional agitation. The microcarriers were allowed to settle, the PBS was removed and a further 1 ml of PBS containing 0.05% (w/v) of trypan blue was added. Both viable and nonviable (stained with trypan blue) cells were then counted on a haemocytometer using an inverted phase contrast microscope.

2.2 Binding assays

2.2.a Assays on endometrial cancer cells

Cells were plated out into 24 well plates (Nunc, Gibco BRL, UK) in 0.5 ml DMEM plus 10% FCS per well at a density of 10^5 to 10^6 depending on the cell line. As the cells reached confluence they were used for binding assays. Three wells were used per assay point and 3 wells were washed as the rest but received no EGF, being used at the end for counting cell numbers.

^{125}I-labelled EGF (Amersham, UK) was diluted in binding buffer (PBS plus 1% BSA) to achieve the required radioactive count number (50-60,000 cpm/well) in 10µl and then further diluted on ice in binding buffer with increasing concentrations of unlabelled growth factor (0-250 ng/ml). The wells were washed with cold binding buffer prior to adding 0.5 ml of cold binding buffer containing the diluted mixture of ^{125}I-EGF (~0.25 ng/ml) and unlabelled EGF in increasing concentrations.

The plate was incubated at 4°C for 4 h with intermittent gentle agitation and the remainder of the experiment was carried out on ice. The medium was aspirated from the 3 wells with the maximal concentration of added EGF and put into counting vials. The average of these 3 counts was taken as the total number of counts added per well (total binding). The remaining wells were aspirated and the medium discarded into radioactive waste. The wells were washed 3 times with cold binding buffer with care to avoid dislodging any cells. Lysis buffer (1 ml/well) was added for a few minutes before the wells were aspirated and the aspirates placed in counting tubes and counted on a γ-counter (Beckman Gamma 5500, Beckman, UK). The average of the counts from the three wells from which the supernatant had previously been taken to determine total binding was used as the non-

specific binding. Lysis buffer was made up in 200 ml quantities to the following composition and stored at room temperature:

NaOH	100 mM
SDS	1%
Na_2CO_3	2%
NaN_3	0.01%

Cells in the counting wells were trypsinized, counted using a haemocytometer and the average of the 3 wells taken as the number of cells per well. The radioactivity counts for each well were entered into the EBDA computer programme (G.A. McPherson, 1983). This programme subtracts the value for non-specific binding from the average of each assay point and generates a Scatchard plot (Scatchard, 1949) of specific binding.

2.2.b Inhibition of ^{125}I-EGF binding to Swiss 3T3 cells

In order to look for the inhibition of EGF binding by EGF-like molecules in the serum-free conditioned medium passed through a FPLC column, the technique for EGF binding assays described in the preceding Section was modified for use on Swiss 3T3 cells as follows. Swiss 3T3 cells were plated into 24 well plates at a density of 3 x 10^4 cells per well and cultured until subconfluent. Sufficient wells were seeded to give triplicate wells for each column fraction to be tested and positive and negative controls.

All wells were washed twice with 0.5 ml of binding medium which was aspirated off and 250 µl of binding medium and approximately 0.05 ng/ml of ^{125}I-EGF (~10,000 cpm) made up to 250 µl in binding medium added to each well. Column fractions or controls made up to 50 µl were added to appropriate wells and the plates incubated for 1 h at 37°C. The wells were washed 4 times with cold PBS (4°C) plus 0.1% BSA and 0.1 µm

KI and lysis buffer added. Thereafter the wells were aspirated and the radioactivity counted as described.

Fresh binding buffer was made in 100 ml quantities of DMEM to which the following had been added:

BSA	0.1%
KI	0.1 µm
BES	50 mM

The pH was corrected to 7 with approximately 1 ml of 5N NaOH.

2.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

2.3.a Preparation of gel

Protein separation was carried out under denaturing conditions using vertical slab SDS-polyacrylamide gels with a discontinuous buffer system. Two clean glass plates were fixed either 0.75 mm or 1 mm apart in the gel apparatus (Bio-Rad Laboratories, Richmond, CA, USA), ensuring complete sealing at the sides and bottom. Thirty ml of the following were made up in the listed order resulting in a 7.5% final concentration of acrylamide:

Acrylamide (30%)	7.5 ml
SDS (10%)	0.3 ml
1M Tris (pH 8.8)	8.4 ml
Water	13.6 ml
TEMED	15 µl
APS (10%)	150 µl

The mixture was drawn up into a pipette and poured between the plates to within 3-4 cm of the top of the plates. A few ml of water saturated propan-

2-ol were gently poured on top of the gel mixture to promote a smooth upper surface. The gel was then left at room temperature until it had polymerized when the propan-2-ol was carefully poured off and the top of the gel washed with water. A comb was placed in the top of the gel apparatus and a 4.5% acrylamide stacking gel was poured using 10 ml of the following mixture:

Acrylamide (30%)	1.5 ml
SDS (10%, w/v)	0.1 ml
1M Tris (pH 6.7)	1.25 ml
Water	7.1 ml
TEMED	7 µl
APS (10%)	100 µl

After the stacking gel had polymerized, the comb was removed and the sample wells washed twice with the running buffer. Running buffer was made in 10 litre quantities to the following composition:

Tris base (pH 8.9)	25 mM
Glycine	200 mM
SDS	0.1% (w/v)

2.3.b Loading of gel

Running buffer was poured into the lower reservoir and the samples were boiled for five min and centrifuged for one min at 10,000g before loading. The samples were individually drawn up and loaded into sample wells in sequence using a Hamilton glass syringe. Prestained molecular weight markers (Sigma, UK) were added to wells on either side of the samples. The top reservoir was attached, carefully filled with running buffer to avoid disturbing the samples and a perspex front cover fixed in place.

2.3.c Electrophoresis

The gel was subjected to electrophoresis for approximately 14 h at 55 V constant DC using a Hoefer PS500X power pack (Hoefer Scientific Instruments, San Francisco, CA, USA) until the dye front reached the base of the gel. The current was then switched off and the gel removed and marked to ensure subsequent correct orientation.

2.3.d Coomassie staining

Gels were stained for 1 h with 0.2% Coomassie Brilliant Blue R in methanol/acetic acid/water (40:10:50 by vol) and destained in methanol/acetic acid/water (20:7:73 by vol). When ^{32}P-labelled samples were being analyzed, the destained gel was dried under vacuum on Whatman 3 mm filter paper and autoradiographed on pre-flashed XAR-5 film (Kodak, UK) at -70°C using DuPont intensifying screens.

2.3.e Reagents used in SDS-PAGE

Acrylamide	Mixture of 30% (w/v) acrylamide and 0.8% (w/v) N-N'-methylenebisacrylamide (Bio-Rad).
TEMED	Tetramethylene diamine (Bio-Rad).
APS (10%)	1 g of ammonium persulphate was dissolved in 10 ml of water (prepared fresh).
SDS (10%)	100 g of electrophoresis grade sodium dodecyl sulphate was dissolved in 1 litre of water by heating to 68°C.
1M Tris	121 g Tris base was dissolved in 800 ml of water.

The pH was adjusted to 6.7 or 8.8 by adding conc HCl, and the volume made up to 1 litre.

2.4 Western blotting of proteins

Transfer of proteins to a nitrocellulose membrane (Schleicher and Schuell BA 85, 0.45μm) was carried out using a LKB 2117 Multiphor II electrophoresis Unit with LKB 2301 Macrodrive I power supply. Before the gel had finished running 250 ml of transfer buffer was degassed for 30 min using a water pump. Transfer buffer was made up in 4 litre quantities as follows:

Glycine	57.6 g
Tris base	12 g
SDS	4 g
Methanol	800 ml
Water	to 4 l

When the gel had finished it was soaked in transfer buffer for 15 min prior to assembling the transfer apparatus. The nitrocellulose membrane and 12 pieces of 1F filter paper were cut to fit the gel exactly and bathed in transfer buffer. The base plate of the transfer apparatus was moistened with transfer buffer and 6 pieces of filter paper and then the nitrocellulose membrane positioned on the base plate taking care to remove any air bubbles from between the layers. The gel was placed directly on top of the nitrocellulose followed by the remaining 6 pieces of filter paper again ensuring that any bubbles were removed. The top plate of the apparatus was then placed in position on top of the filter paper and connected to the negative electrode.

Electrophoresis proceeded at a constant current of R amps for approximately two hours (where R = 0.8 x size of gel [cm x cm]). Complete transfer was confirmed by carefully lifting a corner of the gel and checking that the prestained markers had transferred from gel to nitrocellulose membrane. The transfer apparatus was then disassembled and the nitrocellulose membrane placed in the appropriate blocking buffer.

2.5 Kinase assays

2.5.a Kinase assay using immunoprecipitated EGFR

In experiments on cultured cells, human EGF (Amersham, UK) was added to the medium of one 10 cm dish of a pair to a final concentration of 200 ng/ml and both dishes incubated at 37°C for 10 min. The dishes were washed twice with ice-cold PBS (Ca^{2+}- and Mg^{2+}-free) and then 1 ml of cold solubilization buffer added to each dish. The cells were scraped off using a rubber policeman and homogenized by repeated aspiration through a 19G and then a 25G needle. Solubilization buffer was made up in 200 ml quantities according to the following formula and stored at 4°C until used:

Tris-HCl (pH 8.0)	50 mM
NaCl	150 mM
EDTA	100 mM
Nonidet P-40	1%
Benzamidine	50 mM
PMSF	200 mM

The solubilized cells in 1.5 ml Eppendorf tubes were then centrifuged at 14,000g in a microcentrifuge (Sarstedt MH 2-K) at 4°C for 20 min. 25 µl

of the supernatant was removed for an estimation of the protein content (see Section 2.5.c) and the remainder was transferred to fresh tubes and incubated at 4°C for 10 min. Antibody to the EGFR was then added, either R1 (~10 µg/immunoprecipitation), a mouse monoclonal antibody to the external domain, or ICR9 (~10 µg/immunoprecipitation), a rat monoclonal antibody. The solutions were then incubated at 4°C for 60 min with intermittent agitation.

40 µl of Protein A-Sepharose (Pharmacia, UK) was added per tube if the R1 antibody was used, or Protein A-Sepharose/$F(ab^1)_2$ (see Section 2.5.b) with the ICR9 antibody, and the tubes covered and mixed at 4°C for 60 min. The tubes were then gently, and briefly, centrifuged to settle the Protein A-Sepharose beads. The beads were washed 4 times with ice-cold solubilization buffer to remove unbound proteins. The beads were then washed once in kinase buffer before resuspending them in 50 µl of kinase buffer. The kinase buffer comprised the following:

HEPES (pH 8.0)	50 mM
NaCl	150 mM
Triton	0.02% (w/v)
$MnCl_2$	2 mM
$MgCl_2$	12 mM
Glycerol	10%
Na_3VO_4	100 µM

After dilution in kinase buffer, 5 µCi of γ^{32}P-ATP (>5000 Ci/mmol) were added per tube and the tubes were placed on ice for 10 min. Sample buffer (5x) was then added to a final concentration of 1x to terminate the reaction and equal amounts of homogenate protein run on 7.5% polyacrylamide gels as described in Section 2.3. The gels were stained, dried and autoradiographed at -70°C (see Section 2.3.d).

2.5.b Preparation of Protein A-Sepharose

Approximately 40 µl of Protein A-Sepharose powder was placed in an Eppendorf tube, 1 ml of PBS added and the tube stirred for 60 min. The Protein A-Sepharose beads were then washed 5 times in solubilization buffer (see preceding Section), centrifuging gently each time to settle the beads and pipetting off the supernatant. The hydrated beads were then resuspended in an equal volume of solubilization buffer and stored at 4°C until used.

In experiments using the rat antibody ICR9 it was necessary to modify the preparation to add a sheep anti-rat antibody $F(ab^1)_2$ as Protein A does not bind rat antibodies. For every 30 µl of Protein A-Sepharose, 10 µl of $F(ab^1)_2$ antibody (1 mg/ml) was added to the hydrated beads and incubated for 30 min with intermittent agitation. The beads were washed five times in solubilization buffer to produce the Protein A-Sepharose/$F(ab^1)_2$ mixture added in the experimental method described in Section 2.5.a.

2.5.c Estimation of protein concentration

Protein concentrations in the range of 1-25 µg/ml were assayed using the Pierce Protein Assay Reagent (Pierce & Warriner, UK). This reagent contains Coomassie Brilliant Blue G-250 and is a modification of the Bradford method (Bradford, 1976). Duplicate dilutions of bovine serum albumin (BSA) in solubilization buffer of 5, 10, 20, 25 µg/ml concentrations were prepared in 1 ml aliquots along with unknown protein samples. One ml of Protein Assay Reagent was added to each tube, mixed and the absorbance at 595 nm was read. The absorbance at 595 nm of the

solubilization buffer alone was subtracted from the standards and samples and a standard curve plotted. This was then used to determine the protein concentrations of each unknown protein sample.

2.5.d Kinase assay using antiphosphotyrosine antibodies

Cell lysis

Cells grown to confluence on 10 cm dishes were washed twice with PBS (warmed to 37°C) and starved for two days in DMEM with 0.5% FCS. The conditioned medium was aspirated and the cells washed twice with warmed PBS. Using the previously aspirated medium, growth factor was added to dishes with a control dish receiving medium only. The dishes were then incubated at 37°C for 10 min, the medium aspirated and the cells washed with PBS. Fifty µl of 10 mM Na_3VO_4 was added to 0.9 ml of sample buffer which was boiled, added to each dish and immediately swirled thoroughly round. Sample buffer was made to the following formula:

SDS	2% (w/v)
Na_3PO_4 (pH 7.0)	5 mM
Glycerol	10% (v/v)
DTT	100 mM
bromophenol blue	0.01% (w/v)

Sample buffer was made up in 45 ml quantities, aliquoted into 5 ml universals and kept at -20°C for up to 2 months before use.

The lysed cells were scraped off with a rubber policeman and tipped into a 1.5 ml Eppendorf tube. The DNA was sheared by drawing the solution repeatedly through a 19G and then a 25G needle. β-mercaptoethanol was added to each sample to a final concentration of 10% (v/v) and the samples were then frozen at -70°C or boiled and run on a

7.5% SDS-polyacrylamide gel as previously described (Section 2.3).

Visualization of phosphotyrosine containing proteins

After running a gel, Western transfer was performed as described in Section 2.4 except that the transfer buffer also contained 500 μM Na_3VO_4. The nitrocellulose sheet was incubated in blocking buffer at 37°C for not less than 60 min. Blocking buffer consisted of 5% BSA and 1% ovalbumin in rinsing buffer. The rinsing buffer was made up in 4 litre quantities and contained the following:

Tris-HCl (pH 7.4)	10 mM
NaCl	0.9% (w/v)
NaN_3	0.01%

The blot was then incubated with 5 μl of anti-phosphotyrosine antibody (~20 μg/ml) in 5 ml of blocking buffer for 2 h at room temperature with continuous gentle agitation. This antibody was raised in rabbits by Drs M.J. Fry and G. Panayotou, LICR, against an alanine, glycine, phosphotyrosine mixture coupled to keyhole limpet haemocyanin with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide according to the method described by Kamps and Sefton (1988).

The nitrocellulose filter was washed twice for 10 min in rinsing buffer, once for 10 min in rinsing buffer plus 0.05% NP-40, then twice for 5 min in rinsing buffer again. All these washes were carried out at room temperature under continuous agitation. The filter was then incubated for 60 min at 37°C with 1 μCi of ^{125}I-Protein A (45 mCi/mg, 1 μCi/10 μl, Amersham, UK) in 5 ml of blocking buffer. The filter was washed as before, damped dry on Whatman 3 mm filter paper, placed on another sheet of filter paper, covered with saran wrap and then autoradiographed at -70°C.

2.6 Concentration of conditioned medium

Medium conditioned by the growth of cells and collected from the Techne stirring flasks (see Section 2.1.b) was concentrated ~25-fold using the Minitan-S Ultrafiltration system (Millipore Corporation, Mass, USA). This is a tangential flow system used to filter biological fluids under pressure through the filtration sheets of specific pore size.

The apparatus was assembled as per the manufacturer's instructions using Minitan-S PCAC ultrafiltration sheets with a molecular weight cut-off of 1,000. From a starting volume of 5-600 ml, the conditioned medium passes through the apparatus under a pressure of 5 p.s.i., the filtrate and molecules with a molecular weight less than 1,000 are collected and the retentate containing larger molecules is recirculated to pass through the system again. This cycle is repeated continuously until the retentate volume reaches the limit of the apparatus, approximately 15-20 ml.

To exchange buffers in the concentrated conditioned medium in preparation for column chromatography in this experiment, the final rentate volume was diluted in 20x the volume of PBS and passed through the system again.

2.7 DNA synthesis assay

Stock cultures of Swiss 3T3 mouse fibroblasts were grown in 10 cm Nunc tissue culture dishes until almost confluent. The cells were trypsinized and counted on a haemocytometer as in Section 2.1.b. A suspension of 3.6×10^5 cells per 100 ml of DMEM/10% FCS was prepared and pipetted into a 96 well tissue culture plate (Nunc, UK), 100 µl per well. The plate was then

incubated at 37°C in 95% air : 5% CO_2 for approximately 1 week without changing the medium until the cells were confluent and quiescent.

The medium was aspirated, the cells were washed twice with warmed PBS and 100 µl of incubation medium were added to each well. Incubation medium consisted of:

DMEM	100 ml
Thymidine (100 µm)	1 ml
^{3}H-thymidine	1 ml

The ^{3}H-thymidine solution contained 250 µl of ^{3}H-thymidine (1 mCi/ml, 25 Ci/mmol, TRK 120, Amersham, UK) diluted with 1 ml of 100 µm thymidine. The growth factor or other agents to be tested were added to triplicate wells and the plate was incubated for 40-48 h at 37°C.

The remainder of the assay was performed using a 12 channel cell harvester (Multimash 2000, Dynatech, UK). The incubation medium was aspirated and the cells washed twice with warm PBS (Ca^{2+}- and Mg^{2+}-free, 37°C). 100 µl of a 10x mixture of trypsin (0.25%, w/v) and EDTA (0.02%, w/v) in PBS (Ca^{2+}- and Mg^{2+}-free) were added to each well and the dish incubated at 37°C until the cells detached. If the cells failed to detach completely, the plate was placed on a plate shaker Model 804 Vibrator (Luckman, UK) for 5 min.

The cells were then harvested onto the filter mats provided with the cell harvester and the mats were allowed to dry at room temperature. The filter discs from each channel were removed individually from the mat and placed into scintillation vials (Beckman, UK). Five ml of scintillant (Beckman Protein-+, Beckman, UK) were added to each vial and the radioactivity counted on a LS 1801 scintillation counter (Beckman RIIC Ltd., UK).

2.8 Column chromatography

Separation of the conditioned medium concentrated by the Minitan S (Section 2.6) into molecules of different molecular weight was achieved using an FPLC™ system (Pharmacia, UK). This system is a modification of high performance liquid chromatography (HPLC) with the major difference being the reduced pressures that are needed to pump the columns. The FPLC system is fully automated and connected to a PC computer which controls its various functions. The FPLC column runs were performed by Dr P Stroobant, LICR, using a Superose 12 (Pharmacia, UK) size exclusion column. This column contains a crosslinked, agarose-based medium which is suitable for separating proteins with a molecular weight of 10^3 to 3 x 10^5, which was considered the appropriate size for growth factor-like molecules.

Standards of known molecular weight were run through the column prior to starting the separation of the conditioned medium. Three ml of concentrated conditioned medium (3 mg/ml protein) was then added to the column and the column run at 1 ml per minute using PBS (pH 7.4) (filtered through 0.2 µm) as an eluent. Four min fractions were collected for 100 min following the passage of the column buffer. The column fractions were then stored at -70°C until used for DNA synthesis assays.

2.9 DNA METHODS

2.9.a DNA extraction from cultured cells

Cell lines

Six epithelial endometrial cancer cell lines were utilized in this study. Two were sublines of the oestrogen-responsive Ishikawa cell line. This cell line was originally established in 1980 from a well differentiated endometrial adenocarcinoma (Nishida *et al.*, 1985). The first subline, Ishikawa 1, (Passage No. unknown) obtained was a gift of Dr J White (Department of Obstetrics and Gynaecology, Hammersmith Hospital, London) and the second, Ishikawa 2, (Passage No. unknown) was obtained from Dr E Gurpide (The Mount Sinai Medical Centre, New York, USA). The remaining 4 lines were purchased from the American Type Culture Collection (ATCC), Rockville, Maryland, USA. Two of these, HEC-1-A (Passage No. 127) and HEC-1-B (Passage No. 128), were derived from a single moderately differentiated adenocarcinoma of the endometrium (Kuramoto *et al.*, 1972), with HEC-1-B being a tetraploid subclone of HEC-1-A (Kuramoto, 1972). The RL95-2 cell line (Passage No. 123) was established from a moderately differentiated adenosquamous carcinoma of the endometrium (Way *et al.*, 1983) and the KLE cell line (Passage No. 8) was cultured from an undifferentiated endometrial carcinoma (Richardson *et al.*, 1984).

Cell lysis

Cultured cells grown to confluence on 15 cm dishes (Nunc, Gibco BRL) were washed twice with PBS to remove the medium. Four ml of lysis

buffer was added on to the cells and swirled round the dish. Lysis buffer was made immediately prior to use to the following composition:

NaCl	0.1 M
Tris-HCl (pH 8.0)	10 mM
EDTA (pH 8.0)	1 mM
SDS	0.5% (w/v)

The cells were scraped off using a disposable plastic cell scraper (Costar, Northumbrian Biologicals, UK) and the cell suspension transferred to a 50 ml polypropylene tube. Proteinase K (Sigma, UK) was added to the DNA lysate to a final concentration of 200 µg/ml and the tube was incubated at 37°C on a rotating wheel for 4 h.

Phenol/chloroform extraction

This was carried out largely as described in Maniatis *et al.* (1982). An equal volume of phenol (Gibco-BRL) saturated with 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA was added to the tube and mixed on a wheel for 30 min at room temperature. The aqueous phase (containing the DNA) was separated from the organic phase (containing phenol and protein) by centrifugation at 1200 g for 20 min at 20°C in a Sorvall Technospin R centrifuge (DuPont, UK). The aqueous (upper) phase was carefully removed using a wide-mouthed pipette and placed into another 50 ml polypropylene tube.

An equal volume of chloroform/isoamyl alcohol (24:1, v/v; Applied Biosystems, UK) was added and the mixing and centrifugation steps repeated. The aqueous phase was removed and the extraction with chloroform/isoamyl alcohol repeated. Following centrifugation the aqueous phase was transferred to another 50 ml polypropylene tube ready for precipitation.

Precipitation of DNA

The DNA was precipitated by the addition of 0.1 vol of 3 M Na acetate (pH 5.2) and 2.5 vol of 100% ethanol (Mérck, UK) to the DNA lysate. After gentle mixing, the DNA could be seen as a white gelatinous precipitate which was removed from the precipitation mixture by spooling it onto a hooked glass Pasteur pipette. This helps to separate the high molecular weight DNA molecules from the smaller RNA molecules which are left behind in the precipitation mixture. The DNA adhering to the Pasteur pipette was rinsed in 70% ethanol to remove the Na acetate and placed in a 1.5 ml Eppendorf tube containing 1 ml of TE (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) and dissolved overnight at 4°C.

Quantitation of DNA concentration by spectrophotometry

Serial dilutions of the DNA suspended in TE were subjected to ultraviolet absorption spectrophotometry. Readings were taken at optical densities (OD) of 260 nm and 280 nm on a DU-50 spectrophotometer (Beckman Ltd., UK). The OD_{260} : OD_{280} ratio is an indication of the purity of the DNA preparation (with respect to protein contamination) with a value less than 1.8 indicating a pure preparation. An OD_{260} of 1.0 is equivalent to a double stranded DNA concentration of 50 µg/ml.

2.9.b DNA extraction from solid tumour samples

The tumour sample of approximately 0.5 ml in volume was homogenized on ice in a glass homogenizer in 3 ml of PBS (Ca^{2+}- and Mg^{2+}-free). This resulted in the release of the intact cell nuclei and the homogenizer was rinsed with a further 7 ml of PBS to ensure maximal recovery.

Homogenization was carried out in a class II laminar flow cabinet

which had an external exhaust system. All buffers used in preparation of the DNA prior to the addition of lysis buffer were discarded into a 5% (v/v) chloros solution and left to stand for 6 h in the laminar flow cabinet before disposal.

After homogenization the nuclei were pelleted by centrifugation at 400 g for 15 min at 4°C in a Sorvall Technospin R centrifuge. Preparation of a nuclear pellet prior to adding the lysis buffer is a useful technique in extracting DNA from solid tumours as it reduces the amount of protein and RNA in the lysate. The nuclear pellet was then lysed in 10 ml of lysis buffer and the DNA lysate processed in an identical manner to that described in Section 2.9.a.

2.9.c Digestion of DNA with restriction endonucleases

All restriction enzymes were obtained from Boehringer Corporation (London) Ltd., UK. DNA was digested in a total volume of 40 µl of the following mixture:

DNA (10 µg) in TE	variable volume
10x restriction buffer	4 µl
Restriction enzyme	1-2 µl
Water	to 40 µl total

10x restriction buffers, providing the specific ionic concentration for a particular enzyme, were supplied by Boehringer Corporation. Digestion was allowed to proceed for 3 h at 37°C with inversion of the tubes each hour. Further restriction enzyme was then added to ensure complete digestion and the tubes incubated a further 3 h. At this time a small agarose gel (see following Section) was run with a selection of the digests to confirm that digestion was complete.

2.9.d Agarose gel electrophoresis

Gels were prepared by dissolving the requisite amount of agarose (0.8%, Gibco-BRL Ltd., UK) in 300 ml of TAE buffer by boiling in a microwave oven. TAE buffer (50x) was made up in 10 litre quantities to the following formula:

Tris-acetate	2 M (Tris base plus glacial acetic acid)
EDTA	50 mM

After boiling the mixture was allowed to cool to 60°C and poured into a 20 x 20 cm gel casting tray which was provided with the gel tank apparatus (International Biotechnologies Inc., New Haven, CT, USA). A comb was placed in one end of the gel and the gel was allowed to set.

The gel was submerged in a horizontal electrophoresis tank containing TAE buffer (1x) and the comb was removed. Samples of restriction enzyme-digested DNA (40 µl) were mixed with 4 µl of loading buffer (10x) and loaded into the wells formed by the comb. Loading buffer (10x) was made up to the following composition in 10 ml quantities and stored at -20°C:

Glycerol	50% (v/v)
EDTA	1 mM
Bromophenol blue	0.4% (w/v)

After loading, the samples were subjected to electrophoresis for approximately 18 h at 30 V DC (constant voltage) using a Hoefer PS500X power pack (Hoefer Scientific Instruments, San Francisco, CA, USA). The DNA was then visualized by staining the gel in a 1 µg/ml ethidium bromide solution for 10 min followed by illumination on a long wave UV light box. Photographs of the gel were taken using Polaroid type 57 film with the gel lying alongside a 20 cm ruler to facilitate accurate sizing of DNA

fragments after hybridization.

DNA markers of known molecular weight were co-electrophoresed alongside the tumour and cell DNA samples in order to assess the size of the test sample bands. Lambda phage DNA digested to completion with *Hin*dIII (Gibco-BRL Ltd., UK) was used for this purpose with fragments of the following sizes: 23130, 9416, 6682, 4361, 2322, 2027, 564, and 125 base pairs.

2.9.e Southern blotting of DNA

Southern blotting was used to transfer DNA fragments separated by agarose gel electrophoresis onto nylon-backed nitrocellulose membrane. Once immobilized on the nylon membrane, the DNA could then be probed with labelled DNA probes in order to identify complementary sequences of interest.

Following photography of the ethidium-stained gel, the gel was soaked in 1 litre of denaturation buffer (1.5 M NaCl, 0.5 M NaOH) for 30 min. Denaturation buffer separates the double-stranded DNA and allows them to bind to the nylon membrane, as the membrane only binds single-stranded nucleic acids.

The gel was then neutralized by soaking it in 1 litre of neutralizing buffer (1 M Tris-HCl (pH 8.0), 1.5 M NaCl) for 30 min. The gel was inverted and placed on a piece of filter paper soaked in 10 x SSC (20 x: 3 M NaCl, 0.3 M trisodium citrate pH 7.0) which was lying on a gel casting tray. The ends of the filter paper were in contact with a reservoir of 10 x SSC, to act as a wick to keep the filter moist. A piece of nylon membrane (Hybond-N, Amersham, UK), previously cut to the same size as the gel, was placed on top of the gel. On top of this were placed two pieces of filter

paper the same size as the gel followed by a stack of tissue paper at least 5 cm thick and then a 1 kg weight. Transfer of the DNA takes place by capillary action, and was allowed to proceed for at least 12 h. Following transfer, the nylon membrane was removed, allowed to air dry for 10 min and placed in a UV Stratalinker 1800 (Stratagene, UK) in order to immobilize the DNA to the membrane.

2.9.f Hybridization of DNA immobilized to nylon filters

Preparation of stock solutions used for DNA hybridization

Denhardt's solution was prepared as a 50x concentrated stock to the following formula:

Ficoll	1% (w/v)
polyvinylpyrrolidone	1% (w/v)
BSA (in sterile water)	1% (w/v)

It was filtered through 0.2 µm filters (Nalgene, UK), decanted into 10 ml aliquots and stored at -20°C. The formamide obtained from Fluka Chemicals Ltd., UK, was of sufficient purity to be used without deionization. A stock solution of carrier DNA was prepared by dissolving 5 mg of salmon sperm DNA (Sigma, UK) in 50 ml of TE by gentle agitation overnight. The DNA was sheared by sonication at full power for 10 min in a Soniprep 150 sonicator (MSE, UK), divided into 5 ml aliquots and stored at -20°C until used. The remainder of the stock solutions used in the hybridization process did not require any special preparatory techniques.

Radioactive labelling of DNA probes for hybridization

The DNA probes used to identify specific parts of the human genome were

radioactively labelled by means of the "random primer" reaction, first described by Feinberg and Volgenstein (1983). One µl of hexameric random primers (1 mg/ml, Pharmacia, UK) was added to approximately 200 ng of probe DNA suspended in 10 µl of TE and the tubes heated to 95°C for 5 min followed by immediate cooling on ice. Then the following were added in the listed order to a final volume of 30 µl:

Messing buffer (10x)	3 µl	(0.5 M Tris (pH 7.6), 0.1 M $MgCl_2$, 50 mM DTT)
Water	1 µl	
3x dNTPs	1 µl	(dATP, dGTP, dTTP, each 25 mM)
α^{32}P-dCTP	10 µl	(~3000 Ci/mmol)
Klenow fragment	1 µl	

The reaction mixture was incubated at 37°C for 1 h in a water bath and the unincorporated nucleotides separated from the labelled probe by spinning the mix through a G50 Sephadex column (see below). The separation centrifugation spins were performed at 500 rpm for 4 min at 20°C using an HB-4 swing bucket rotor in a Sorvall RC5C centrifuge (DuPont, UK). A 1 µl aliquot of probe was removed for estimation of radioactive label incorporation by means of liquid scintillation using an LS 1801 scintillation counter (Beckman RIIC Ltd., UK). Levels of radioactivity in excess of 1 x 10^6 cpm/µl of probe reaction mixture were routinely obtained.

The handling and disposal of radioactive materials was performed according strict safety procedure followed at the LICR.

Preparation of G50 Sephadex columns

The distal 0.5 cm of a 1 ml plastic syringe was loosely packed with siliconized glass wool. G50 Sephadex (Pharmacia Ltd., UK) equilibrated in TE was added to the top of the syringe ensuring that no air bubbles

formed. The syringe was then placed in a 50 ml tube and centrifuged at 500 rpm for 4 min in a Sorvall centrifuge. The syringe was topped up with G50 Sephadex in TE, centrifuged again and the top covered with saran wrap until used. Columns were made in sets of 4 and stored upright at 4°C.

Hybridization of ^{32}P-labelled DNA probes to nylon filters

Prior to hybridization, a prehybridization step was necessary to prevent radioactive probe DNA binding randomly to the nylon membrane. After immobilizing the DNA the nylon membrane was removed from the Stratalinker and soaked in 3x SSC for 5 min. It was then placed in a plastic bag in which 0.5 ml of prehybridization solution had been added per square cm of membrane. The prehybridization solution as made as follows:

Formamide	50% (v/v)
SSC	5x
Denhardt's	5x
$NaPO_4$ (pH 6.7)	50 mM
Na pyrophosphate	1 mM
ATP	0.1 mM
SDS	0.1% (w/v)

The solution was made up in 500 ml quantities, aliquoted into 50 ml tubes and stored at -20°C until use. Immediately prior to use, sonicated salmon sperm DNA (0.1 mg/ml) was denatured by heating to 95°C for 10 min, followed by instant cooling on ice for 3 min, and added to the prehybridization mixture (10 µl/ml of prehybridization solution). The bag was sealed ensuring complete expulsion of all air bubbles and placed in an incubator (Leec, UK) on a R100 rotary shaker (Luckham, UK) at 42°C for 1 h.

The prehybridization solution was discarded and an equal volume of

the same buffer added to the bag for hybridization. A volume of labelled probe DNA equivalent to 10^6 cpm/ml of hybridization buffer was denatured by heating to 95°C for 10 min followed by immediate cooling on ice for 3 min. The labelled DNA was then added to the plastic bag and the bag was sealed and incubated as before for 18 h on a rotary shaker.

Washing of nylon filters after hybridization

After hybridization the membrane was subjected to 2 washes of 10 min each in 2x SSC at room temperature with gentle shaking on a rotary shaker. The final high stringency washes were in 0.1x SSC/0.1% SDS at 60°C in a shaking water bath (Gallenkamp, UK, model BKS 350).

Autoradiography

Following the high stringency washes the filters were blotted dry on 3 mm filter paper (Whatman, UK), covered with saran wrap and exposed to Kodak XAR-5 film at -70°C using a single intensifying screen. The initial exposure was for 18 h and longer exposures were then performed if necessary. The maximum exposure for a satisfactory result was 5 days.

Removal of the radioactive probe from the nylon filter

If the same Southern blot membrane was required for re-probing with a different labelled DNA probe, the previous probe was stripped from the blot. This was achieved by immersing the membrane in 250 ml of a stripping solution for 45 min at 65°C with vigorous agitation every 15 min. The membranes were then ready for rehybridization. The stripping solution was made up as follows:

Formamide	50% (v/v)
Tris-HCl (pH 7.6)	10 mM

EDTA	1 mM

2.9.g Preparation of stock solutions

Unless otherwise stated in the text, all reagents were obtained from BDH Ltd., Dagenham, Essex, UK.

1 M Tris	This was made to the appropriate pH as described in Section 2.3.e and the solution was sterilized by autoclaving.
0.5 M EDTA	186.1 g of disodium EDTA was added to 800 ml of water and stirred vigorously while the pH was adjusted to 8 with NaOH pellets. The volume was made up to 1 litre and the solution sterilized by autoclaving.
5 M NaCl	292.2 g of NaCl was dissolved in 1 litre of water. The solution was sterilized by autoclaving.
3 M Na acetate	408.1 g of Na acetate.$3H_2O$ was dissolved in 800 ml of water. The pH was adjusted to 5.2 with glacial acetic acid. The solution was made up to 1 litre and sterilized by autoclaving.
1 M $MgCl_2$	203.3 g of $MgCl_2$ was dissolved in 1 litre of water and the solution sterilized by autoclaving.
10% SDS (w/v)	This was made as described in Section 2.3.e..
SSC (20x)	175.3 g of NaCl and 88.2 g of sodium citrate were dissolved in 1 litre of water. The solution was sterilized by autoclaving.
Ethidium bromide	1 g of ethidium bromide was dissolved in 100 ml of water.

3 IS THERE AMPLIFICATION OR REARRANGEMENT OF GROWTH FACTOR RECEPTOR GENES IN ENDOMETRIAL CANCER?

3.1 Introduction

To date there have been few reports in the literature of abnormalities in growth factor receptor genes in endometrial cancer (Zhang *et al.*, 1989; Borst *et al.*, 1990). This contrasts with other oestrogen-dependent malignancies, breast and ovarian carcinoma, where both amplification and rearrangement of growth factor receptor genes have been demonstrated to be present and associated with the biological behaviour of the tumours (Slamon *et al.*, 1989; and see Section 1.6). Abnormalities of the growth factor receptor genes appear to be related to tumour progression and it is assumed that the abnormalities are important in the genesis of these cancers in an analogous manner to the activation of oncogenes and inactivation of tumour suppressor genes described in other cancers (reviewed in Bishop, 1991). Endometrial cancer is similar to breast and ovarian cancer in its dependence on oestrogen and it is therefore possible that similar abnormalities in growth factor receptor genes could play a role in the development of endometrial cancer.

One of the major obstacles to the investigation of endometrial cancer is related to the way in which these tumours develop. In contrast to both breast and particularly ovarian carcinomas, endometrial cancer presents early in its natural history because of its tendency to cause vaginal bleeding, typically in postmenopausal women. Whereas 75% of women with endometrial cancer have disease confined to the uterus at the time of

diagnosis (Morrow *et al.*, 1991), 60% of women with ovarian cancer have widespread disease on abdominal peritoneal surfaces or distant metastases at presentation (Morrow, 1992).

In practical terms this means that it is relatively easy to obtain an adequate sample from an ovarian cancer without interfering with the subsequent pathological assessment. In contrast, many endometrial cancers are either not macroscopically visible at the time of surgery or so small that biopsy would significantly impair the pathologist's assessment of the extent and invasive potential of the tumour. For this reason, it follows that reports of studies on endometrial cancer utilizing tissue biopsies have an unavoidable selection bias in that samples are only obtained from specimens with sufficient tumour volume. This has significant implications in terms of data interpretation as tumour volume has been proposed as an independent prognostic variable in endometrial cancer (Schink *et al.*, 1991). In addition, tumour samples contain variable and unpredictable proportions of normal tissue, which contribute to the results obtained.

While recognizing these limitations, it was decided to screen a series of endometrial cancers for amplification and rearrangement of growth factor receptor genes. This work was undertaken in two parts: the first was performed in association with Dr D Venter from the LICR using frozen samples of endometrial cancers obtained from Dr J White (Department of Obstetrics and Gynaecology, Hammersmith Hospital, London, UK). DNA was extracted from these samples and the genes for c-*erb*B-2 and EGFR examined. In the second part of the work, the EGFR, PDGFR, and c-*fms*/(CSF-1R) genes were studied using DNA from a selection of the tumour samples previously used, and from endometrial cancer cell lines grown in culture.

STUDY USING FROZEN TUMOUR SAMPLES

3.2 Sample analysis and results

3.2.a Samples

Tissue samples from 50 endometrial cancers stored in liquid nitrogen were obtained from Dr J White (Department of Obstetrics and Gynaecology at Hammersmith Hospital, London). These samples had been collected from a number of hospitals in the greater London area (see Table 3.1). The samples were collected by clinicians at the time of curettage or hysterectomy and passed to a technician from Dr White's laboratory who immediately placed the samples in cryotubes and snap froze the specimens in liquid nitrogen prior to transportation back to the laboratory for storage.

To obtain a histological diagnosis for each of the samples, pathologists at the individual hospitals from which the samples were collected were contacted and a copy of the report for the operative specimens obtained.

3.2.b Southern analysis of the c-*erb*B-2 and EGFR genes

DNA was extracted from the tissue samples as described in Section 2.9.b. In 18 specimens it was considered that there was insufficient tissue present to perform the extraction. After DNA extraction, a further 4 samples had no measurable DNA leaving 28 specimens with adequate amounts of DNA for analysis.

Southern blots were made after digesting 10 μg of tumour DNA with

*Eco*R1 and separation of the DNA fragments on a 0.8% agarose gel as described in Sections 2.9.c-e. Tumour samples were divided into 2 groups of 14 and run on 2 gels with the DNA from two normal subjects and molecular weight markers and will be discussed as ENDO 1 (samples 1-14) and ENDO 2 (samples 15-28). The gels were blotted on to nylon membranes and then probed sequentially with probes to the c-*erb*B-2, EGFR and the collagen genes.

The c-*erb*B-2 gene was examined using a 4.6 kb full-length human c-*erb*B-2 cDNA probe (Yamamoto *et al.*, 1986). The EGFR gene was assessed with the p64.1 probe, which is a 1838 base pair *Eco*R1 human cDNA fragment (Ullrich *et al.*, 1984).

The blots were probed for c-*erb*B-2 initially and washed at high stringency as described in Section 2.9.f. Following autoradiography, the bound radioactivity was removed and the blot re-probed.

To determine the degree of gene amplification the blots were hybridized with probes to the α-1-I (Solomon *et al.*, 1984) and α-2-I (Sykes and Solomon, 1978) collagen genes following hybridization to the c-*erb*B-2 and EGFR probes. Each of the probes gives rise to a single band at 6 and 4 kb, respectively. The blots were washed at high stringency and autoradiographed as described in Section 2.9.f.

The strength of the signal obtained from each probe was quantified using a soft laser scanning densitometer (Joyce-Loebl, UK).

3.2.c Results

Data on the histology of the samples were gathered after most of the work described in this chapter was completed. The specimens had been stored for a number of years in some cases but not correlated with a histological

diagnosis until this thesis was undertaken.

Following histological correlation, of the 28 samples from which DNA was extracted, only 13 were malignant with 1 of these being an ovarian cancer, albeit endometrioid in histological type (No. 8), and 1 poorly differentiated adenocarcinoma of the cervix (No.15). Of the remainder, 8 were completely normal and of different phases of the menstrual cycle (No.s 2,6,9,11,14,17,23,28) and 4 showed varying degrees of hyperplastic change (No.s 5,10,13,19). No histological diagnosis could be obtained on 3 cases because of inadequate record collection in 2 (No.s 4,24) and failure of the pathologist to respond to both telephone and written enquiries in 1 case (No. 21).

Of the 11 endometrial cancers, 4 were well differentiated (No.s 16,18,25,27), 4 were moderately differentiated (No.s 3,7,12,20), 2 were poorly differentiated (No.s 1,22) and 1 a clear cell carcinoma (No. 26).

3.2.d Southern analysis

The probe for c-*erb*B-2 is a full length cDNA and the band sizes normally detected are ~20, ~12, 7.7, 6.5, 5.0 and 2.1 kb in length.The p64.1 probe to the EGFR hybridizes to the part of the gene encoding most of the external (EGF binding) domain, the transmembrane region and the 5' part of the region coding for the cytoplamic domain (Ullrich *et al.*, 1984). The band sizes of the EGFR gene normally detected on Southern blots of EcoR1-digested DNA by the p64.1 cDNA probe are as follows: 7.7, 6.9, 5.9, 2.7, 2.4, 2.3, 2.0 and 1.6 kb in length (Libermann *et al.*, 1985).

There was no evidence of either amplification or rearrangement of either the EGFR or c-*erb*B-2 genes (see Table 3.2 and Figs 3.1 & 3.2).

3.3 Discussion

The technique described in this chapter, whereby the density of the autoradiographic signal produced by the gene under investigation is compared to the signal produced by known single copy gene sequences, is suitable for detecting amplification with certainty when the target gene is present in at least 3 times the normal copy number (Slamon *et al.*, 1987; Gullick *et al.*, 1989).

This study compared the levels of the EGFR and c-*erb*B-2 genes with the single copy collagen genes. The α-1-I collagen gene is situated on chromosome 17 (Solomon *et al.*, 1984) as is the gene for c-*erb*B-2, while the α-2-I collagen gene (Sykes and Solomon, 1978) and the gene for the EGFR are both located on chromosome 7. This allowed a comparison of the strength of the autoradiographic signals produced by the EGFR and α-2-I genes which was independent of any duplication of chromosome 7. A similar comparison was possible with the c-*erb*B-2 and α-1-I genes. Duplication of either chromosome was further excluded by the similarity of the signal intensities of the α-1-I and α-2-I bands in each sample (densitometry data not shown but see Figs 3.1 & 3.2).

There was no evidence of amplification of the c-*erb*B-2 gene, but it is possible that a more sensitive technique such as serial dilution of the DNA and dot blot analysis (Ro *et al.*, 1988) for detecting gene amplification may have confirmed this.

This lack of amplification is of interest as it contrasts with a previous report of c-*erb*B-2 amplification in 11 out of 16 (69%) endometrial carcinomas (Borst *et al.*, 1990). In their study amplification of the gene was associated with advanced and poorly differentiated lesions and the authors suggested that c-*erb*B-2 amplification may be a predictor of

biologically aggressive endometrial carcinomas. Possible criticisms of the study are that there was no single copy gene to control for variations in DNA loading and the signal was not quantified in any way. These concerns make it difficult to compare the results of Borst *et al.* (1990) and the current work.

Amplification of the c-*erb*B-2 gene with associated overexpression of c-*erb*B-2 protein is a common phenomenon in adenocarcinomas and has been reported in adenocarcinoma of the breast (King *et al.*, 1985), salivary gland (Semba *et al.*, 1985), stomach (Yokota *et al.*, 1986b), colon (Meltzer *et al.*, 1987), kidney (Yokota *et al.*, 1986b) and ovary (Slamon *et al.*, 1989). However, c-*erb*B-2 amplification is present in only 30% of breast cancers (Slamon *et al.*, 1987) and in less than 10% of a variety of adenocarcinomas (Yokota *et al.*, 1986b). Although unlikely, it is possible that there were insufficient numbers in this study to reliably detect c-*erb*B-2 amplification.

Another explanation is that amplified c-*erb*B-2 sequences from epithelial endometrial cancer cells were diluted by DNA of stromal cells which contain only single copy genes. Cancers are usually heterogeneous and contain variable proportions of malignant and stromal components and this is one of the unavoidable sources of error in studies of tumour DNA. The error can only be minimized by scrupulous attention to collection techniques.

Another explanation for the lack of amplification observed was that degradation of the DNA had occurred prior to electrophoresis. This problem was avoided by checking the integrity of the DNA prior to digestion.

Although no definite abnormality in either copy number or gene arrangement of c-*erb*B-2 was detected, this does not exclude mutations in

the DNA. The oncogenic rat homologue of c-*erb*B-2, *neu*, was originally identified as a transforming gene in a NIH3T3 transfection assay (Shih *et al.*, 1981). Comparison with the normal rat *neu* gene revealed an identical point mutation in these and subsequent cases, which results in a single base substitution of glutamic acid for a valine residue at position 664 in the transmembrane region of the protein (Bargmann *et al.*, 1986).

No evidence of mutations analogous to the point mutation in the transmembrane region of *neu* have been detected in the amplified c-*erb*B-2 gene in human tumours although rearrangement of the gene was demonstrated in a small proportion of breast cancers with amplified c-*erb*B-2 genes (Slamon *et al.*, 1987). The mere presence of increased quantities of structurally normal c-*erb*B-2 protein is sufficient to induce cell transformation when overexpressed in NIH3T3 cells (DiFiore *et al.*, 1987a). It is therefore likely that overexpression of normal protein is the primary mechanism involved in tumourigenesis in humans. It is possible that structural mutations in c-*erb*B-2 may yet be detected in human tumours but it was felt to be beyond the scope of this work to look for possible mutations.

In contrast to the report by Zhang and colleagues (1989) who showed rearrangement in the 5' region of the EGFR in the one endometrial cancer they studied, we were unable to demonstrate any evidence of genetic rearrangement of the EGFR. The structural alteration they have reported is interesting because of its similarity to the avian oncogene v-*erb*B, where the gene product is a 5' truncated EGFR that is constitutively activated (Kris *et al.*, 1985). However, only 11 endometrial tumours were analysed in this study so it is possible that rearrangement of the EGFR gene occurs in endometrial adenocarcinomas, but at a low frequency.

Another explanation is that rearrangements were present in these

samples but have not been detected. A possible cause for this is that the rearrangement occurred towards the 3' end of the EGFR gene without any net loss of genetic material. The p64.1 cDNA probe used in this study spans a large segment of the 5' end of the EGFR gene so theoretically could fail to detect a rearrangement in the 3' end although this is unlikely. This issue could be decided by using additional probes to the 3' end of the EGFR gene.

Alternatively, although the rearrangement reported by Zhang *et al.*, (1989) was detected in an *Eco*R1 digest, the *Eco*R1 digest of these tumours may not have cut the DNA at suitable sites to demonstrate the rearrangement. In this case there may be a rearrangement of DNA within a fragment cut at either end by the enzyme, with retention of sufficient of the correct DNA sequence for hybridization of the probe to occur and consequently no alteration in band size on the Southern blot. This is very unlikely but could be resolved by cutting the DNA with further restriction enzymes.

The lack of amplification of the EGFR gene is not surprising as amplification of this gene is a characteristic of squamous cell carcinomas but not adenocarcinomas (Ozanne *et al.*, 1986).

In Section 3.1 some of the problems associated with collecting endometrial cancer specimens were discussed. A further problem arose during the study which proved to be a major methodological flaw. The endometrial samples were collected by non-clinical technicians who had inadequate understanding of the importance of confirming the diagnosis of malignancy. However, the responsibility rests with the investigators for ascertaining the clinical data and the consequence of this omission is that only one third of the samples are taken from malignancies, thus reducing the probability of detecting any abnormalities in the genes studied.

Despite this drawback, the 28 samples included examples of normal, hyperplastic, cystic hyperplastic and atypical hyperplastic endometrium, in addition to the cancers. This represents a progressive neoplastic transformation of the endometrium similar to that seen in colon carcinoma. One of the reasons that the study of genetic abnormalities in colon cancer is perhaps the furtherest advanced of all the solid tumours is the availability of tissue from each of the steps along this neoplastic progression (reviewed in Fearon and Vogelstein, 1990). We therefore inadvertently ended up with a similar situation which potentially could have been advantageous had abnormalities been detected.

STUDY USING FROZEN TUMOUR SAMPLES AND ENDOMETRIAL CANCER CELL LINES

3.4 Sample analysis and results

The six epithelial endometrial cancer cell lines utilized in this study are described in detail in Section 2.9.a. They consisted of 2 Ishikawa cell lines, designated 1 and 2, derived from the same tumour. Two lines, HEC-1-A and HEC-1-B, also derived from a single tumour and 2, RL95-2 and KLE, cultured from different endometrial carcinomas. Cell culture followed by lysis of the cells and subsequent extraction of the DNA were performed as described in Section 2.9.a.

In addition, 6 DNA samples extracted from solid tumour specimens in the previous study (see Section 3.2) were also used in this study. The following samples were used (numbered from Section 3.2):

No.	Diagnosis
3	moderately differentiated adenocarcinoma
5	atypical hyperplasia
8	endometrioid carcinoma of ovary
10	cystic hyperplasia
16	well differentiated adenocarcinoma
17	proliferative endometrium

3.4.a Preparation of cDNA probes

A 3.9 kb *Hin*dIII restriction fragment of the 5' end and a portion of the

untranslated flanking sequence of the EGFR gene from the plasmid vector LIII (Haley and Waterfield, 1991) was radiolabelled using random primers and DNA polymerase Klenow fragment as described in Section 2.9.f and used to probe for the EGFR gene.

The PDGF-αR cDNA 1-1 from a human osteosarcoma line cloned into pBluescript SK+ as a 2.6 kb *Not* I fragment (approximately 1 μg DNA/μl TE buffer) was obtained from Zymogenetics (Seattle, Washington, USA). 5 μl of plasmid DNA was digested in 20 μl final volume with 10x high salt buffer, 2 μl of *Not* I and water to volume for 4 h in a water bath at 37°C. The DNA concentration was measured (see Section 2.9.a) and the fragments separated on a small 1% low melting point agarose gel (Gibco-BRL Ltd., UK) with ethidium bromide (1 μg/ml) run in 1x TAE at 70V constant voltage. The appropriate band was cut out of the gel and placed in an eppendorf tube. This probe was then radioactively labelled using the random priming method (see Section 2.9.f).

The cDNA RP-41 for the PDGF-βR comprises most of the coding region of the gene (Gronwald et al., 1988). The cDNA was cloned into the plasmid pUC118 as a 2.8 kb *Eco*RI fragment from a human fibroblast line was obtained from Zymogenetics. It was prepared for radiolabelling in a similar manner to the alpha receptor using *Eco*RI to excise the fragment from the plasmid.

The c-*fms* (CSF-1R) cDNA was a gift from Dr C Sherr (St. Judes Hospital, Memphis, TN, USA) and consisted of a 3 kb fragment coding for the whole of the receptor and a small portion of the 3' nontranslated sequence (Coussens *et al.*, 1986). The cDNA was cloned into the plasmid pSMc as a *Bam*HI fragment and excised in the manner described for the PDGF-αR.

3.4.b Southern analysis

20 μg of genomic DNA from the 6 cell lines and 6 tumour samples were digested with 3 restriction emzymes, *Eco*RI, *Hin*dIII and *Bam*HI, in the appropriate salt buffer at a final volume of 80 μl. The digestion mixtures were incubated in a water bath at 37°C for 3 h and the tubes inverted to mix the contents hourly. The respective enzymes were then added again and the incubation procedure repeated for a further 3 h. At this time a random selection of digests was run on a small 0.8% agarose gel with ethidium bromide (1 μg/ml) to ensure that digestion was complete.

The digests were divided in half and run simultaneously using ^{35}S-DNA markers (22-0.6 kb, Amersham, UK) as hybridization size markers overnight on 2 large 0.8% agarose gels as described in section 2.9.d.. The resulting fragments were transferred onto nylon filters (Section 2.9.e) and the filters probed and washed at high stringency. After autoradiography, the labelled probe was removed (Section 2.9.f) and the blot re-probed.

3.4.c Results

Six endometrial adenocarcinoma cell lines, 2 endometrial adenocarcinomas, 2 endometrial hyperplasias, an endometrioid carcinoma of the ovary and normal proliferative endometrium were studied for alterations in the 4 proto-oncogenes EGFR, PDGF-αR, PDGF-βR and c-*fms* (CSF-1R). The autoradiographs of the Southern blots probed for these receptors are reproduced in Figs 3.3 to 3.6, respectively. A further autoradiograph of genomic DNA from normal endometrium digested with the same 3 restriction enzymes and probed for c-*fms* is shown in Fig 3.7. None of the tumours or cell lines contained any evidence of amplification or

rearrangement of the genes studied. However, lanes 7-12 of the *Eco*R1 digest of the EGFR (Fig 3.3.A) are uninterpretable because of low band intensity and high background.

Additional bands were detected in the αPDGFR and c-*fms* blots. In lanes 3, 5, 7, 11, and 12 of the *Hin*dIII digest of the PDGF-αR there is a band at 5.8 kb (Fig 3.4.C). Otherwise the pattern of banding in these lanes is similar in intensity and size to the lanes in which the 5.8 kb band is absent.

All three restriction enzyme digests revealed additional bands in lanes 3, 7, 8, 11, and 12 in the tumour blot probed with c-*fms* (Fig 3.6). In the *Eco*RI digest (Fig 3.6.A) there is an additional band at 9.4 kb in these lanes while lanes 3, 4, 7, 9, and possibly 10 lack a 3.2 kb band which is present in the remaining lanes. A 1.8 kb band is absent from lanes 5, 9, and 11 but present in the remaining lanes. In the *Bam*HI digest (Fig 3.6.B) there is an extra 2.2 kb band while the *Hin*dIII digest (Fig 3.6.C) has an additional band in lanes 3, 7, 8, 11, and 12 at 2.1 kb.

Fig 3.7 shows DNA from 7 normal endometrial samples digested with the same 3 restriction enzymes and probed with c-*fms*. This demonstrates similar, but not identical, variability in banding pattern. In lanes 1, 3, 4, and 5 of the *Eco*RI digest (Fig 3.7.A) the extra 9.4 kb band is present. However, the 3.2 kb band lacking in some of the tumours is absent in lanes 3 and possibly 6. Although it also appears to be absent in lanes 1 and 2, it was visible but very faint on the original autoradiograph. The *Bam*HI digest (Fig 3.7.B), although less clear, has sufficient resolution to detect the 2.2 kb band in lanes 1, 3, 4, and 5 that was detected in the tumour blot. The *Hin*dIII digest (Fig 3.7.C) demonstrates the additional 2.1 kb band, again in lanes 1, 3, 4, and 5, seen in the tumour blot. A 3.6 kb band not seen in the tumour blot is visible in lanes 1, 2, and 3.

3.5 Discussion

Cultured cell lines were chosen for this study in addition to endometrial samples because it was felt that they would be likely to demonstrate genetic abnormalities if these are an important mechanism in the development of endometrial cancer. A transformed, immortalized cell line is more likely to contain genetic abnormalities than the parent tissue from which it was derived (reviewed in Newbold, 1985) thereby increasing the chances of detecting lesions of this type. Conversely, any lesion detected may be a result of the immortalization process rather than an intrinsic abnormality of the tumour itself. This study also complemented the rest of the work in this thesis which utilized these cell lines.

Apart from the EGFR, amplification or rearrangement of the proto-oncogenes studied have not been reported in adenocarcinomas. Amplification of the EGFR gene is found in gliomas (Libermann *et al.*, 1985) and in squamous carcinomas derived from a number of tissues (Ozanne *et al.*, 1986) and rearrangement of the gene is detected in some of those tumours that have amplified genes (Libermann *et al.*, 1985; Ekstrand *et al.*, 1991). Amplification of the EGFR gene has also been reported in 9% of adenocarcinomas of the lung (Shiraishi *et al.*, 1989). However, proto-oncogene amplification and rearrangement are consistent findings in tumours of all types (see Section 1.2) and it was therefore felt to be appropriate to look at these genes of interest in endometrial cancers.

This study is an extension of the work described in Section 3.2. Using 3 restriction enzymes to digest the DNA could reasonably be expected to detect a major rearrangement of the genes studied. Although lanes 7-12 of Fig 3.3.A showing the *Eco*R1 digest of the EGFR are not sufficiently clear to permit this assertion without reservation, the chance of

an undetected rearrangement is very small in view of the lack of rearrangements in the *Eco*R1 digests of the EGFR in the previous study (Section 3.2). This work supports the finding from that study that rearrangement of the EGFR gene is not a common event in endometrial cancer. Thus the truncated EGFR gene in a single case of endometrial cancer reported by Zhang and colleagues (1989) is unlikely to be a significant factor in the genesis of adenocarcinoma of the endometrium. However, this work has not excluded other genetic abnormalities such as smaller rearrangements or point mutations which would not be detected using these methods.

Although the additional bands detected in the blots probed for PDGF-αR and c-*fms* might on initial inspection appear to be due to genetic rearrangement, closer scrutiny does not support this. Additional bands were detected in the DNA from the samples in lanes 3, 7, 11, and 12 in both the blots probed for PDGF-αR (*Hin*dIII digest only) and c-*fms* (all enzyme digests). These samples were derived, respectively, from the two Ishikawa cell lines, the KLE cell line and a case of cystic hyperplasia of the endometrium. In addition, lane no. 5 (atypical hyperplasia of the endometrium) had an abnormal 5.8 kb band on the *Hin*dIII digest probed with PDGF-αR, and lane no. 8 (RL95-2 cell line) showed abnormal bands in the blot probed with c-*fms*. It seems unlikely that two non-malignant conditions of the endometrium would contain gene rearrangements although an oncogene abnormality in a premalignant adenoma of the colon absent in a colon cancer from the same patient has been described (Meltzer *et al.*, 1987).

To confirm that gene rearrangement was not the explanation for the variable banding, DNA from 7 normal endometrial samples was digested, electrophoresed and probed for c-*fms* (Fig 3.7). This also showed variable

banding which was similar, but not identical to, that observed with the tumour DNA.

The most likely explanation for these findings is that this variability is a result of restriction fragment length polymorphisms (RFLPs, Hayes *et al.*, 1989). RFLPs arise because genomic DNA contains variable numbers of repeated sequences termed tandem repeats that are thought to be of little functional significance. Two alleles of the same gene may contain different numbers of these tandem repeats and cleavage of the DNA by endonucleases in this instance will result in fragments of different length, hence RFLP. The RFLP may also create or abolish a restriction enzyme recognition site, resulting in variation in DNA fragment length.

To confirm that RFLP is the explanation for the variation in banding observed, DNA obtained from normal tissue such as peripheral leukocytes from these patients could be digested, electrophoresed and probed in the same manner. It would be expected that the normal DNA would demonstrate the same banding pattern as the endometrial specimen. Unfortunately in this instance this was not possible because the specimens had been stored for several years and it was not felt to be appropriate to follow up these patients after such a long delay. Multiple RFLPs of the EGFR gene have been reported and the authors questioned the validity of studies reporting EGFR gene rearrangements in which there was no comparison with normal DNA (Lee *et al.*, 1988).

It is difficult to explain the coincidence that 4 of the lanes showed abnormal banding when probed for both PDGF-αR and c-*fms* except by chance. The genes are found on different chromosomes, 4 and 5, respectively (see Section 1.3), so should behave independently. These 2 chromosomes are thought to have a common ancestral origin (Comings, 1972) but it is speculative to propose that this is of significance in this case.

The variable banding was present in only the *Hin*dIII digest of the blot probed with PDGF-αR suggesting that a cleavage site for *Hin*dIII was affected, while all 3 digests showed abnormal banding in the blot probed with c-*fms*. This indicates that the RFLP had introduced a much larger segment of DNA into the c-*fms* gene, therefore altering the fragment size of the DNA digested by all of the enzymes.

The possibility of amplification of any of the genes studied was not completely assessed. The blots were not probed for a single copy gene and densitometry was not performed. The differences observed in band intensity between lanes were adequately explained by differences in sample loading. The gels were run in duplicate with identical quantities of digested DNA added to the corresponding lanes on each gel. After electrophoresis, the ethidium bromide-stained gels showed similar loading patterns which were confirmed by the autoradiographs of the probed blots. There was no obvious amplification and no further assessment for amplification was made. The blot probed for the EGFR was of poor resolution due to high background but as no evidence of amplification of the EGFR gene had been found previously (Section 3.2) and the autoradiograph was adequate to exclude rearrangement, it was not repeated.

Sample No.	Hospital	Histological diagnosis
1	St Helier's	***poorly diff adeno**
2	Central Middlesex	benign polyp
3	Hammersmith	***mod diff adeno**
4	†	
5	Hammersmith	atypical hyperplasia
6	Hammersmith	mid-secretory
7	Ashford	***mod diff adeno**
8	Orpington	***endometrioid (ovary)**
9	Central Middlesex	post-menopausal
10	Central Middlesex	cystic hyperplasia
11	Greenwich District	proliferative
12	St Helier's	***mod diff adeno**
13	Hammersmith	hyperplasia
14	Central Middlesex	endocervical tissue
15	King Edward VII	***poorly diff adeno (cervix)**
16	Hammersmith	***well diff adeno**
17	Hammersmith	proliferative
18	Wexham Park	***well diff adeno**
19	St Helier's	atypical hyperplasia
20	St Helier's	***mod diff adeno**
21	Beckenham	no report
22	Hammersmith	***poorly diff adeno**
23	Hammersmith	early secretory
24	†	
25	Ashford	***well diff adeno**
26	Hammersmith	***clear cell carcinoma**
27	Ashford	***well diff adeno**
28	Hammersmith	proliferative

† = no data recorded

* = malignancy

mod diff adeno = moderately differentiated adenocarcinoma

Table 3.1 List of endometrial samples from which DNA was obtained with hospital of origin and histological diagnosis

ENDO 1

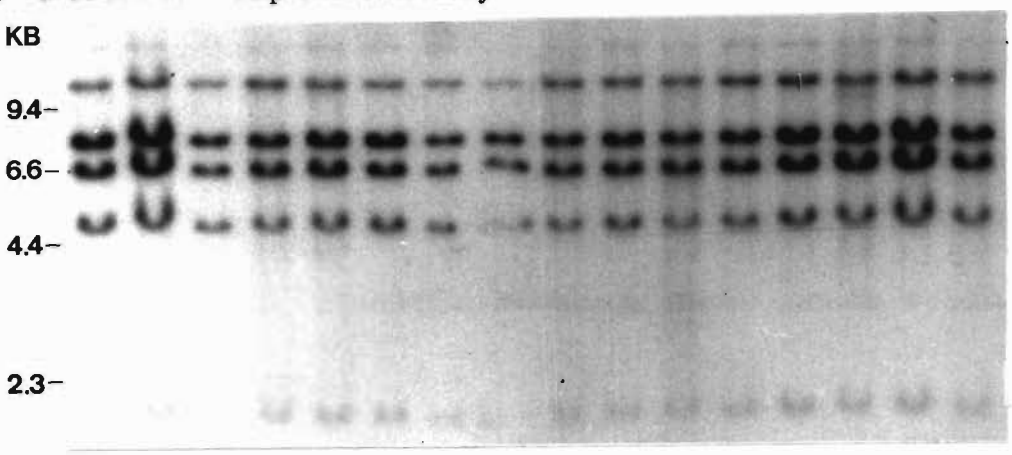
Sample	EGFR α-1-I	EGFR α-2-I	c-*erb*B-2 α-1-I	c-*erb*B-2 α-2-I
Normal	1.22	1.45	0.63	0.74
Normal	1.35	1.51	0.94	1.05
1	1.47	2.21	0.53	0.80
2	1.58	2.05	0.62	0.81
3	1.70	2.06	0.65	0.80
4	1.56	2.00	0.54	0.69
5	1.46	1.60	0.56	0.61
6	1.41	1.82	0.62	0.80
7	1.53	1.75	0.56	0.64
8	1.46	1.63	0.61	0.68
9	1.33	1.46	0.59	0.65
10	1.30	1.52	0.59	0.69
11	1.34	1.47	0.74	0.81
12	1.27	1.41	0.74	0.82
13	1.27	1.41	0.83	0.92
14	0.94	1.11	0.70	0.79

ENDO 2

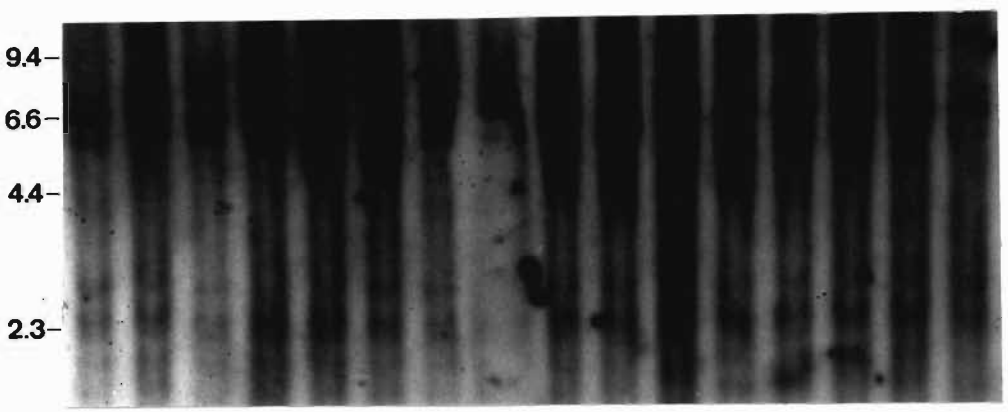
Normal	1.40	1.47	1.34	1.40
Normal	1.50	1.68	1.32	1.47
15	2.01	2.07	1.71	1.76
16	1.84	1.91	2.20	2.28
17	1.99	2.04	2.02	2.07
18	2.19	2.41	1.55	1.70
19	2.06	2.14	1.92	2.00
20	1.91	1.17	1.64	1.00
21	1.90	1.92	1.54	1.56
22	2.38	2.16	2.98	2.71
23	1.90	1.86	1.67	1.64
24	2.20	0.80	0.86	0.31
25	2.19	2.12	1.15	1.12
26	1.65	1.76	1.17	1.24
27	1.84	2.12	1.44	1.66
28	2.07	2.48	1.65	1.97

Table 3.2 Readings of autoradiographic signal intensity using scanning laser densitometer for the hybridized probes for the EGFR and c-*erb*B-2 genes expressed as ratios of the respective genes to the intensity of the α-1-I and α-2-I hybridization signals for each sample. Each autoradiograph was scanned horizontally through the point of maximum band intensity by the densitometer with an aperture width of 0.5 mm. The areas under the individual peaks of the resultant curve were integrated by the densitometer and these figures were used to calculate the above ratios. Because these results were obtained by sequentially probing the same blots, the amount of DNA in each lane was constant and the major source of variability was in the difference in overall background signal between each probing. However, as this was reasonably constant over each blot as a whole, the differences in background were eliminated by deriving intensity ratios rather than absolute values.

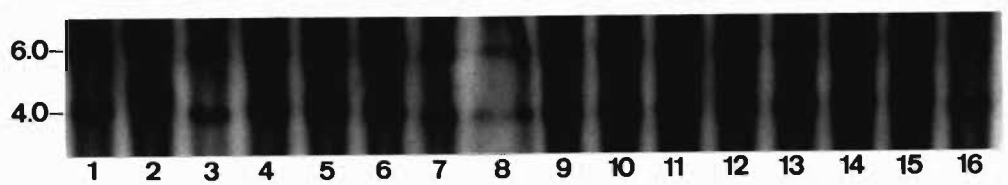
A) c-*erb*B-2 Exposure time 1 day



B) EGFR Exposure time 3 days



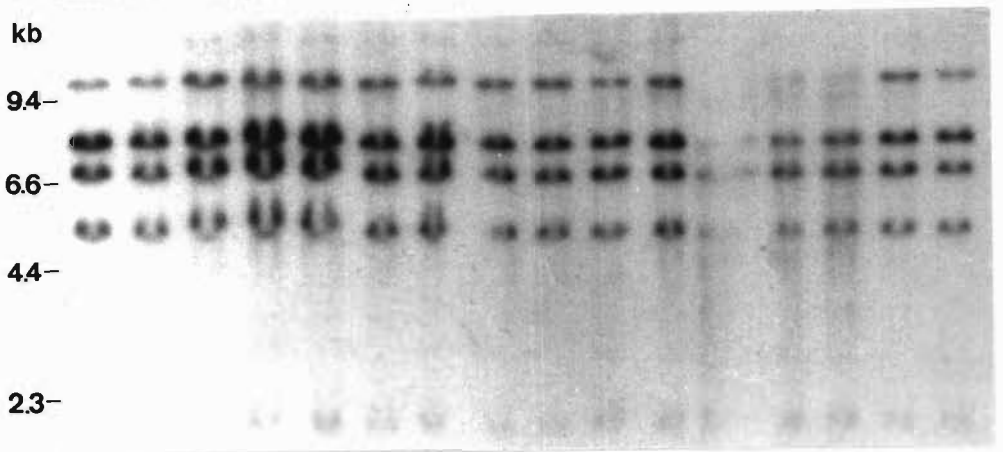
C) α-1-I and α-2-I Exposure time 2 days



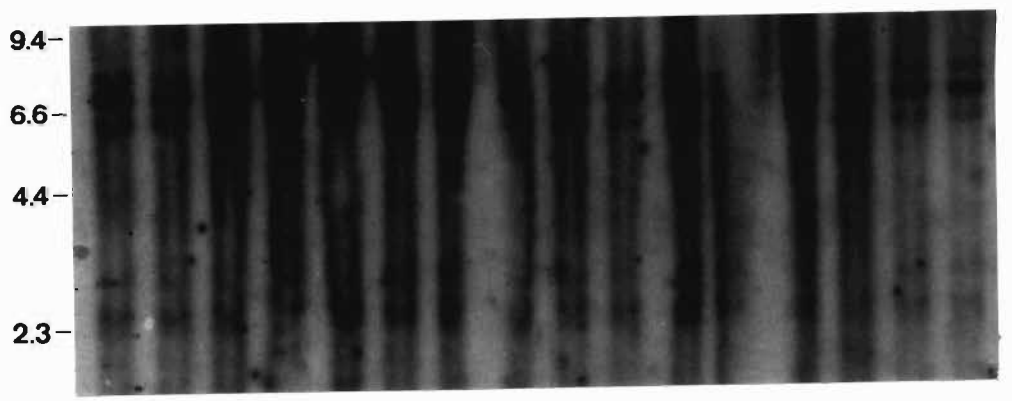
Lane				
	1	Normal	9	Mod diff adeno
	2	Normal	10	Endometrioid (ovary)
	3	Poorly diff adeno	11	Postmenopausal endometrium
	4	Benign polyp	12	Cystic hyperplasia
	5	Mod diff adeno	13	Proliferative endometrium
	6	Unknown	14	Mod diff adeno
	7	Atypical hyperplasia	15	Hyperplasia
	8	Secretory endometrium	16	Endocervical tissue

Fig 3.1 ENDO 1 Genomic DNA from endometrial samples digested with *Eco*RI and probed with labelled cDNAs from the genes as marked. Specific activity of probes: c-*erb*B-2 - 3.4 x 10^8; EGFR - 9.2 x 10^7; α-1-I - 6.5 x 10^8; and α-2-I - 8.5 x 10^7 cpm/μg DNA.

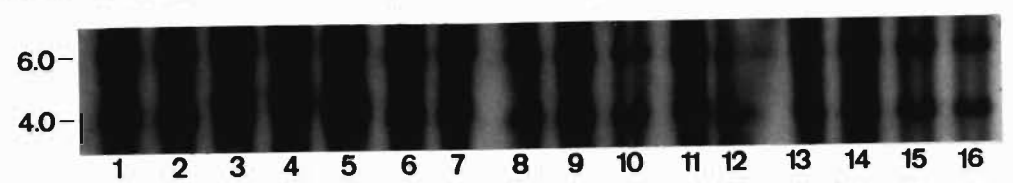
A) c-*erb*B-2 Exposure time 1 day



B) EGFR Exposure time 3 days



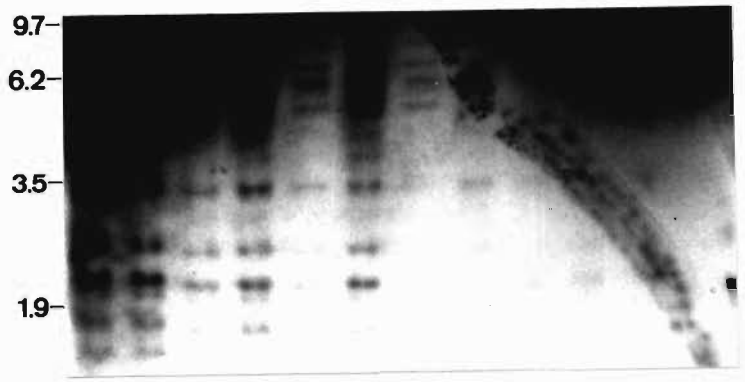
C) α-1-I and α-2-I Exposure time 2 days



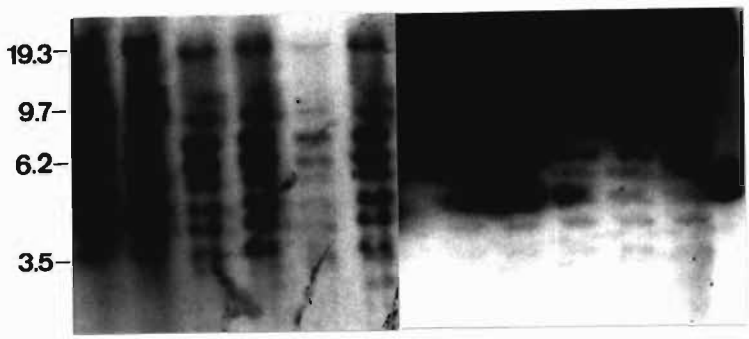
Lane				
	1	Normal	9	No report
	2	Normal	10	Poorly diff adeno
	3	Poorly diff adeno (cervix)	11	Secretory endometrium
	4	Well diff adeno	12	Unknown
	5	Proliferative endometrium	13	Well diff adeno
	6	Well diff adeno	14	Clear cell carcinoma
	7	Atypical hyperplasia	15	Well diff adeno
	8	Mod diff adeno	16	Proliferative endometrium

Fig 3.2 ENDO 2 Genomic DNA from endometrial samples digested with *Eco*RI and probed with labelled cDNAs from the genes as marked. Specific activity of probes: c-*erb*B-2 - 3.4 x 10^8; EGFR - 9.2 x 10^7; α-1-I - 6.5 x 10^8; and α-2-I - 8.5 x 10^7 cpm/μg DNA.

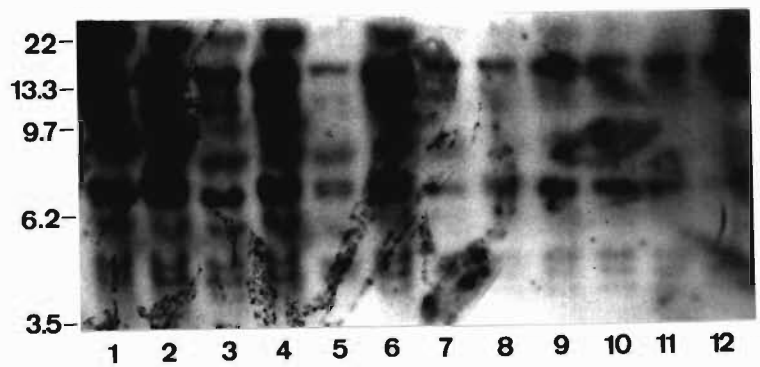
A) *Eco*RI digest



B) *Bam*HI digest: The discrepancies in band position and exposure between lanes 1-6 and 7-12 are a result of electrophoresis of the 2 groups on separate parts of the gel.



C) *Hin*dIII digest

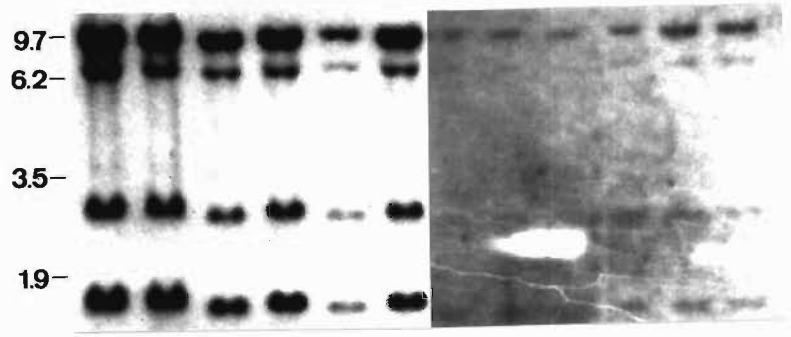


Lane				
	1	Normal (proliferative endometrium)	7	KLE
	2	Well diff adeno	8	RL95-2
	3	Cystic hyperplasia	9	HEC-1-B
	4	Endometrioid carcinoma (ovary)	10	HEC-1-A
	5	Atypical hyperplasia	11	Ishikawa 2
	6	Mod diff adeno	12	Ishikawa 1

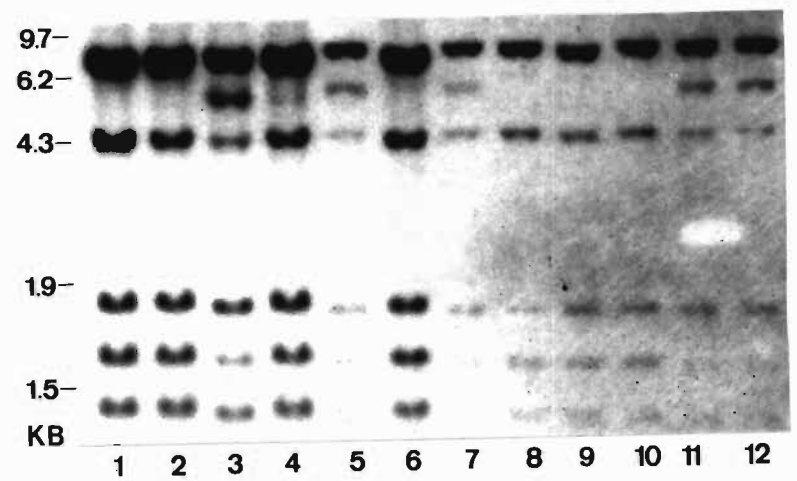
Fig 3.3 EGFR Genomic DNA from 6 endometrial cancer cell lines, 2 endometrial cancers, 2 endometrial hyperplasias, an endometrioid carcinoma of the ovary and normal endometrium digested with *Eco*RI, *Bam*HI and *Hin*dIII and probed with labelled cDNA (specific activity 1.1 x 10^8 cpm/μg DNA) derived from the EGFR gene. Exposure time 6 days.

A) *Eco*RI digest

B) *Bam*HI digest: The discrepancies in band position and exposure between lanes 1-6 and 7-12 are a result of electrophoresis of the 2 groups on separate parts of the gel.



C) *Hin*dIII digest: In lanes 3, 5, 7, 11, and 12 an additional 5.8 kb band is present.

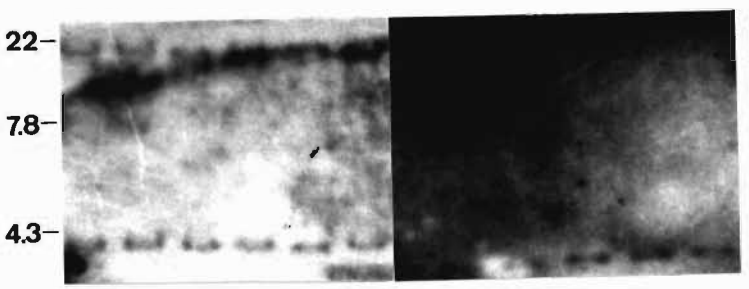


Lane	1	Normal (proliferative endometrium)	7	KLE
	2	Well diff adeno	8	RL95-2
	3	Cystic hyperplasia	9	HEC-1-B
	4	Endometrioid carcinoma (ovary)	10	HEC-1-A
	5	Atypical hyperplasia	11	Ishikawa 2
	6	Mod diff adeno	12	Ishikawa 1

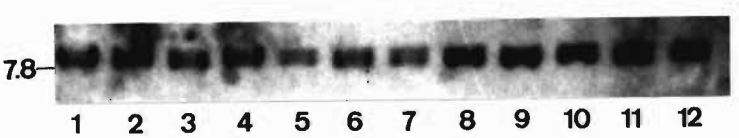
Fig 3.4 PDGF-αR Genomic DNA from 6 endometrial cancer cell lines, 2 endometrial cancers, 2 endometrial hyperplasias, an endometrioid carcinoma of the ovary and normal endometrium digested with *Eco*RI, *Bam*HI and *Hin*dIII and probed with labelled cDNA (specific activity 1.1 x 10^6 cpm/μg DNA) derived from the PDGF-αR gene. Exposure time 7 days.

A) *Eco*RI digest

B) *Bam*HI digest: The discrepancies in band position and exposure between lanes 1-6 and 7-12 are a result of electrophoresis of the 2 groups on separate parts of the gel.



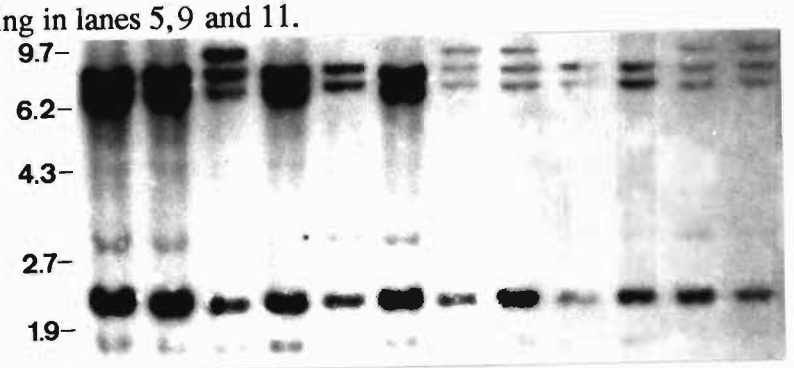
C) *Hin*dIII digest



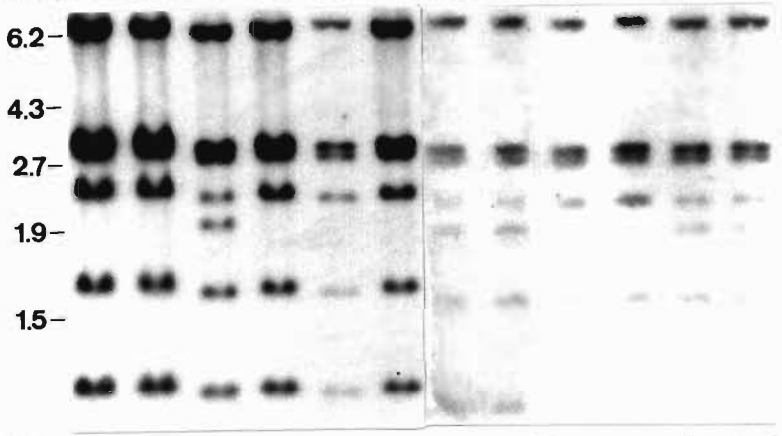
Lane				
	1	Normal (proliferative endometrium)	7	KLE
	2	Well diff adeno	8	RL95-2
	3	Cystic hyperplasia	9	HEC-1-B
	4	Endometrioid carcinoma (ovary)	10	HEC-1-A
	5	Atypical hyperplasia	11	Ishikawa 2
	6	Mod diff adeno	12	Ishikawa 1

Fig 3.5 PDGF-βR Genomic DNA from 6 endometrial cancer cell lines, 2 endometrial cancers, 2 endometrial hyperplasias, an endometrioid carcinoma of the ovary and normal endometrium digested with *Eco*RI, *Bam*HI and *Hin*dIII and probed with labelled cDNA (specific activity 3.5 x 10^6cpm/μg DNA) derived from the PDGF-βR gene. Exposure time 5 days.

A) *Eco*RI digest: There is an additional 9.4 kb band in the designated lanes (see below), there is a 3.2 kb band absent in lanes 3, 4, 7, 9, and possibly 10, and a 1.8 kb band missing in lanes 5,9 and 11.



B) *Bam*HI digest: In the designated lanes an additional 2.2 kb band is present. The discrepancies in band position and exposure between lanes 1-6 and 7-12 are a result of electrophoresis of the 2 groups on separate parts of the gel.



C) *Hind*III digest: An extra 2.1 kb band is seen in the designated lanes.

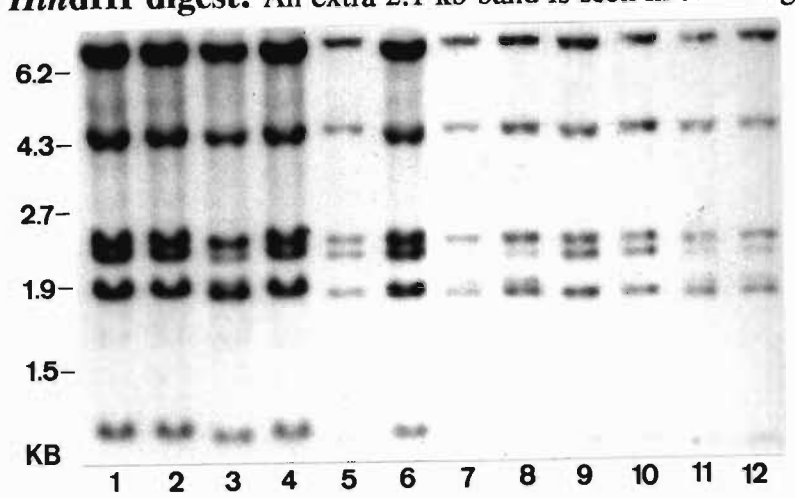
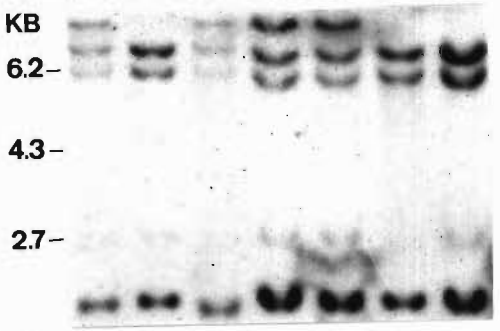
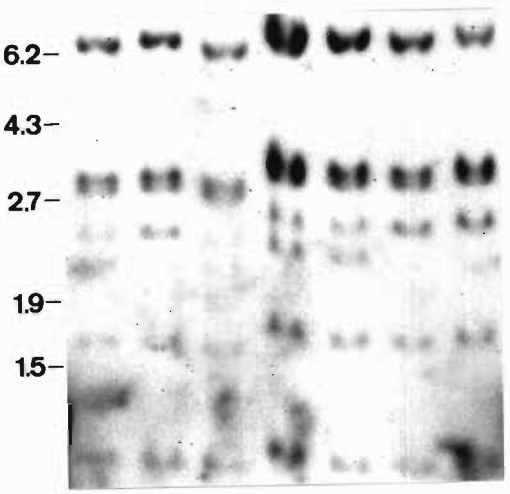


Fig 3.6 c-*fms*/CSF-1R Genomic DNA from 6 endometrial cancer cell lines, 2 endometrial cancers, 2 endometrial hyperplasias, an endometrioid carcinoma of the ovary and normal endometrium digested with *Eco*RI, *Bam*HI and *Hind*III and probed with labelled cDNA (specific activity 4.5 x 10^8 cpm/µg DNA) derived from the c-*fms* gene. Lanes 3, 7, 8, 11, and 12 show abnormal banding in all 3 digests. Exposure time 7 days.

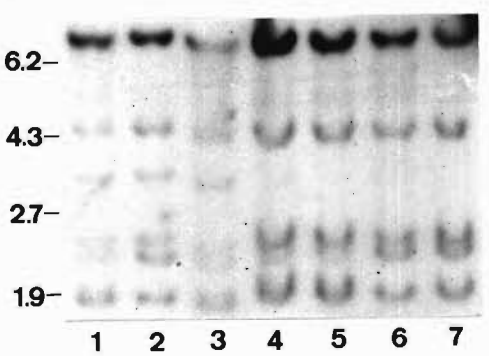
A) *Eco*RI digest: There is an additional 9.4 kb band in lanes 1, 3, 4, and 5. There is a 3.2 kb band absent in lane 3 and possibly lane 6.



B) *Bam*HI digest: In lanes 1, 3, 4, and 5 an additional 2.2 kb band is present.



C) *Hind*III digest: An extra 2.1 kb band is seen in lanes 1, 3, 4, and 5. A 3.6 kb band is seen only in lanes 1, 2, and 3.



Lane	1	Proliferative	5	Postmenopausal
	2	Early secretory	6	Mid-secretory
	3	Proliferative	7	Benign polyp
	4	Proliferative		

Fig 3.7 c-*fms*/CSF-1R Genomic DNA from 7 normal endometrial samples digested with *Eco*RI, *Bam*HI and *Hind*III and probed with labelled cDNA (specific activity 6.7 x 10^7 cpm/μg DNA) from the c-*fms* gene. Exposure time 5 days.

4 DO ENDOMETRIAL CANCER CELL LINES POSSESS FUNCTIONAL EGF AND PDGF RECEPTORS?

4.1 Introduction

Although receptors for EGF have been demonstrated by immunohistochemistry in endometrial cancers (see Section 1.8), little is known of the function of these receptors. The level or absence of expression of this receptor does not appear to be related to any of the known prognostic variables. One possible explanation is that those tumours in which EGFRs are not detected by immune staining express structurally altered receptors, e.g., truncated receptors similar to v-*erb*-B (Downward *et al.*, 1984), not recognized by the antibody. The phenomenon of structurally altered EGFRs is seen in cells of the vulval carcinoma cell line A431 which express an aberrant 2.8 kb mRNA in addition to the 10.5 and 5.8 kb mRNA detected in normal tissue (Ullrich *et al.*, 1984). This 2.8 kb mRNA encodes a 70,000 MW protein which is secreted and comprises almost the entire extracellular domain of the EGFR (Mayes and Waterfield, 1984).

There have been no reported studies on the expression of receptors for PDGF in endometrial cancer. The presence of PDGF-βR in normal endometrium (see Section 1.7.c) suggests that they are also likely to be present in endometrial cancers. In normal endometrium, as in most tissues, PDGF-βR are detected in stromal cells adjacent to endometrial glands and it is possible they have a role in the induction of the stromal reaction to cancers (Bronzert *et al.*, 1987). However, the endometrial cancer cell lines used in this study are epithelial in origin and it is less likely that PDGFR

are present in these cells.

As little has been described about the function of growth factor receptors in endometrial cancer it was felt to be appropriate to study both EGF and PDGF receptors. Other receptors such as those for insulin, the insulin-like growth factors and CSF-1 may also be important in endometrial cancer and await further study. While recognizing the limitations of relating the properties of immortalized cells in culture to cells *in vivo*, the cell lines were used because of their relative reliability in culture compared to primary explants. In this chapter the investigation of the function of EGFR and PDGFR in 2 endometrial cancer cell lines, HEC-1-A and Ishikawa 1 cells, is described.The chapter is divided into 2 parts: firstly, examination of the function of these receptors using assays for receptor phosphorylation; and secondly, the demonstration of the presence and affinity of receptors using binding studies.

4.2 Assays of tyrosine phosphorylation

4.2.a Materials and methods

Assays for the phosphorylation of growth factor receptor tyrosine kinases in both the Ishikawa 1 and HEC-1-A cell lines were performed as described in Section 2.5, using either the EGFR extracted from cells with anti-EGFR antibodies, or antiphosphotyrosine antibodies to detect tyrosine phosphorylation on Western blots. Cells from the A431 vulval carcinoma cell line were used as a comparison in all experiments. Three different forms of recombinant human PDGF were used in these experiments: PDGF-BB, PDGF-AD1 and PDGF AA13 (Chiron Corporation, USA).

PDGF-AD1 is a recombinant full length homodimer of PDGF A, and PDGF AA13 is a mutant form of PDGF-AA in which both molecules in the homodimer lack 15 amino acids from the C-terminus. The latter is biologically active *in vitro* and has identical dose-response curves to PDGF-AA (personal communication, Dr S. Gale, Chiron Corporation). In all these experiments, physiological concentrations of the various agents were used as given in the figures.

Solutions used

EGF — Human recombinant EGF, lyophilized and stored at -20°C.

PDGF — Three different forms as described (Section 4.2.a) stored as 50 µg lyophilized aliquots at -20°C and made up in PBS immediately prior to use.

bFGF — Human recombinant basic fibroblast growth factor (Sigma, UK), lyophilized and stored at -20°C. Made up prior to use in 6 mM Tris-HCl buffer with 1% BSA.

Oestradiol — Stock solution (Sigma, UK) made up to 10^{-4} M and 10^{-5} M in ethanol, stored at -40°C and diluted to the appropriate concentration for use.

Insulin — Stock solution of 1 mg/ml in 6 mM HCl stored at 4°C (Sigma,UK).

4.2.b Results

The results of 2 experiments using the immunoprecipitation of the EGFR from Ishikawa 1 and HEC-1-A cells are shown in Fig 4.1.A and 4.1.B, respectively. In Fig 4.1.A in both the Ishikawa and A431 cells the EGFR appears as a double band at 170 and 150 kD and there is an increase in tyrosine phosphorylation in the presence of physiological concentrations of EGF. In Fig 4.1.B the EGFR is identified as a single band at 170 kD in HEC-1-A and A431 cells with considerably enhanced phosphorylation in all cells with the addition of EGF. These results confirm that EGFRs of the expected molecular weight are present in both Ishikawa 1 and HEC-1-A cells and that the addition of ligand leads to phosphorylation of this receptor.

Autoradiographs of Western blots probed with antiphosphotyrosine antibodies which were then labelled with ^{125}I-Protein-A are shown in Figs 4.2 and 4.3. The effects of EGF and recombinant forms of PDGF-A and PDGF-B on Ishikawa 1 cells are shown in Fig 4.2. A431 and Swiss 3T3 cells are used as positive controls for the EGFR and PDGFR, respectively. A431 cells overexpress the EGFR with ~2 x 10^6 receptors per cell (Carpenter, 1987), while Swiss 3T3 cells express approximately equal numbers of α and β forms of the PDGFR (Gronwald *et al.*, 1989). No increase in phosphorylation is seen with the addition of either PDGF-A or PDGF-B. However, EGF appears to cause increased signal intensity on 3 bands at lower than expected molecular weight. This is in contrast to the A431 cells in which the major signal was seen at the expected molecular wieght although the A431 cells also showed an increase in 1 of the lower molecular weight bands in the EGF stimulated cells. This experiment was repeated and an identical banding pattern was obtained (results not shown).

The Swiss 3T3 cells show increased phosphorylation in response to PDGF-A and -B, consistent with activation of the α- and β-type receptors for PDGF, respectively.

In Fig 4.3 the effect of various agents on tyrosine phosphorylation of both Ishikawa 1 and HEC-1-A cells is demonstrated. The only definite response is seen with the addition of EGF to HEC-1-A cells, with increased signal in bands at 170, 115 and 45 kD when compared to the control. Therefore using this method of measuring receptor tyrosine phosphorylation also confirms that EGF activates the EGFR in these cells. In addition, in the Ishikawa cells in Lane 3 (insulin 10 μg/ml in medium) there appears to be a slight increase in signal intensity compared to the control in a number of bands between 160 and 114 kD.

4.3 Binding studies

The previous work on phosphorylation of tyrosine by growth factors in the Ishikawa and HEC-1-A cells failed to show activation of the PDGFR. This is consistent with the epithelial origin of the endometrial cancer cells and, for these reasons, only the binding of labelled EGF to the 2 cell lines was studied.

4.3.a Materials and methods

EGF binding assays were performed as described in Section 2.2 using Ishikawa 1 and HEC-1-A cells. The Ishikawa and HEC-1-A cells were plated at a density of 2.5×10^5 cells and 2×10^5 cells per well, respectively, while the specific activities of the ^{125}I-EGF used in the 2 assays were

162μCi/μg and 170 μCi/μg.

In a further experiment the effect of steroid hormones and other growth factors on the EGF-binding of Ishikawa 1 cells was assessed. The cells were plated at a density of 1.5 x 10^5 cells per well into 24 well culture dishes in DMEM plus 10% FCS and grown to confluence. The cells were kept serum-free for 48 h then washed with PBS (37°C). Physiological concentrations of the various agents were then added to the culture medium for 24 h in triplicate wells. Controls contained the medium plus 0.01% ethanol.

10 μl of ^{125}I-labelled EGF (~0.5 μCi, specific activity 108 μCi/μg) were diluted in binding buffer on ice. The wells were washed once with binding buffer on ice before adding 250μl of the solution containing labelled EGF (~23,500 cpm/well). Excess unlabelled EGF (500 ng/well) was added to 3 wells to measure the total and non-specific binding. The plates were then incubated at 4°C and the remainder of the assay carried out as described.

Solutions used

R5020 Synthetic progestin (Sigma, UK) made up to stock solution of 10^{-6} M in ethanol, stored at -40°C and diluted to the appropriate concentration for use.

Dexamethasone Synthetic glucocorticoid (Sigma, UK) made up to stock solution of 10^{-5} M in ethanol, stored at -40°C and diluted to the appropriate concentration for use.

Tamoxifen 4-hydroxytamoxifen (Sigma, UK) made up to stock solution of 10^{-5} M in ethanol, stored at -40°C in the dark and diluted to the appropriate concentration for use.

TGF-β_1 Transforming growth factor-β_1 (Sigma, UK) purified from human platelets, lyophilized and stored at -20°C

4.3.b Results

EGF-binding

The results of the EGF-binding experiments with both Ishikawa 1 and HEC-1-A cells are shown in Figs. 4.4 and 4.5, respectively. The raw counts for each cell line have been transformed into Scatchard plots using the EBDA programme. Each point is the mean of the counts from triplicate wells. Both cell lines showed a single affinity receptor with affinity constants of 1.49 nM and 1.39 nM for the Ishikawa 1 and HEC-1-A cells, respectively. Using the curves plotted in Figs 4.4 and 4.5, the receptor numbers were estimated to be 69,300 and 48,300 receptor sites per cell, respectively. These values were calculated from the point on the Scatchard analysis where the regression curve passed through the x-axis (B_{max}), representing the total binding of labelled EGF by the total cell number per well.

Effect of steroid hormones and growth factors on EGF-binding

The effect of various agents at physiological concentrations on EGF-binding of HEC-1-A cells is shown in Fig 4.6. Significant reduction in EGF-binding is seen with the addition of EGF and medium containing 10% FCS. There is a smaller reduction in EGF-binding with TGF-β1 but

repetition of this experiment failed to confirm this result (data not shown).

4.4 Discussion

Two of the endometrial cancer cell lines, the Ishikawa 1 cells containing oestrogen receptors and the HEC-1-A cells lacking oestrogen receptors, were used for this work. This was felt to be important because of the differences in growth regulation observed between breast cancer cell lines responsive and nonresponsive to oestrogen (reviewed in Dickson *et al.*, 1990).

The Scatchard analysis of experimental data allows the estimation of affinity constants (K_D) and receptor numbers (B_{max}). The affinity constants obtained for these two cell lines, 1.49 nM (Ishikawa 1) and 1.39 nM (HEC-1-A), and receptor numbers, 69,300 and 48,300, respectively, are within the range of values generally reported (reviewed in Carpenter, 1987). The experiments which produced this data were all carried out on ice as binding parameters obtained on intact cells at temperatures above 10°C are seriously compromised by the intracellular events that occur after ligand binding such as receptor clustering and internalization and ligand degradation (Carpenter, 1987).

There have been a number of reports suggesting that in some cells subpopulations of EGFR with high and low affinities are present giving rise to nonlinear Scatchard plots (King and Cuatrecasas, 1982; Kawamoto *et al.*, 1983). It has been proposed that it is the numerically fewer receptors of high affinity which are responsible for the mitogenic effects of EGF (Shechter *et al.*, 1978). However, there is considerable doubt as to the statistical validity of this interpretation of nonlinear Scatchard plots (Norby

et al., 1980; Klotz, 1982). To be convincing the affinities of the two receptor sites should differ by at least 200-fold when the high affinity receptors comprise 10% or less of the total number (Carpenter, 1987). The EBDA computer programme used to generate the plots in this study can assume that there are two sites of differing affinity and plot the curves appropriately. Using this facility on the data presented in this study results in two lines on the Scatchard plot with an ~20-fold difference in affinity constant (data not shown). This is comparable with other studies that report receptors of different affinities where the high affinity receptors usually comprise 10% or less of the total receptor population and the difference in K_D values is only about 10-20 fold (Carpenter, 1987). The evidence for discrete high and low affinity receptor subtypes is much stronger for other receptors such as the interleukin-2 receptor where the K_Ds for the high and low affinity sites differ by 1000-fold and the subtypes can be manipulated biochemically (reviewed in Greene and Leonard, 1986).

The effects of other agents on EGF binding to Ishikawa 1 cells were also investigated as part of this study. Transmodulation of the EGFR by PDGF acting through protein kinase C is known to occur (see Section 1.4.a) and these cells were screened to look for this type of effect. As these cells possess functional oestrogen receptors, a number of steroid hormones were used in addition to growth factors. Dexamethasone was also used as an increase in EGF binding in response to 24 h exposure to glucocorticoids has been described in HeLa S_3 cells (Fanger *et al.*, 1984).

No response was demonstrated with this prolonged exposure (24 h) to physiological concentrations of these agents except EGF and serum. The expected decrease in binding with EGF due to down regulation of the EGFR confirms that experimental procedures were performed correctly. The reduction in EGF binding observed with the addition of serum is

explained by the presence in serum of small quantities of EGF.

The addition of steroid hormones to the Ishikawa 1 cells had no effect on EGF binding despite the presence of oestrogen and progesterone receptors on these cells (Nishida *et al.*, 1985). In particular, Ishikawa cells contain functional progesterone receptors but the addition of a synthetic progesterone, R5020, to the cells had no effect on EGF binding. This finding contrasts markedly with results in breast cancer cell lines MCF-7 and T-47D which contain progesterone receptors. Although incubation with oestrogen, androgens and glucocorticoids for 24 h had no effect on EGF binding to MCF-7 and T-47D cells, progesterone specifically increased EGF binding without affecting cell numbers or the specific binding of other ligands with cell surface receptors (Murphy *et al.*, 1986). This increase in binding is due to a 2- to 3-fold increase in receptor numbers rather than an effect on receptor metabolism. In another breast cancer cell line, BT 20, that lacks progesterone receptors, no change in EGF binding was observed suggesting that this effect was mediated via the progesterone receptor.

Although progestins inhibit the growth of Ishikawa cells (Terakawa *et al.*, 1987) and therefore potentially the number of cells expressing EGFR, the growth inhibition occurs over a time course of several days and would not be apparent in 24 h.

The lack of PDGF binding to endometrial cancer cells contrasts with the findings in endometrium and myometrium from porcine uteri which have been shown to bind PDGF-B (Terracio *et al.*, 1988). However, in their study the binding of PDGF was primarily to stromal cells adjacent to endometrial glands and not to the epithelial cells of the glands. As the cell lines used in the current study are derived from epithelium rather than stroma it is perhaps not surprising that they lack PDGFRs. This is

consistent with the notion that PDGF is a mitogen primarily for cells of mesenchymal origin (reviewed in Ross *et al.*, 1986).

Two different methods were used to study the activity of the protein tyrosine kinases in these cells. The first utilized an antibody to the EGFR to extract the receptor from the cellular proteins and then measured its *in vitro* tyrosine kinase activity. The second stained phosphotyrosine residues in the total cellular proteins and compared the intensity of signals before and after the addition of growth factors or other agents. The advantage of the former method is that it permits better resolution of the receptor by removing most of the background proteins. The latter method is a more "physiological" one and theoretically more representative of intracellular events. In addition, these experiments were carried out in the absence of serum in order to avoid the possibility of receptor down-regulation and thereby increase the sensitivity of the experiments.

The effects of preparative techniques are clearly demonstrated in this study. In Fig 4.1, using immunoprecipitation of the receptor, the EGFR is seen as a double band in Fig 4.1.A and a single band of appropriate molecular weight in Fig 4.1.B. Identical methodology was used in both experiments.

In Fig 4.2 the findings are more complex. The addition of EGF to Ishikawa cells clearly leads to phosphorylation of proteins at 112 and 45 kD. In the A431 cells the EGFR is also phosphorylated, but at the normal molecular weight of 170 kD, with less intense phosphorylation of the 45 kD protein. Similarly, in Fig 4.3 there is a band present in both Ishikawa and HEC-1-A cells at 112 kD which shows increased phosphorylation with EGF in the HEC-1-A cells. However, the HEC-1-A cells also demonstrate a normal 170 kD band which shows phosphorylation with EGF. These results should be compared to those of the immunoprecipitation experiments in

which the EGFR in both cell lines were demonstrated to be of expected size.

The simplest and most likely explanation is that degradation of the receptor by proteases occurred during preparation although it is difficult to understand the preservation of the receptor size in the A431 cells prepared at the same time in an identical manner. An aglycosylated 115 kD form of the EGFR in A431 cells has been described, however this was thought to be an intermediary metabolite and was not present in a hepatocellular carcinoma cell line also studied (Carlin and Knowles, 1986).

A number of smaller proteins are phosphorylated in response to EGF of which some have been identified. As mentioned previously (see Section 1.4.a), EGF causes phosphorylation of lipocortin I, an anti-inflammatory protein with a molecular weight of 40,000 (Pepinsky and Sinclair, 1986). Another protein which is phosphorylated by the EGFR kinase is a 35 kD calcium- and phospholipid-binding protein which is similar to Lipocortin I and appears to have a role in neural morphogenesis (McKanna and Cohen, 1989). In A431 cells EGF also increases tyrosine phosphorylation of 2 unidentified proteins of molecular weights 39 and 81 kDa (Hunter and Cooper, 1981). The multiple bands observed in this study in response to EGF are therefore consistent with previous work but were not studied further.

Using antiphosphotyrosine antibodies the Ishikawa 1 and HEC-1-A cells were also screened for the presence of other growth factor receptors with tyrosine kinase activity (Fig 4.3). No increase in density of signal was observed with bFGF or insulin. Receptors for both these agents are members of the protein-tyrosine kinase family (see Section 1.4) and, in addition to binding to its own receptor, insulin binds to receptors for IGF-1 with 100-fold less affinity (Massagué and Czech, 1982). These findings

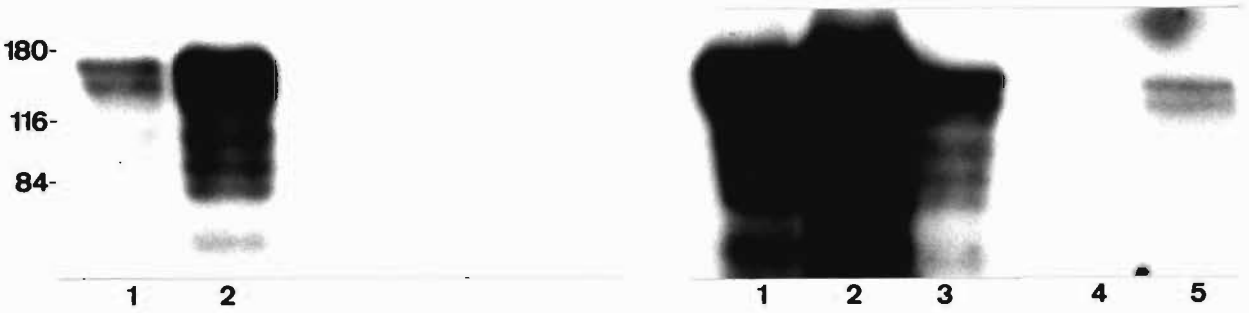
suggest that neither cell line possess receptors for bFGF, IGF-1, or insulin although it is not possible to exclude the expression of non functional receptors from this work.

Also in Fig 4.3 the lack of effect of oestradiol on intracellular phosphotyrosine in both cell lines is demonstrated. No increased phosphorylation of tyrosine residues was expected in the HEC-1-A cells as they lack oestrogen receptors. However, as Ishikawa cells possess functional oestrogen receptors it was felt to be of interest to see if addition of oestrogen led to a change in tyrosine phosphorylation, specifically of the EGFR. This finding confirms the lack of effect of oestradiol on EGF binding by Ishikawa 1 cells shown in Fig 4.6.

4.5 Summary

The results of these experiments confirm that these two epithelial endometrial cancer cell lines bind EGF with moderate affinity but not the 2 A and B isoforms of PDGF. Binding of EGF also activates the EGFR protein tyrosine kinase which is of normal molecular weight. However it is not possible to assess from these experiments whether phosphorylation of the EGFR tyrosine kinase leads to activation of subsequent intermediaries in the signal transduction pathway. Other growth factors and steroid hormones had no effect on EGF binding or EGFR tyrosine kinase activity.

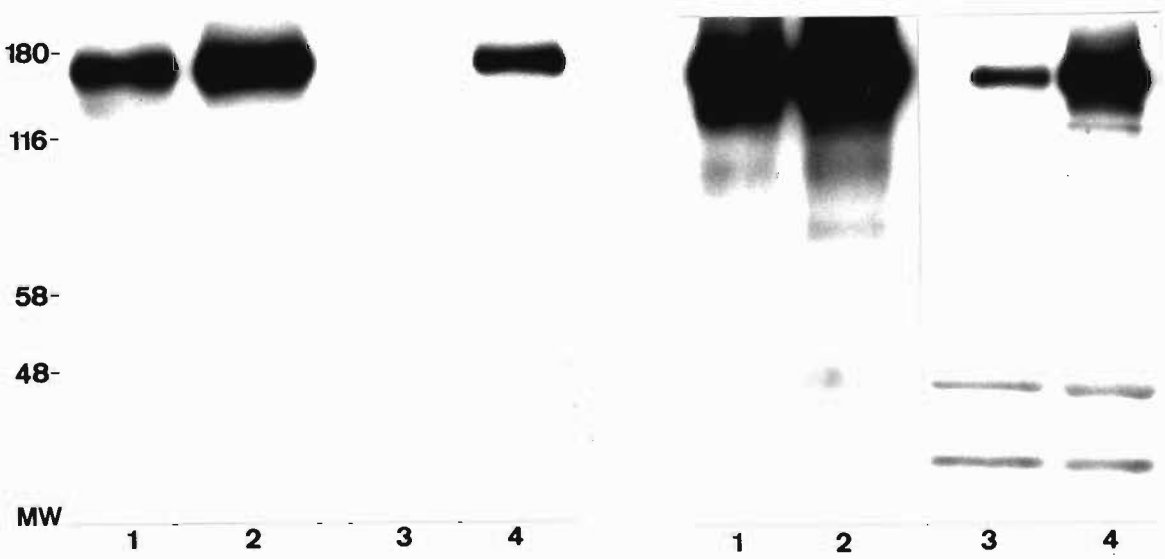
A) Ishikawa cells



Short exposure (45 min) Long exposure (24 h)

Lane	1	A431	4	Ishikawa
	2	A431 + EGF	5	Ishikawa + EGF
	3	HEC-1-A + EGF		

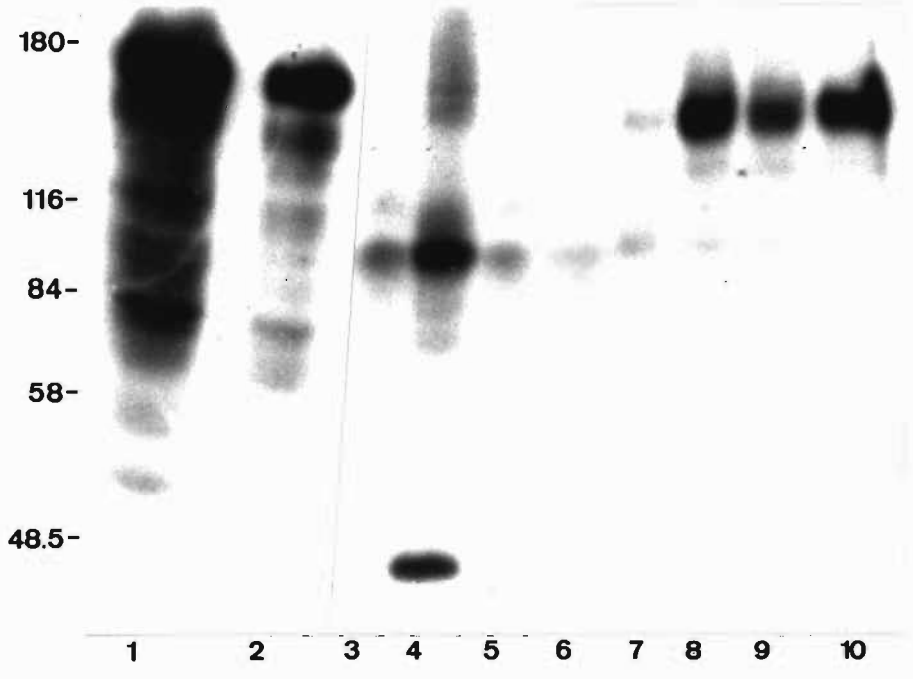
B) HEC-1-A cells



Short exposure (90 min) Long Exposure (12 h)

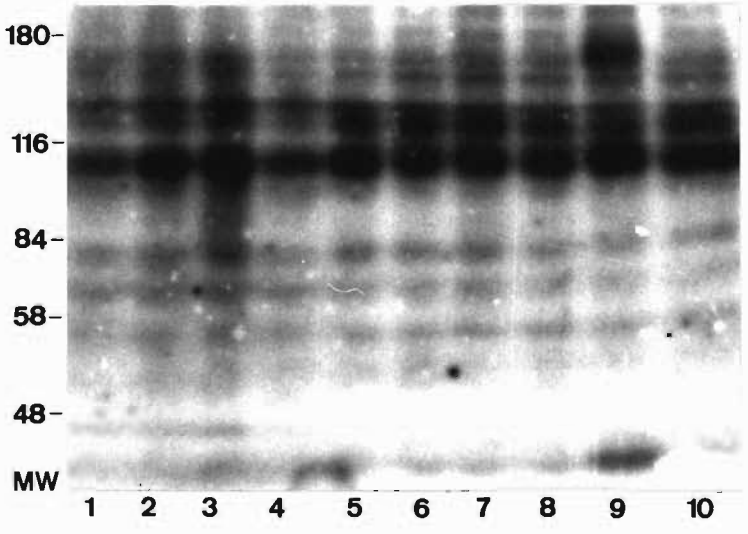
Lane	1	A431	3	HEC-1-A
	2	A431 + EGF	4	HEC-1-A + EGF

Fig 4.1 Immunoprecipitation of the EGFR from Ishikawa (A) and HEC-1-A (B) cells using A431 squamous carcinoma cells as positive controls. EGF was added to a final concentration of 200 ng/ml in each case.



Lane	1	A431 + EGF (200 ng/ml)	6	Ishikawa + PDGF-BB
	2	A431	7	Swiss 3T3
	3	Ishikawa	8	Swiss 3T3 + PDGF-AA13
	4	Ishikawa + EGF (200 ng/ml)	9	Swiss 3T3 + PDGF-AD1
	5	Ishikawa + PDGF-AD1	10	Swiss 3T3 + PDGF-BB

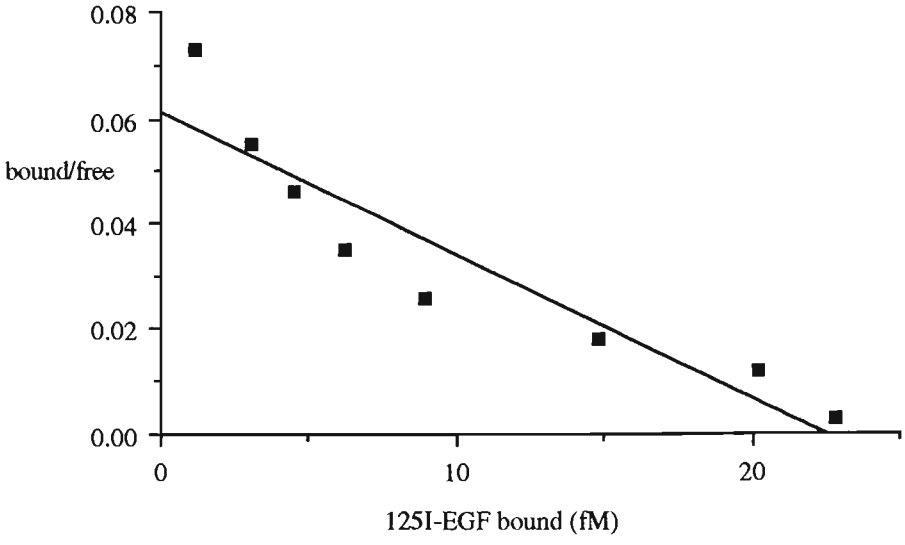
Fig 4.2 Protein tyrosine kinase activity in Ishikawa cells detected by antiphosphotyrosine antibodies labelled with ^{125}I-Protein-A. A431 and Swiss 3T3 cells were used as positive controls for activation of the EGF and PDGF receptors, respectively. The 3 forms of PDGF are described in Section 4.2.a and were used at a concentration of 20 ng/ml in each case. Molecular weight markers were not used in this experiment.



Lane	1	Ishikawa + oestradiol (10^{-8} M)	6	HEC-1-A + bFGF (200 ng/ml)
	2	Ishikawa + bFGF (200 ng/ml)	7	HEC-1-A + insulin (10 µg/ml)
	3	Ishikawa + insulin (10 µg/ml)	8	HEC-1-A + PDGF-BB (20 ng/ml)
	4	Ishikawa + 0.01% alcohol (control)	9	HEC-1-A + EGF (200 ng/ml)
	5	HEC-1-A + oestradiol (10^{-8} M)	10	HEC-1-A + 0.01% alcohol (control)

Fig 4.3 Effect of growth factors and hormones on the protein tyrosine kinase activity of Ishikawa and HEC-1-A cells detected by antiphosphotyrosine antibodies labelled with ^{125}I-Protein-A.

A)



B)

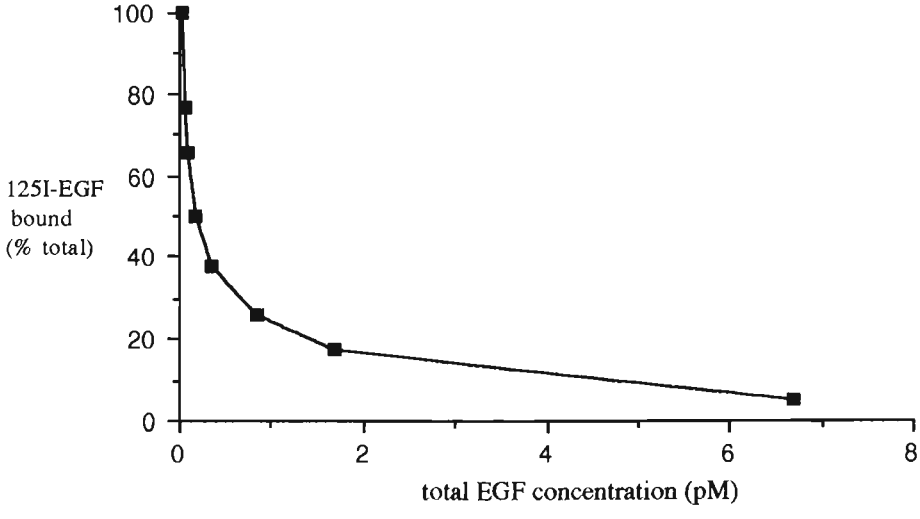


Fig 4.4 Binding of ^{125}I-EGF to Ishikawa cells. A) Scatchard plot of the competitive inhibition of ^{125}I-EGF binding by unlabelled ligand. B) Competitive inhibition of ^{125}I-EGF binding by unlabelled EGF.

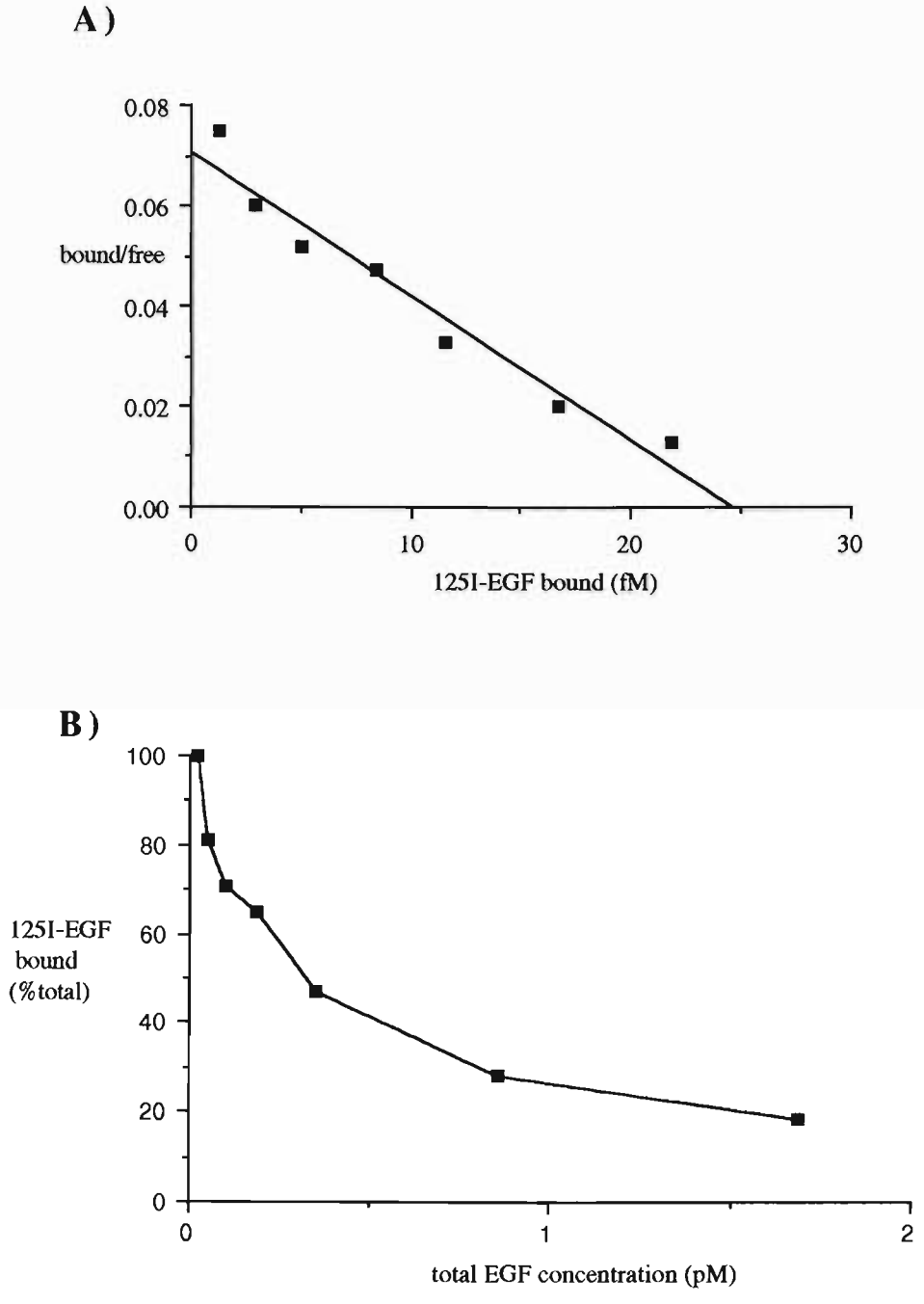


Fig 4.5 Binding of ^{125}I-EGF to HEC-1-A cells. A) Scatchard plot of the competitive inhibition of ^{125}I-EGF binding by unlabelled ligand. B) Competitive inhibition of ^{125}I-EGF binding by unlabelled EGF.

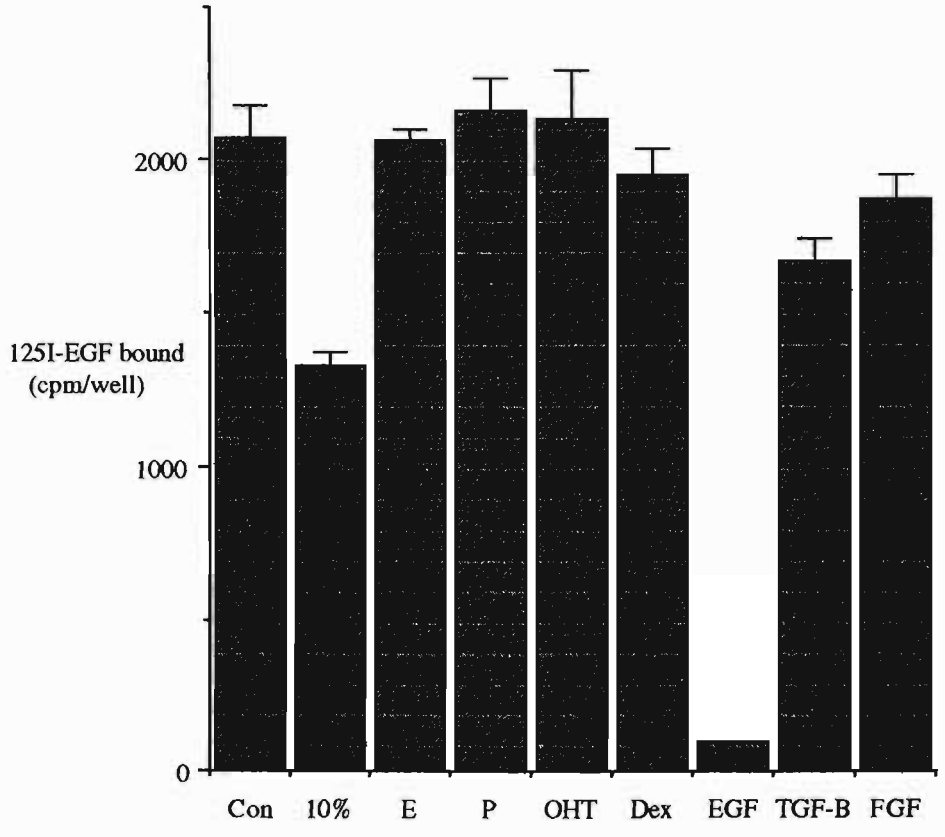


Fig 4.6 The effect of physiological concentrations of various growth factors and steroid hormones on the total binding of 125I-EGF to Ishikawa cells.

Con = medium + 0.01% ethanol
10% = medium + 10% FCS
E = oestradiol (10 nM)
P = R5020 (1 nM)
OHT = 4-hydroxytamoxifen (10 nM)

EGF = epidermal GF (1 nM)
TGF-B = transforming GF-beta 1 (1 ng/ml)
FGF = basic fibroblast GF (0.1 nM)
Dex = dexamethasone (10 nM)

5 THE REGULATION OF GROWTH OF ENDOMETRIAL CANCER CELL LINES IN CULTURE

5.1 Introduction

EGF and PDGF are growth regulators to a wide variety of cells derived primarily from epithelial and mesenchymal cells (reviewed in Sporn and Roberts, 1988). A number of mammary carcinoma cell lines secrete a range of growth factors including TGF-α, TGF-β and PDGF, and in oestrogen-responsive breast carcinoma cells, oestrogen treatment stimulates TGF-α synthesis (Dickson *et al.*, 1987). Indeed TGF-α is thought to be the primary effector of oestrogen-dependent stimulation of growth in these cells, while TGF-β functions as a hormonally regulated growth inhibitor (reviewed in Dickson *et al.*, 1990). These secreted growth factors act on the carcinoma cells in an autocrine and/or paracrine fashion resulting in a complex system of growth regulation involving the interaction of hormones and growth factors.

Much less is understood of the role of growth factors in the regulation of the growth of endometrial cancer cells *in vivo* or *in vitro* but the similarity between breast and endometrial cancer in terms of steroid hormone responsiveness raises the possibility that growth factors may also be involved in the regulation of growth of endometrial cancer cells.

Evidence supporting a growth regulatory role for growth factors in endometrial cancer was first reported from work on RL95-2 cells, one of the endometrial cancer cell lines described in Chapter Three. EGF was demonstrated to bind to these cells and, at a concentration of 0.83 nM, to inhibit their growth (Korc *et al.*, 1986). A further report from this group

extended this work and showed that the effects of EGF were more complex and dependent on the concentration of EGF and the density at which the RL95-2 cells were plated (Korc *et al.*, 1987). When the cells were seeded at a low density (4.7 x 10^3 cells/cm^2), a low concentration (16.6 pM) of EGF enhanced cell proliferation while higher concentrations were inhibitory. At a higher plating density (2.5 x 10^4 cells/cm^2), a higher concentration of EGF (0.83 nM) had a mild stimulatory effect. No further information was presented in this report on the dose-response relationship of the cells plated at higher density to different concentrations of EGF. The actions of EGF were mimicked by TGF-α which had a greater inhibitory effect at equimolar doses. This study also reported that, irrespective of seeding density, TGF-β inhibited the proliferation of these cells in a dose-dependent manner and caused a change in cellular morphology.

The effects of TGF-β on the growth of human endometrial cancer cells have been further investigated (Boyd and Kaufman, 1990). In 8 endometrial cancer cell lines examined, 5 were inhibited by TGF-β including the RL95-2, HEC-1-A, HEC-1-B and KLE cells described in Chapter Three, and 3 were not. Those cells that were inhibited by TGF-β developed a larger, flatter, more contact inhibited phenotype while the cell lines not inhibited by TGF-β showed no morphological changes. Northern analysis was used to determine TGF-β mRNA levels and revealed that the 3 cell lines not inhibited by TGF-β expressed relatively large quantities of TGF-β mRNA, while the cell lines which responded to TGF-β expressed much lower levels of TGF-β mRNA. The explanation and relevance of these findings with respect to the growth of endometrial cancer is unknown, but a possible interpretation is that one of the mechanisms whereby the growth of endometrial cells is deregulated is that the overexpression of TGF-β mRNA leads to insensitivity to the inhibitory

effects of TGF-β.

Cells from the HEC-1-A and HEC-1-B cell lines have been shown to respond to bFGF with an increase in cellular proliferation and increased secretion of plasminogen activators (Presta *et al.*, 1988). bFGF has angiogenic properties both *in vivo* and *in vitro* and is secreted by a range of normal and tumour-derived cultured cells (Moscatelli *et al.*, 1986). HEC-1-A and HEC-1-B cells also secrete bFGF-like molecules (Presta, 1988) suggesting that bFGF may act in an autocrine manner in these endometrial cancer cells. The secretion of bFGF by the HEC-1-A and HEC-1-B cells is increased by oestrogen and this stimulatory effect of oestrogen is abolished by progesterone. The fact that the secretion of a potent angiogenic molecule like bFGF by endometrial cancer cells is hormonally regulated suggests that its secretion may have a role in tumour development, although the concentrations of oestrogen used in these studies were markedly supraphysiological. In addition, it is difficult to understand this response of HEC-1-A and HEC-1-B cells to oestrogen as they have been reported to lack functional oestrogen receptors (Holinka *et al.*, 1986a).

In contrast, oestrogen at physiological doses (10^{-8} M) has been reported to induce the proliferation of, and the synthesis of progesterone receptors in, Ishikawa cells in culture (Holinka *et al.*, 1986a). In these cells, oestrogen also stimulated the activity of the enzymes alkaline phosphatase (Holinka *et al.*, 1986b) and DNA polymerase α (Gravanis and Gurpide, 1986). These results confirm that oestrogen induces a spectrum of intracellular events in Ishikawa cells consistent with the normal physiological actions of oestrogen.

Ishikawa cells are also responsive to antioestrogens and the addition of 4-hydroxytamoxifen (4-OHtam) stimulated the proliferation of Ishikawa

cells under certain conditions (Anzai *et al.*, 1989). Simply by altering the culture conditions, however, 4-OHtam lost its stimulatory effect and inhibited the proliferative effects of oestradiol. In contrast to oestradiol, 4-OHtam only had a minor effect on alkaline phosphatase activity suggesting that the antioestrogen does not act via the oestrogen receptor in these cells.

Conflicting data have been presented from a study of primary cultures of endometrial cancers (Terakawa *et al.*, 1988). 4-OHtam was found to bind to oestrogen receptors and caused a dose-dependent inhibition of growth in 2 out of 3 cell lines grown from oestrogen receptor positive tumours, but had no effect on 2 cultures from tumours lacking oestrogen receptors. The latter findings are consistent with the data from breast cancer where a response to endocrine therapy (including tamoxifen) is more likely in those patients with oestrogen receptor positive tumours (reviewed in McGuire, 1979).

It therefore appears that in endometrial cancer antioestrogens have tumour-specific actions that may or may not be mediated by the oestrogen receptor. The stimulatory effects of 4-OHtam on Ishikawa cells may provide an *in vitro* explanation of the reports of the development of endometrial carcinoma in breast cancer patients receiving antioestrogens (Killakey *et al.*, 1985; Fornander *et al.*, 1989).

It therefore appears that growth factors may play a role in the regulation of growth of endometrial cancers. *In vitro* work on established endometrial cancer cell lines confirms that EGF, TGF-α and bFGF have important mitogenic effects while TGF-β is inhibitory. The Ishikawa cells display both mitogenic and metabolic responses to oestrogen in physiological doses making them a suitable model of hormone-dependent endometrial cancer comparable with the more extensively studied hormone-dependent breast carcinoma cell lines. The Ishikawa cells provide

a good opportunity to compare growth regulatory mechanisms between steroid hormone sensitive and insenstive cell lines and with the complex growth control of the breast carcinoma cell lines of both types.

The work described subsequently in this chapter deals with the HEC-1-A and Ishikawa cell lines and falls into 3 parts; first, the determination of growth characteristics for the 2 cell lines; second, the assessment of the effects of various agents on cell proliferation; and third, the effects of various steroid hormones and their antagonists on the growth of Ishikawa cells.

5.2 Materials and methods

5.2.a Cell culture

Cells to be used in experiments were maintained in culture as described in Section 2.1 until required. At the beginning of the experiment, the cells were trypsinized, suspended and counted as described in Section 2.1. The appropriate numbers of cells were seeded into 24-well tissue culture dishes in phenol red-free medium containing 5% charcoal-stripped FCS (see Section 5.2.d below). Sufficient wells were plated to provide triplicate wells for each set of culture conditions at the required number of time points plus 3 wells to determine seeding density. After 18-20 hr incubation to allow the cells to attach adequately the medium was removed and the wells carefully washed 4 times with warmed (37°C) PBS. The 3 wells for counting were then prepared and counted as described in Section 5.2.b below. One ml of serum-free medium (SFM, see Section 5.2.c below) without phenol red containing the appropriate additives was then added to

each well.

In the experiment studying the effects of steroids on Ishikawa cells (Figs 5.6-5.8) the cells were starved prior to plating. 48 hr before the start of the experiment the culture medium was aspirated from cells growing exponentially and the cells were washed 4 times with warmed (37°C) PBS to remove all traces of medium. Fresh SFM was then added to the culture dishes and the plates returned to the incubator. The plates were incubated in this medium until the time of the experiment in order to up regulate all receptors.

Steroids were added as stock solutions in ethanol to the medium at the appropriate concentration but such that the ethanol was less than 0.01% final concentration in culture. Control wells contained SFM with 0.01% ethanol only.

The 24-well plates were incubated at 37°C in humidified atmosphere with 95% air/5% CO_2 until needed for counting at each time point. The culture medium was replaced every 3 to 4 days with medium to which the appropriate agents had been freshly added.

5.2.b Cell counting

Because of the large numbers of wells counted at each time point a Model ZB1 Coulter counter (Coulter Electronics Ltd, UK) was used. The wells to be counted were washed 3 times with warmed (37°C) PBS and the cells in each well were then lysed in 2 ml 0.01 M HEPES containing 1.5 mM $MgCl_2$ plus four drops of Zaponin (Coulter Electronics Ltd., UK) for 5 min. The nuclei released were suspended in Isoton (Coulter Electronics Ltd., UK) and counted.

5.2.c Preparation of serum-free medium (SFM)

SFM was prepared weekly and comprised phenol red-free RPMI 1640 [plus sodium bicarbonate (2.4 g/l) and glutamine (292 mg/l)], penicillin (0.06 g/l), streptomycin (0.1 g/l), with 15 mM HEPES buffer (BDH Chemicals Ltd., UK), transferrin (10 µg/ml, Sigma), fibronectin (2 µg/ml, Sigma), and sodium selenide (3 µm, Sigma).

5.2.d Preparation of dextran:charcoal-stripped fetal calf serum

The dextran:charcoal-stripped fetal calf serum (DC-FCS) was made up in 500 ml quantities. The dextran:charcoal (DC) mixture was made to a final volume of 1 litre as follows:

Charcoal (Sigma)	10 g
Dextran T40 (Pharmacia)	1 g
Tris (pH 7.4)	10 mM
EDTA	1 mM

After vigorous agitation of the DC mixture, 125 ml was centrifuged at 5,000 rpm for 20 min and the supernatant completely removed. 250 ml of FCS was added to the DC and mixed vigorously in a shaking water bath at 55°C for 30 min. The charcoal was removed by centrifuging at 10,000 rpm for 20 min and the supernatant (serum) was added to fresh DC mixture and the process repeated. The serum was filter sterilised through a 0.45 µ and then a 0.22 µ filter and stored in 50 ml aliquots at -20°C.

5.2.e Agents used

Oestradiol Stock solution (Sigma, UK) made up to 10^{-4} and 10^{-5} M in ethanol, stored at -40°C and diluted to the appropriate concentration for use.

4-hydroxytamoxifen Stock solution (Sigma, UK) made up to 10^{-5} M in ethanol, stored at -40°C and diluted to the appropriate concentration for use. As it is unstable in light, all solutions containing tamoxifen were stored in the dark.

R5020 Synthetic progestin 17,21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione (Sigma, UK), stock solution made up to 10^{-6} M in ethanol, stored at -40°C and diluted to the appropriate concentration for use.

RU486 Antiprogestin and antiglucocorticoid 17β-hydroxy-11β-(4-dimethylamino-phenyl)-17α-(1-propynyl)-estra-4,9-dien-3-one (Centre de Recherche Roussel-Uclaf, France), stock solution made up to 10^{-5} M in ethanol, stored at -40°C and diluted to the appropriate concentration for use.

ICI 164,384 Antioestrogen N-n-butyl-11-3, 17β-dihydroxyoestra-1,3,5(10)-trien-7α-yl-N-methylundecamide (ICI Pharmaceutical, UK), stock solution made up to 10^{-5} M in ethanol, stored at -40°C

and diluted to the appropriate concentration for use.

5.3 Results

5.3.a Effect of fetal calf serum

The serum used in all these experiments was all DC-FCS. The growth of HEC-1-A and Ishikawa cells in SFM with increasing concentrations of DC-FCS is shown in Fig 5.1. In the HEC-1-A cells there is a 2 day period when cell numbers decrease before exponential growth occurs. Serum appears to increase cell survival during this time and increases growth rate in the recovery phase from day 2 to 6. After day 6, the growth rates in SFM alone and at each serum concentration are similar.

A corresponding growth pattern is exhibited by the Ishikawa cells. Although there is no early reduction in cell numbers, growth is slow for the first 3 days with serum improving growth rate in a dose-dependent manner. As with the HEC-1-A cells, the most dramatic effects of serum are shown between day 3 and 7 where growth rate increases in response to rising serum concentration. After day 7 the growth rates are similar at all serum concentrations, however rate of growth of the cells in SFM continues to increase up to day 13 before plateauing. This fall off in growth is not seen in the presence of serum.

5.3.b Effect of oestradiol

Results from the same experiments described in the previous section carried out in the presence and absence of a physiological concentration of

oestradiol (10^{-8} M) are depicted in Fig 5.2. In the HEC-1-A cells oestradiol has no effect on the growth of cell in the presence of different serum concentrations. In SFM, oestradiol appears to increase the growth rate markedly between day 2 and 6 and thereafter the growth rate is identical.

In the Ishikawa cells no real difference is seen with oestradiol in the presence of the different serum concentrations. In SFM, however, the effect of oestradiol is the reverse to that observed in the HEC-1-A cells. Oestradiol appears to delay the onset of exponential growth to at least 13 days although the final numbers at day 18 are almost the same.

5.3.c Effect of plating density

The growth of HEC-1-A and Ishikawa cells at 2 different plating densities in SFM and 0.5% and 5% DC-FCS is shown in Fig 5.3. Essentially this is the same experiment as described in Section 5.3.a at 2 seeding densities. No error bars are shown in this figure as they obscured the figures excessively. Error on these measurements was of comparable magnitude to that shown in previous figures.

At the higher plating density (~72,000 cells per well), the HEC-1-A cells no longer demonstrate an initial fall off in cell numbers but begin growing exponentially from the start. The growth in serum is marginally better and cell numbers plateau after 13 days. At this stage there is a small decrease in cell numbers in SFM and 0.5% serum but cell numbers remain stable in 5% serum.

At the lower plating density (~6,200 cells per well) the lag phase is again evident to 3 days with serum showing no effect. After day 3 exponential growth occurs with a dose-dependent increase in rate with increasing serum concentration. As with the higher plating density there is

a plateau in growth at day 13 and a minor loss of cell numbers in SFM and 0.5% serum but a slight increase in numbers in 5% serum.

The data at the lower plating density are directly comparable with those from Fig 5.1. It is notable that in this experiment there was no initial fall off in cell numbers and the growth rate in SFM was closer to that in serum than in Fig 5.1.

The Ishikawa cells plated at high density (44,500 cells per well) show a similar pattern of growth to the HEC-1-A cells plated at high density. The cells grow exponentially from plating with those cells in serum showing a modest dose-dependent increase in growth over those in SFM. The Ishikawa cells also plateau, but at 7 days rather than 13 days as with the HEC-1-A cells. Again there is a fall off in cell numbers in SFM after12 days, but maintenance of numbers in the presence of serum.

Serum improves early survival in a dose-dependent manner when Ishikawa cells are plated at low density (5,300 cells per well), similar to the previous experiment with Ishikawa cells shown in Fig 5.1. After the first 4 days growth rate is identical in SFM and both serum concentrations with the higher numbers in 5% serum due to the improved growth over the first 4 days. The effects of serum are also demonstrated after 12 days when there is a decrease in cell numbers in SFM, but no decrease in 0.5% or 5% serum.

These experiments confirmed that plating density was of great importance in determining the subsequent growth pattern of both HEC-1-A and Ishikawa cells. It also made it clear that the effects of addition of any other factor would be unlikely to be apparent at higher plating densities.

5.3.d Screening experiments

The effects of a number of different agents on the growth of HEC-1-A and Ishikawa cells in the system already outlined are shown in Figs 5.4 and 5.5, respectively. The agents were chosen for their known growth affecting potential and were used in physiological concentrations as indicated in the figure legends. The cells were plated as described in Section 5.2.a and at day 0 triplicate wells were counted to determine the plating density. SFM and the appropriate additives were added to the wells, the plates cultured with medium changes every 3 days and the wells counted after 8 or 9 days.

Fig 5.4 shows the results of 2 consecutive experiments looking at the effects of a number of agents on the growth of HEC-1-A cells. In the first experiment (Fig 5.4.A) the only effects seen are a marked inhibition of growth with the phorbol ester 12-*O*-tetradecanoyl-13-acetate (TPA) and a possible slight inhibition with a combination of EGF and insulin. To assess whether these effects were real, the experiment was repeated (Fig 5.4.B) with the addition of the inactive alpha form of TPA. In this experiment there was no effect of EGF and insulin suggesting that the result from the first experiment were spurious. The αTPA reversed the inhibitory effect of TPA confirming that TPA does inhibit the growth of these cells in culture.

In Fig 5.5 the results from similar experiments on Ishikawa cells are depicted. In Fig 5.1.A the only significantly different result was the marked inhibition of the growth of cells by TGF-β_1. The experiment was repeated in the presence and absence of 10^{-8} M oestradiol (Fig 5.5.B). In this experiment the only significant finding was the inhibition of growth by 25 mM HEPES buffer. Oestradiol at this concentration appeared to have no consistent effect on the growth of Ishikawa cells.

5.3.e Effects of steroids and their antagonists on the growth of Ishikawa cells

In a further experiment to assess the steroid responsiveness of the Ishikawa cells, Ishikawa cells starved for 48 hr prior to the experiment were grown in different concentrations of various steroid hormones and their antagonists. The results from this experiment are shown in Figs 5.6 to 5.8.

The effects of different concentrations of serum are shown in Fig 5.6.A. Although the cells were first counted at day 6 and therefore information on numbers in the first 2-3 days in culture is not available, there does not appear to be an obvious lag phase in growth in this experiment. Of more interest is the growth of the cells in SFM, where there is only very slow growth over the course of the experiment. This finding contrasts markedly with those from previous experiments where the growth rate in SFM was similar to that in serum after an initial lag (Fig 5.1.B, Fig 5.3.B).

In Fig 5.6.B the response to various concentrations of oestradiol is shown. There is an increase in growth rate at all concentrations and there appears to be dose response effect with 10^{-7} M giving maximal growth and 10^{-10} M the least increment in growth rate.

The effects of the antioestrogen, 4-hydroxytamoxifen (4-OHtam), and the synthetic progesterone, R5020, shown in Fig 5.7 are more difficult to interpret. 4-OHtam at 10^{-10} M and 10^{-8} M has a small stimulatory effect but at 10^{-9} M has an inhibitory effect overall. The results on R5020 are equally inexplicable with 10^{-10} M causing inhibition of growth and 10^{-8} M and 10^{-9} M improving growth rate.

The progesterone antagonist, RU486, and the nonsteroidal

antioestrogen, ICI 164,384, both caused an increase in growth rate over SFM alone (Fig 5.8). In the case of ICI 164,384 (Fig 5.8.A), this was dose-dependent with 10^{-10} M and 10^{-9} M having an equal stimulatory effect and 10^{-8} M less influence. However, the results from RU486 were confusing with 10^{-10} M and 10^{-8} M causing a similar increase in growth rate and 10^{-9} M a smaller increment.

5.4 Discussion

As outlined in the introduction there are good reasons for expecting that growth factors have a role in the regulation of growth of endomertial cancer cells in culture and *in vivo*. The results of culturing the HEC-1-A and Ishikawa cell lines demonstrated that these cells proliferate even in the complete absence of serum. After an initial lag phase, the growth rate of cells in SFM is equal to that in serum although final numbers are not as high in SFM. The main effect of serum appears to be improving the survival and growth of cells in the first few days after plating and when there is plateauing of numbers after 13 days. In addition, very little serum is required for this action as there is only a minor difference between the effect on growth of the 0.5% and 5% serum concentrations.

This growth pattern suggests that serum provides a factor or factors that the cells initially lack, but are producing in significant quantities by day 6 or 7. The factor(s) secreted by these cells acts in an autocrine or paracrine fashion and it stimulates these cells to proliferate at near maximal rate in the absence of other factors. Thus at higher plating densities serum has a minimal effect on growth as the cells present in the culture secrete enough factor to enable all cells in the culture to grow exponentially. Consequently any other modulators of the growth of these cell lines would

be more likely to have an effect at lower rather than higher plating densities.

Oestradiol at a physiological concentration had unexpected effects on both the HEC-1-A and Ishikawa cells. In both cell lines oestradiol had no effect on growth in the presence of serum. However in SFM the HEC-1-A cells achieved higher numbers in the presence of oestradiol although the growth rates were identical after 6 days. Repetition of this experiment (data not shown) failed to confirm this effect. In the Ishikawa cells oestradiol appeared to have an inhibitory effect but this difference was only present in the cell counts at 13 days. This suggests that this finding represents experimental error and the effects of oestradiol on Ishikawa cells will be discussed in more detail below.

The screening experiments failed to show any consistent response to the agents commonly used in cell cultures: EGF, insulin or the synthetic glucocorticoid, dexamethasone. The tumour promoter TPA had a marked inhibitory effect on HEC-1-A cells and TGF-β_1 had a simlar effect on Ishikawa cells. TPA, an activator of protein kinase C, is known to inhibit the growth of a wide range of cell types (Roos *et al.*, 1986) so its action on these cells is not surprising. As discussed in Section 5.1, TGF-β_1 has been shown to inibit growth of a range of cell lines derived from endometrial cancers including HEC-1-A cells and this work demonstrates that it also inhibits Ishikawa cells. None of the observed effects on Ishikawa cells were altered by the presence of 10^{-8} M oestradiol confirming the findings of the previous experiment on Ishikawa cells.

In order to look at the action of the steroids and their antagonists on Ishikawa cells an experiment was undertaken using these agents at different concentrations around the physiological range (Figs 5.6 - 5.8). One explanation for the lack of responsiveness of the Ishikawa cells to

oestradiol was that there had been a down regulation of the oestrogen receptors. Therefore the cells used for this experiment were prepared differently to those in previous experiments and were starved in SFM for 48 hr prior to the start of the experiment in order to maximize receptor numbers.

The results from this experiment differ from those in previous experiments. Although there was a dose-response to serum, growth in SFM was very slow unlike that in previous experiments. However the plating density was similar to that used in previous experiemts (~10^4 cells per well) and the maximum number of cells reached in 5% serum was also similar (~5 x 10^5 cell per well). The growth in all additives was better than in SFM apart from the lowest concentration of R5020 and the middle concentration of 4-OHtam which were slightly inhibitory. These results are difficult to explain as anything other than experimental error. The most convincing results were obtained with oestradiol where there was a dose-response and the effect of greatest magnitude of all the agents used.

Overall, a consistent stimulatory effect of oestrogen on Ishikawa cells was not seen in this system. In an effort to overcome this problem, a further supply of Ishikawa cells was procured from Dr J White, Department of Obstetrics and Gynaecology, Hammersmith Hospital, London. This laboratory had been performing similar experiments on Ishikawa cells under different culture conditions and had obtained reproducible results. In their system oestradiol and 4-OHtam were mitogenic, progesterone was inhibitory and the inhibitory effect of progesterone on growth was abolished by the addition of its antagonist, RU486 (Croxtall *et al.*, 1990; personal communication, Dr J White).

Initially the new cells were studied in the same system used in previous experiments and failed to show any response to oestrogen (data

not shown). Following this, the cells were cultured in an identical manner to that used in Dr White's laboratory. Again the cells showed rapid growth even in serum-free medium and no effect of oestradiol, a similar pattern of growth to that shown in this Chapter. There was no obvious cause for the failure to reproduce Dr White's results using the same cells and culture conditions.

The lack of response to oestradiol and the rapid growth of Ishikawa cells in serum-free medium are difficult to explain. Other groups who have worked with this cell line and reported steroid responses routinely culture the cells in medium containing phenol red, a pH indicator with weak oestrogenic activity, and FCS (Holinka *et al.*, 1986a; Terakawa *et al.*, 1987). When using the cells for experiments the cells were grown in phenol red-free medium and DC-FCS, sometimes for up to a week prior to the start of the experiment (Anzai *et al.*, 1989). The culture conditions therefore appear to vary considerably while still retaining the steroid sensitivity. This contrasts with data from the oestrogen-sensitive breast carcinoma cell line, ZR-75-1, which loses its oestrogen sensitivity if grown in the absence of oestrogen for 7 to 14 days (Darbre *et al.*, 1983)

In summary, these studies of HEC-1-A and Ishikawa cells showed that, in the absence of serum, these cells proliferate rapidly after an initial lag phase. TPA and TGF-β_1 were shown to inhibit growth of HEC-1-A and Ishikawa cells, respectively, but no agent consistently had a mitogenic effect. This is possibly due to the high basal level of growth in these cells which may be the result of a secreted growth factor acting in an autocrine and/or paracrine fashion. Despite the reported steroid sensitivity of Ishikawa cells, no consistent response to oestrogen or other steroids was demonstrated.

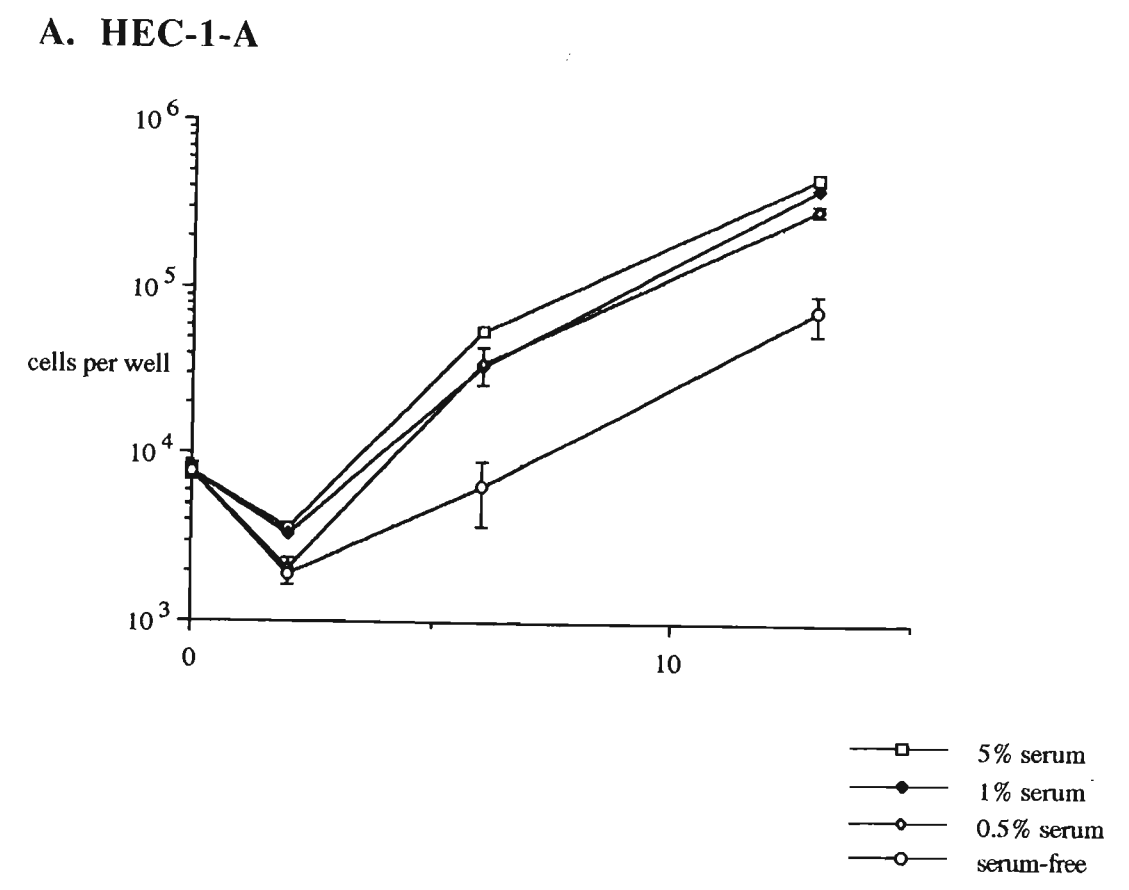


Fig 5.1. Growth of HEC-1-A (A) and Ishikawa (B) cells in SFM with increasing concentrations of DC-FCS. Symbols, mean ± SE. No error bars are shown if the variation was too low for display.

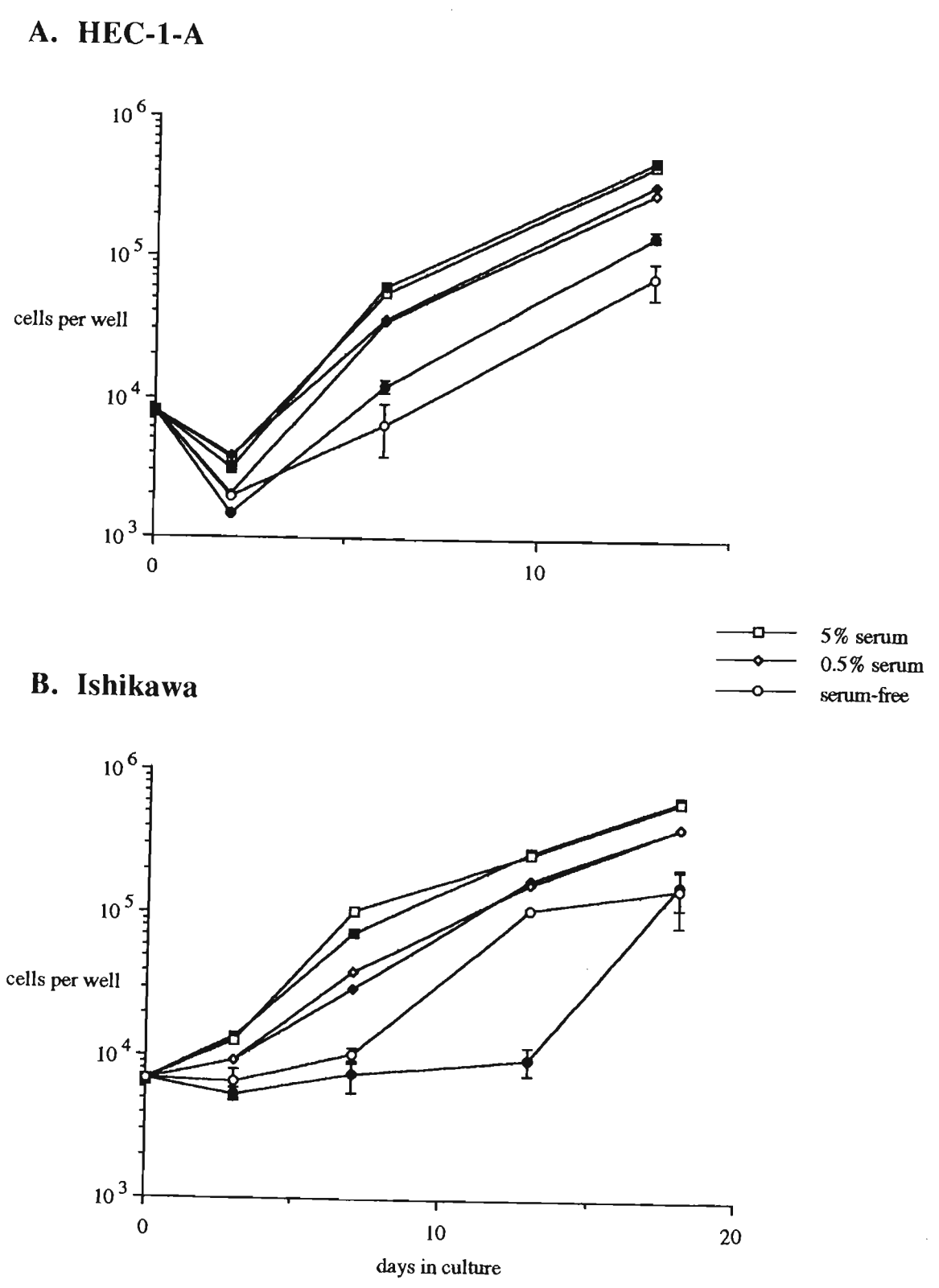


Fig 5.2. Growth of HEC-1-A (A) and Ishikawa (B) cells in SFM in the presence (closed symbols) and absence (open symbols) of 10 nM oestradiol and different concentrations of DC-FCS. Symbols, mean ± SE. No error bars shown if variation too low for display.

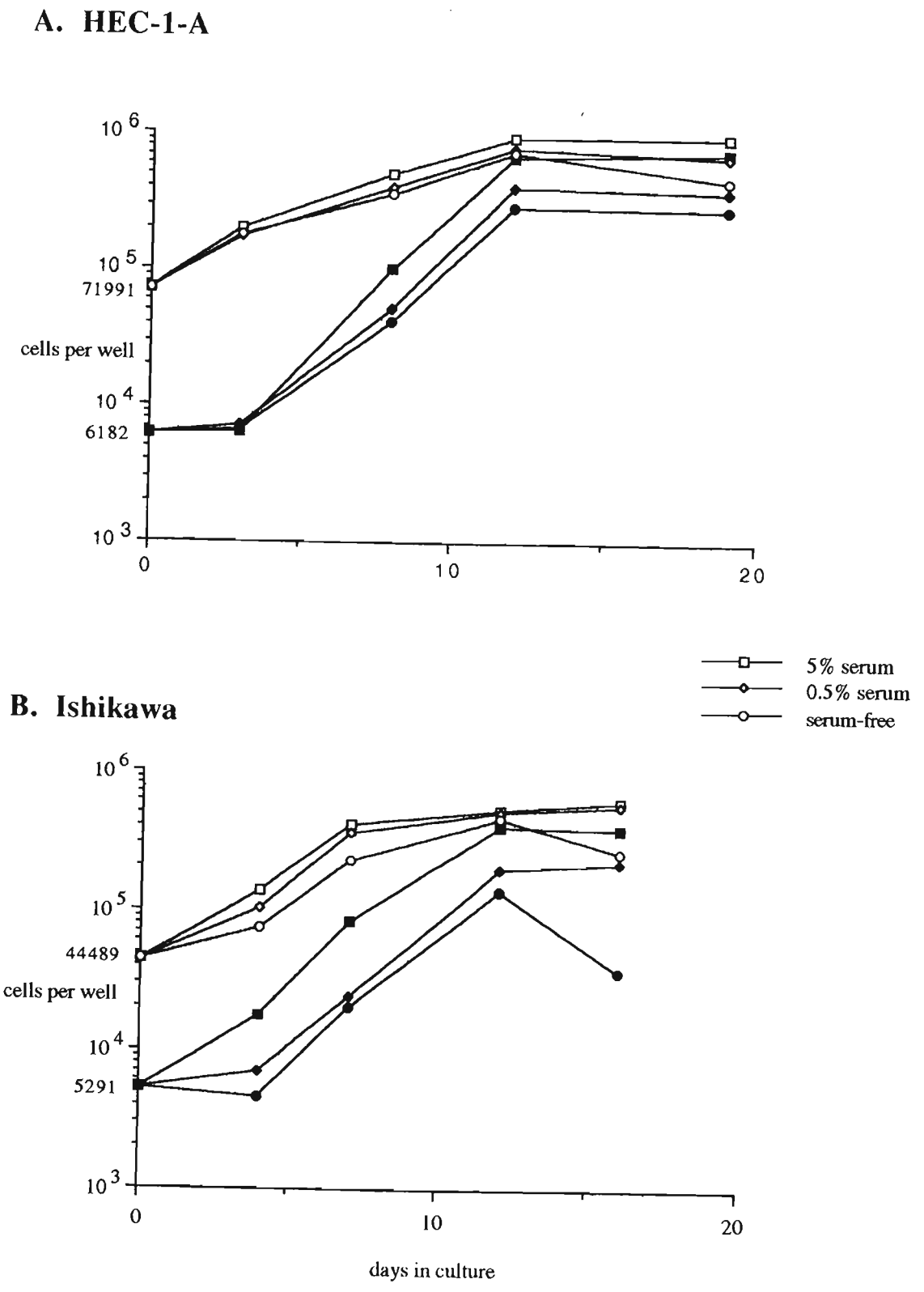
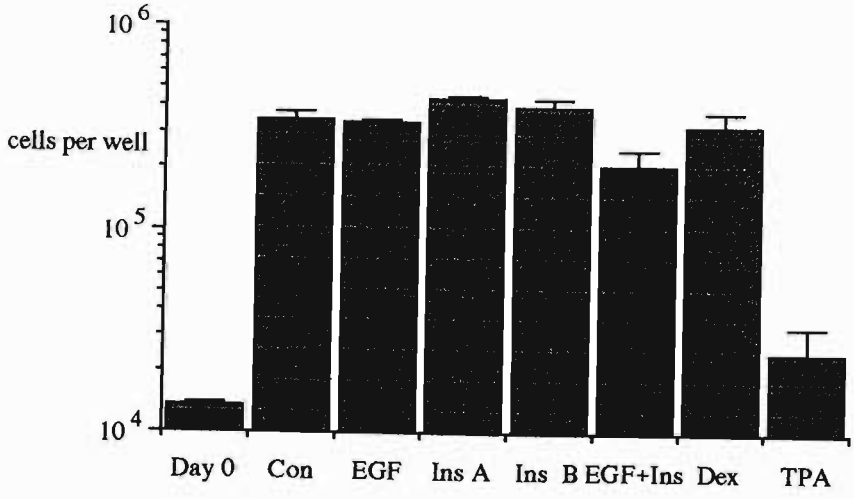


Fig 5.3. Growth of HEC-1-A (A) and Ishikawa (B) cells in SFM plated at low density (closed symbols) and high density (open symbols) and different concentrations of DC-FCS as indicated. Symbols, mean. Error bars not shown (see results text)

A. Experiment 1



Con = serum-free medium
EGF = EGF (100 ng/ml)
Ins A = Insulin (0.1 μg/ml)
Ins B = Insulin (10 μg/ml)
EGF+Ins = EGF (100 ng/ml) + Insulin (10 μg/ml)
Dex = Dexamethasone (10 nM)
TPA = phorbol ester (10 nM)
aTPA = alpha TPA (10 nM)

B. Experiment 2

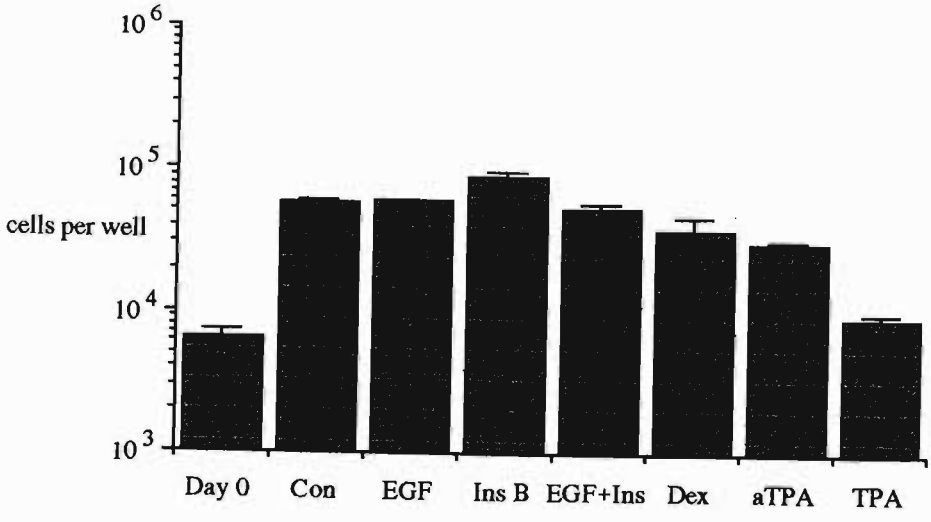
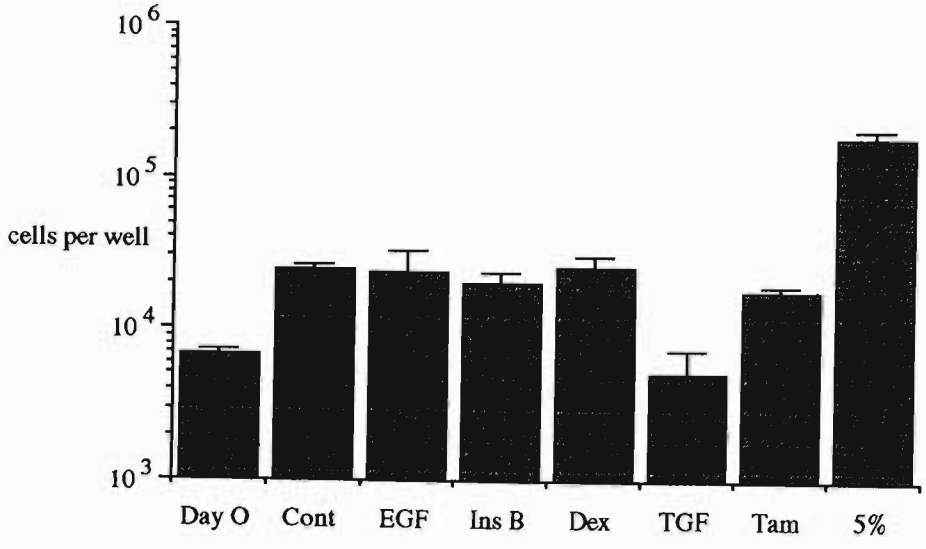


Fig 5.4. Effects of various agents in SFM (concentrations shown in legend) on the growth of HEC-1-A cells in consecutive experiments. Cells were plated (day 0) and then counted in triplicate wells at Day 7. Columns, mean ± SE

A. Experiment 1



Con = serum-free medium
EGF = EGF (100 ng/ml)
Ins A = insulin (0.1μg/ml)
Ins B = insulin (10μg/ml)
Dex = dexamethasone (10 nM)
TGF = TGF beta 1 (1 ng/ml)
Tam = 4-hydroxytamoxifen (10 nM)
5%, 0.5% = 5% and 0.5% DC-FCS
PDGF = PDGF BB (200 ng/ml)
HEPE = 25 mM HEPES buffer
Tran = transferrin (10μg/ml)

B. Experiment 2

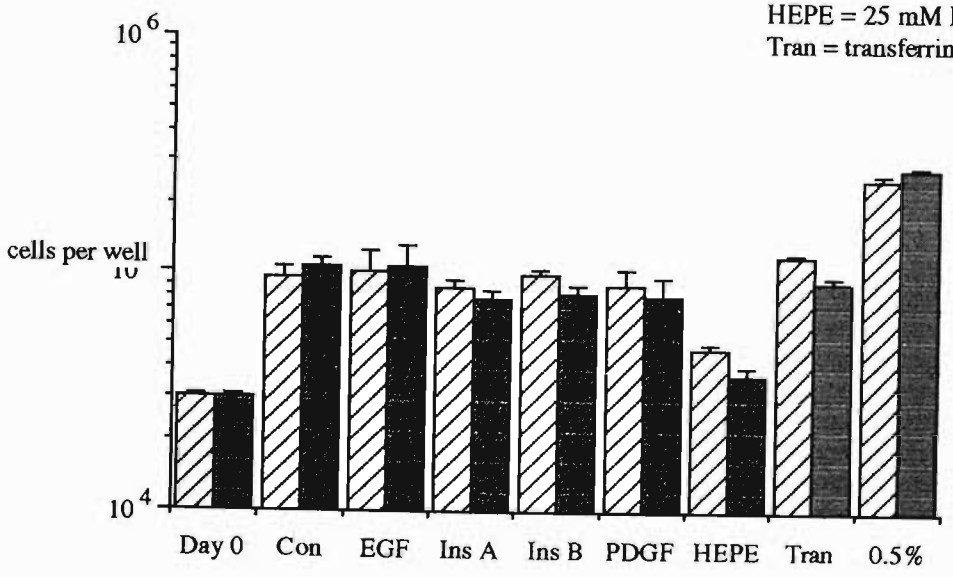


Fig 5.5. Effects of various agents in SFM (concentrations shown in legend) on the growth of Ishikawa cells in consecutive experiments. cells were plated (day 0) and then counted in triplicate wells at Day 9. Columns, mean ± SE.
In experiment 2, cells were grown in the presence (shaded columns) and absence (hatched columns) of 10 nM oestradiol.

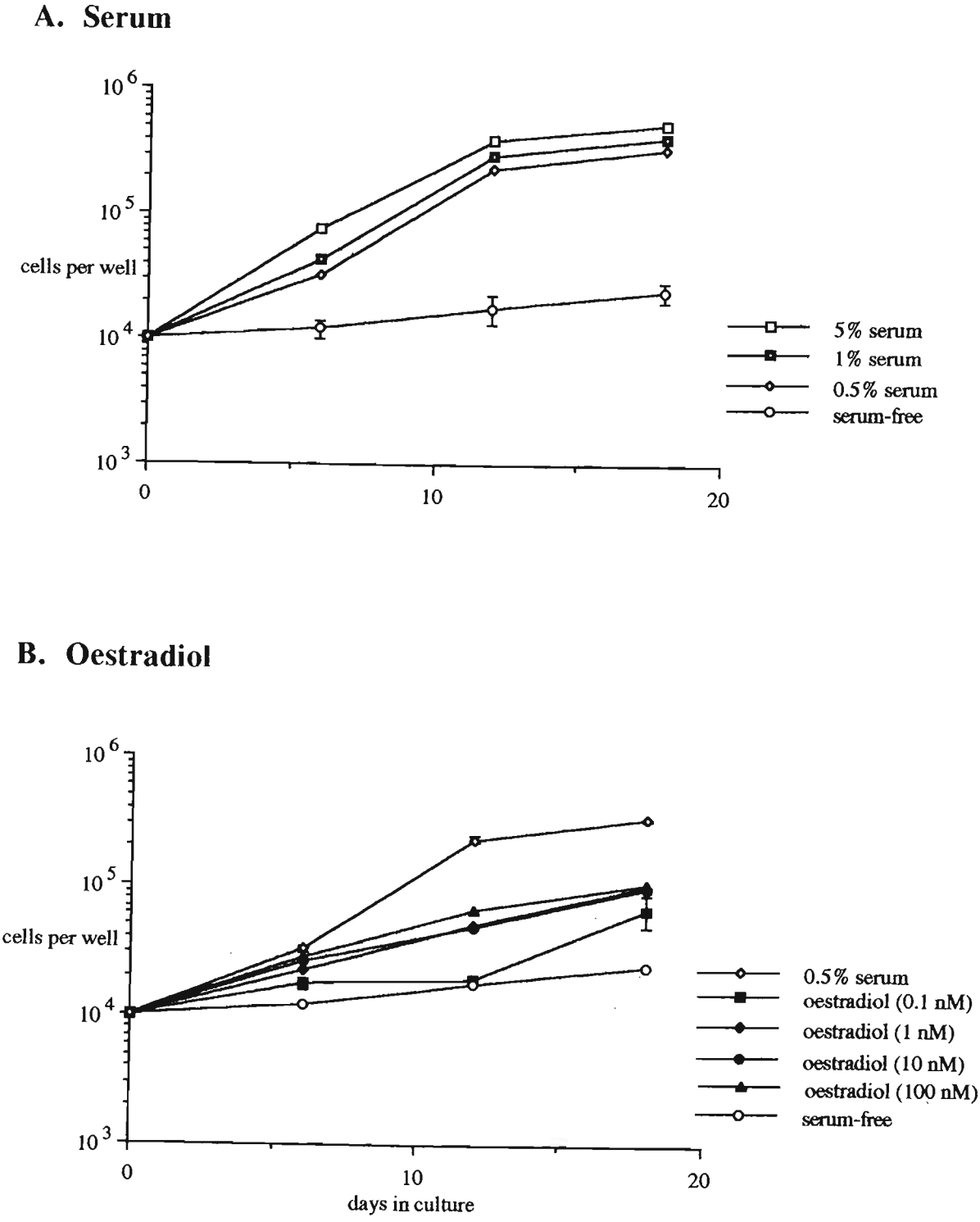


Fig 5.6. Growth of Ishikawa cells in SFM containing different concentrations of DC-FCS (A) and oestradiol (B) as indicated. Symbols, mean ± SE. Error bars not shown if they interfere with display Error for symbols where error bars not shown was similar to those where error bars shown.

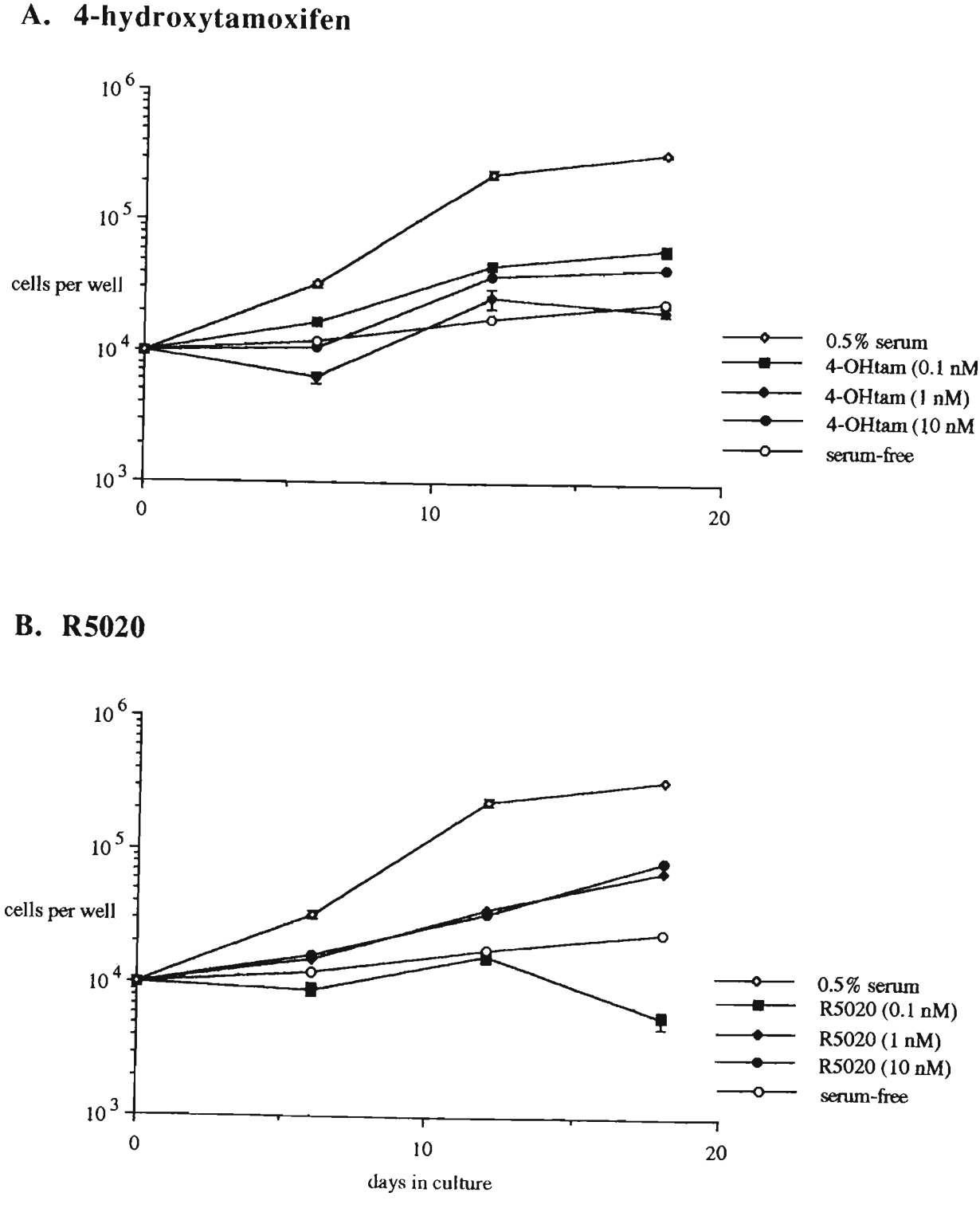
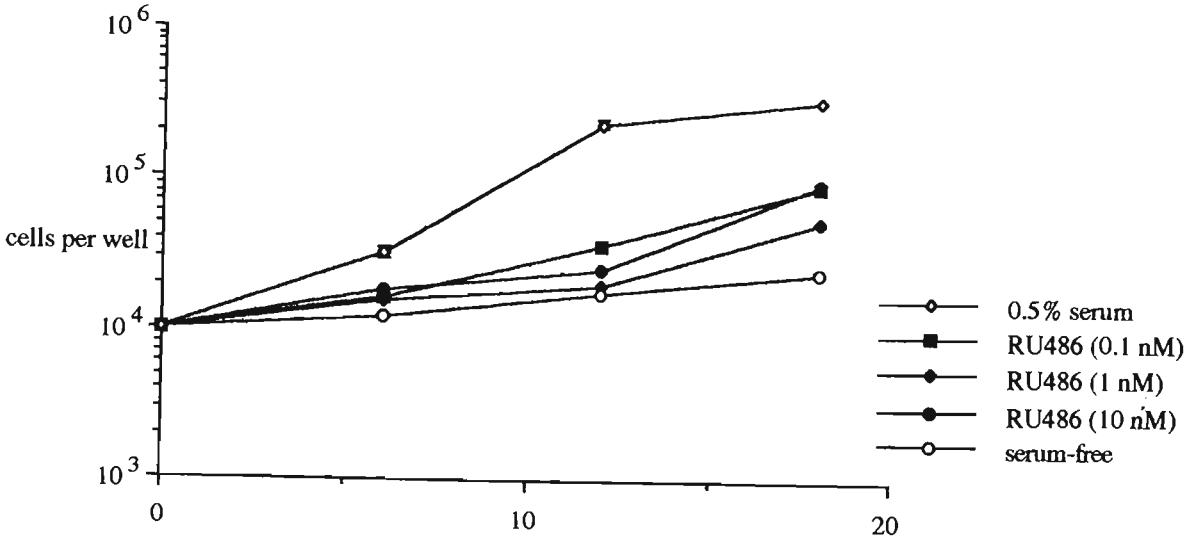


Fig 5.7. Growth of Ishikawa cells in SFM containing different concentrations of 4-hydroxy-tamoxifen (A) and R5020 (B) as indicated. Symbols, mean ± SE. Error bars not shown if they interfere with display.

A. RU486



B. ICI 168,384

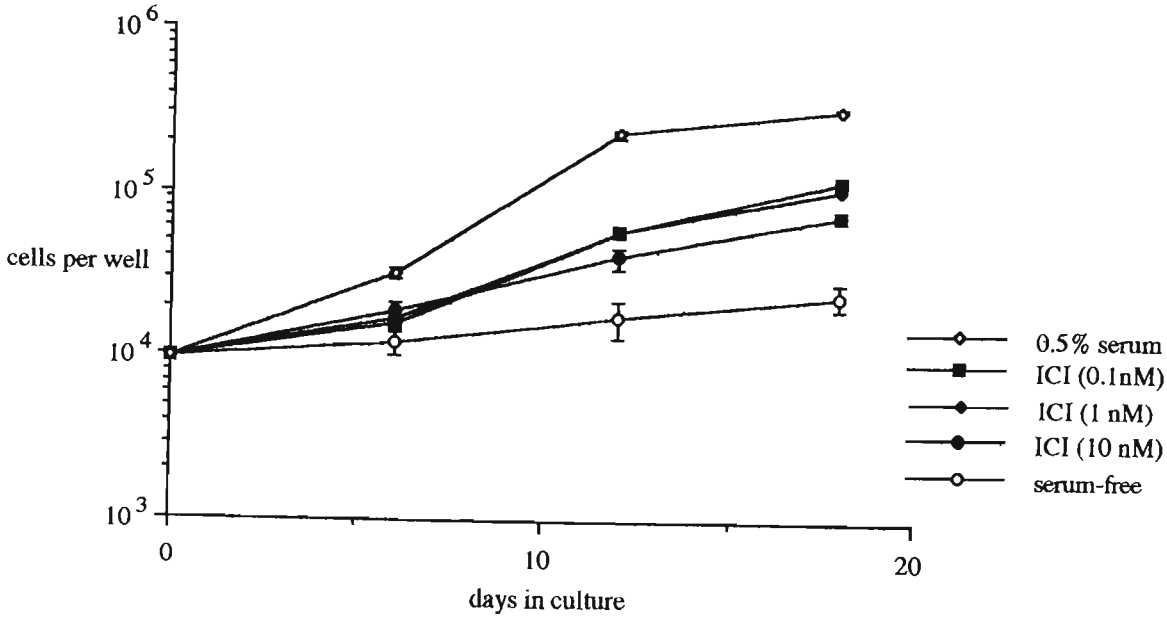


Fig 5.8. Growth of Ishikawa cells in SFM containing different concentrations of RU486 (A) and ICI 168,384 (B) as indicated. Symbols, mean ± SE. Error bars not shown if they interfere with display.

6 DO ENDOMETRIAL CANCER CELL LINES IN CULTURE SECRETE GROWTH FACTORS?

6.1 Introduction

The work described previously in this thesis on the growth of the HEC-1-A and Ishikawa cells in culture suggested that both of these cell lines secreted growth factors which were mitogenic. The evidence for this was the initial lag in growth of the cells in culture followed by vigorous growth of the cells in the absence of serum and the absence of this lag phase with the addition of serum.

The secretion of growth factors which act in an autocrine and paracrine fashion is a common property of transformed cells (Sporn and Roberts, 1985) and a number of growth factors have been identified in the conditioned media from cancer cells in culture. These include TGF-α, TGF-β, PDGF-A and B, CSFs, bombesin and interleukin 2 (reviewed in Goustin *et al.*, 1986). Of these, TGF-α secretion seems to be a common feature of many tumour types and the biological activity of the TGF-α detected in effusions from breast, ovary and lung primaries is indistinguishable (Hanauske *et al.*, 1988). In ovarian carcinomas, some studies have reported that high levels of TGF-α are related to poor prognostic variables (Arteaga *et al.*, 1988; Kohler *et al.*, 1989), while others have shown no association (Owens *et al.*, 1991).

To date the only growth factor reported to be secreted by endometrial cancer cell lines is bFGF (Presta, 1988) but its role in the regulation of growth in these cells has not been determined. Using a biological assay for bFGF activity and Western blot analysis of proteins

immunoprecipitated with antibodies to human placental bFGF, the presence of bFGF-like molecules was detected in extracts from the AN3CA, HEC-1-A, and HEC-1-B endometrial adenocarcinoma cell lines. The synthesis of these molecules was increased by supraphysiological doses of oestradiol and the stimulatory effect of oestradiol was abolished by progesterone. One of the functions of bFGF appears to be the induction of angiogenesis in receptive cells (Gospodarowicz *et al.*, 1987) and it is therefore unlikely that the secreted bFGF acts on the cancer cells directly, rather the vessels supplying them. Its role in endometrial cancer cells is, as yet, unknown.

The lack of information on growth factor secretion from endometrial cancer cells contrasts with the data from breast cancer where it is known that multiple growth factors are produced (reviewed in Dickson *et al.*, 1990). In hormone responsive breast cancer cell lines the secretion of a TGF-α-like growth factor is induced by oestrogens and TGF-α is mitogenic for both normal and malignant breast epithelial cells. Similarly, IGF-like proteins are secreted by all breast cancer cell lines and their production in hormone responsive cell lines is regulated by oestrogen, TGF-α and -β, glucocorticoids and antioestrogens (Dickson *et al.*, 1990). Other growth factors secreted by breast cancer cells *in vitro* include PDGF-like (Bronzert *et al.*, 1987) and FGF-like proteins (Halper and Moses, 1987).

The ubiquitous nature of growth factor secretion by cancer cells in culture and the similarities between endometrial and breast cancers together with the observed growth properties of the cultured Ishikawa and HEC-1-A endometrial cancer cell lines suggested that these cells might be secreting growth factors into their growth medium. For similar reasons it was thought that the most likely candidate would be a TGF-α-like protein and the work outlined below was directed along these lines.

The work described in this chapter involved the large scale culture of HEC-1-A and Ishikawa cells to produce sufficient quantities of serum-free conditioned medium suitable for molecular weight fractionation on a FPLC column. The system for large scale culture on microcarriers using Techne flasks and stirring rods was already in use in the LICR for other epithelial cell lines and it was expected to be suitable for the endometrial cancer cells.

6.2 Materials and Methods

6.2.a Outline

The procedures used in this work are described in detail in Chapter 2. Briefly, a large quantity of serum-free conditioned medium was produced by culturing HEC-1-A and Ishikawa cells on Cytodex 3 microcarriers in Techne stirring flasks. When the cells were growing exponentially the serum concentration was reduced serially at each change of medium. When the concentration of serum in the conditioned medium was less than 0.3%, at each subsequent change of medium the conditioned medium was aspirated, collected and frozen at -70°C. The volume in the Techne flask was maintained by replacing an equal amount of fresh, serum-free medium.

The conditioned medium was concentrated ~25-fold using a Minitan-S Ultrafiltration System (Millipore Corporation, Mass., USA) and buffer exchanged using the same system. The concentrated conditioned medium was then passed through a preparative Superose 12 size exclusion HR 16/50 column (Pharmacia, UK) using the FPLC system (Pharmacia, UK). The

presence of growth stimulatory activity was assayed by measuring the ability of the column fractions to stimulate DNA synthesis in quiescent Swiss 3T3 cells using tritiated thymidine (described in Section 2.7). The presence of either EGF- or PDGF-like growth factors was determined by assaying the fractions for competition with ^{125}I-EGF for binding to Swiss 3T3 cells at 37°C.

The ability of the conditioned medium to stimulate the phosphorylation of tyrosine residues in Ishikawa and HEC-1-A cells was analyzed using antiphosphotyrosine antibodies on Western blots from SDS-PAGE gels.

6.2.b Comparison of microcarriers

Two microcarriers, Cytodex 1 and Cytodex 3, had been used successfully at the LICR in the system described in Section 2.1.b. Cytodex 1 microcarriers consist of a cross-linked dextran matrix with positively charged N,N-diethylaminoethyl groups dispersed through the matrix to facilitate cell attachment and growth. The Cytodex 3 microcarriers are also composed of a cross-linked dextran matrix which is covered with a denatured Type I collagen coat to which the cells attach. In order to assess on which microcarrier growth of the endometrial cells was more prolific, a pilot study comparing the growth on the 2 types of microcarriers was undertaken. Two aliquots of Ishikawa cells (1.1×10^7) in 100 ml of DMEM plus 10% FCS were seeded into two 250 ml Techne flasks, 1 containing Cytodex 1 (0.3 g dry weight) and the other, Cytodex 3 (0.3 g dry weight), prepared as described in Section 2.1.b. The cells were then cultured and the numbers of cells in each flask counted and the medium changed every 3-4 days.

6.2.c Stimulation of tyrosine phoshorylation

The effect of conditioned medium on tyrosine phosphorylation in Swiss 3T3 and NR6$^+$ cells was studied using the methods described in detail in Section 2.5.d. NR6$^+$ cells are a clone of Swiss 3T3 mouse fibroblasts which overexpress the EGFR (Di Fiore *et al.*, 1987b; symbolized by NR6$^+$, obtained from Dr G Panayotou, LICR). Four 10 cm dishes of the 2 cell lines were grown to subconfluence and the cells were starved for 48 h and washed as described in Section 2.5.d.

Conditioned media from HEC-1-A, Ishikawa 1 and Ishikawa 2 cells were obtained by collecting the medium from these cells prior to passaging and frozen at -20°C until required. The media were thawed, warmed to 37°C and 2 ml of each conditioned medium were added to the confluent dishes of 3T3 and NR6$^+$ cells, with control dishes receiving fresh culture medium only. As a positive control for the tyrosine phosphorylation of the EGFR, EGF (200 ng/ml) was added to a confluent dish of NR6$^+$ cells. As a positive control for receptor phosphorylation in response to PDGF, PDGF-BB (200 ng/ml) was added to a dish of Swiss 3T3 cells, which express receptors for both PDGF and EGF.

The medium and additives were left on the cells for 6 min at 37°C before aspirating the medium, washing with PBS (4°C) and stopping receptor phosphorylation by the addition of boiling sample buffer. The cells on the dishes were lysed, prepared, electrophoresed on SDS-PAGE gels, the gels blotted and the blots incubated with antiphosphotyrosine antibodies as described in Section 2.5.d.

This experiment was repeated and widened in scope to look at the effect of concentrated conditioned medium from HEC-1-A and Ishikawa cells on the Swiss 3T3 and NR6$^+$ cells and the HEC-1-A and Ishikawa cells

themselves. The experimental procedure was similar to that just described except that, 0.5 ml of concentrated conditioned medium diluted in 1.5 ml of fresh medium was added to each dish with control dishes receiving fresh medium only.

6.3 Results

The growth of HEC-1-A cells appeared to be slightly better on Cytodex 3 than Cytodex 1 (Fig 6.1.A) although growth on both microcarriers was prolific. Cytodex 3 microcarriers were used in subsequent experiments.

Serum-free conditioned medium was obtained fom both HEC-1-A and Ishikawa cells. HEC-1-A cells were cultured in a 250 ml Techne flask which was maintained in culture for 99 days. At day 19 the residual serum concentration was 0.31% and the conditioned medium was collected from this timepoint and immediately frozen. Ishikawa cells were cultured in a 1 litre Techne flask for 53 days and the conditioned medium collected and frozen from Day 22. The total cell numbers in each flask are shown in Fig 6.1.B.

Three ml of concentrated, buffer-exchanged, conditioned medium (protein concentration 3.5 mg/ml) from the Ishikawa cells were passed through the Superose 12 size exclusion column. The run-off profile from the column (A_{280}, i.e., absorbance at 280 nm) is shown in Fig 6.2.A. The run-off profiles for molecular weight standards are shown for comparison in Fig 6.2.B.

The ability of the column fractions to stimulate DNA synthesis in Swiss 3T3 cells in the presence and absence of insulin (1 µg/ml) is shown in Fig 6.3 using 10 µl (Fig 6.3.A) and 40 µl (Fig 6.3.B) of each fraction.

Overall the results are inconclusive although fractions 2-12 inclusive appear to have some stimulatory activity, particularly when 40 µl of each fraction were used (Fig 6.3.B). The expected dose response curve to increasing concentrations of serum was obtained in this experiment (Fig 6.3.C), suggesting that the assay was performed correctly.

Fractions 3-12 were assayed for their ability to compete with ^{125}I-EGF binding to Swiss 3T3 cells. Only fractions 3, 9 and 10 showed inhibition of ^{125}I-EGF binding (Fig 6.3.D). The EGF used as a positive control failed to compete with the labelled growth factor and the lack of biological activity of this batch of EGF was later confirmed in further binding assays.

Because of the uncertain results obtained from the DNA synthesis and ^{125}I-EGF binding assays on the fractions, the FPLC run was repeated. The column run-off profile (A_{280}) and DNA synthesis assay of column fractions (40 µl +/- insulin, 1 µg/ml) are shown in Fig 6.4.A and 6.4.B, respectively. Although the UV profile is identical with that of the first run, the DNA synthesis assay is quite different. The DNA synthesis assay was repeated (data not shown) and similar results obtained.

The effect of conditioned medium from HEC-1-A and Ishikawa cells on tyrosine phosphorylation in the NR6$^+$ and Swiss 3T3 cells is shown in Fig 6.5.A. The only increases in uptake were seen in the positive controls, with increased signal intensity at 170 kD in the NR6$^+$ cells in the presence of EGF and at 180 kD in the Swiss 3T3 cells in the presence of PDGF-BB, corresponding to the EGF and PDGF receptors, respectively. This experiment was repeated on a broader scale to look at the effect of the concentrated conditioned medium on the tyrosine phosphorylation of both HEC-1-A and Ishikawa cells in addition to the NR6$^+$ and Swiss 3T3 cells (Fig 6.5.B). Although the autoradiograph is of poorer quality, once again

the only increases in signal intensity were seen in the positive controls.

6.4 Discussion

The system used in this project for the large scale production of conditioned medium was effective. It yielded adequate volumes for further investigation and, in particular, for concentration via the Minitan-S ultrafiltration system to provide sufficient of the concentrate at the maximum protein content for the FPLC size exclusion column. The principal problem encountered in culture was fungal and bacterial contaminations, the frequency of which was related to the length of time that the cultures were maintained. This necessitated scrupulous attention to culturing technique and it was only after considerable practice and a number of failures that the long term cultures described in this chapter were achieved. At the LICR the establishment and maintenance of cultures using this system is restricted to individuals who have developed sufficiently rigorous culturing practices to consistently sustain long term cultures.

The FPLC size exclusion column used for molecular weight separation appeared to give consistent results in that the UV run-off profiles were identical on both runs.

The DNA synthesis assay using the incorporation of tritiated thymidine into Swiss 3T3 cells was chosen as a nonspecific measure of mitogenic effectiveness. This assay is widely used and gives consistent results. The addition of insulin (1 μg/ml) to the assay is a means of increasing the sensitivity of the assay by allowing for the cooperative effect that insulin has with various growth factors in many sytems (reviewed in Rozengurt, 1986). The reliability of the assay was confirmed by the dose-

response curve obtained with increasing concentrations of serum (Fig 6.3.C).

The major problem encountered in this study was the inability to obtain convincing and reproducible evidence of biological activity in the run-off fractions using this assay. There was a suggestion of some activity in fractions 3-12 on the first run but the peaks were broad and at a high molecular weight. Although large growth factors are secreted by some cells, in particular large forms of TGF-α arising through differential proteolytic cleavage and glycosylation (reviewed in Derynck, 1988), these results made it less likely that a growth factor was present in the conditioned medium from Ishikawa cells. The data from the competition with ^{125}I-EGF binding to Swiss 3T3 cells suggested the possibility of some either EGF- or PDGF-like activity in Fractions 3, 9 and 10.

Because of the inconclusive nature of the DNA synthesis assay data, a further aliquot of concentrated, conditioned medium from the same cells was run through the size exclusion column. This yielded an identical UV profile of protein content in the run-off fractions to the first run but a quite dissimilar curve from the DNA synthesis assay (Fig 6.4). In view of this, and the results obtained from experiments looking at the tyrosine phosphorylation in response to conditioned medium which were being run concurrently (see below), this work was not pursued any further.

The conditioned medium from the HEC-1-A and Ishikawa cells had no effect on tyrosine phosphorylation in either cell type or the positive controls, Swiss 3T3 and NR6$^+$ cells. The integrity of the experimental method was confirmed by the increased phosphorylation of tyrosine in the PDGFR in the Swiss 3T3 cells and the EGFR in the NR6$^+$ cells in response to PDGF and EGF, respectively. The lack of effect of the conditioned medium on the cell lines used as positive controls indicates that the medium

does not contain PDGF-like, EGF-like or TGF-α-like proteins, at least in a biologically active form. A similar absence of a response in the HEC-1-A and Ishikawa cells to the concentrated conditioned medium suggests that these cells do not secrete growth factors for which the cells also express tyrosine kinase containing receptors. Receptors of this type include the FGF family of receptors, meaning that, if these cells do secrete bFGF (see Section 6.1), then the secreted bFGF probably does not have an autocrine or paracrine action in these cells.

Other explanations for the lack of effect of the conditioned medium on tyrosine phosphorylation are that any growth factor present was inactive or that it was present in insufficient quantity to allow detection by the methods used. Growth factor may have been secreted in an inactive form and require specific conditions or structural modification to be activated. This phenomenon has been described for TGF-β which is secreted in a latent form that must be activated by acidification (*in vitro*) before it can interact with its receptor (Lawrence *et al.*, 1985). The evidence of some stimulation of DNA synthesis by the higher molecular weight column fractions would be consistent with the production of a large molecular weight form of growth factor which required further enzymatic processing, analogous to the proteolysis of the EGF and TGF-α precursors (Gray *et al.*, 1983; Derynck *et al.*, 1984, respectively).

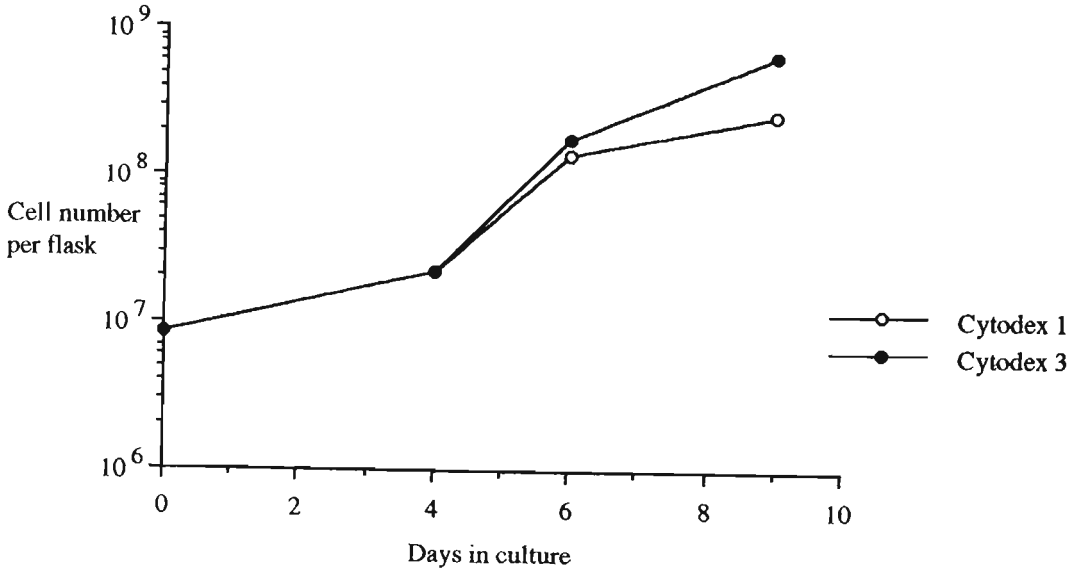
Alternatively, the experimental conditions under which the medium was prepared may have led to inactivation of any growth factor present. This is a potential problem in the isolation of any protein and this was not investigated further.

It is unlikely that growth factor was present in insufficient quantity to allow detection as the medium was concentrated 25-fold making it unlikely that any growth factor in the medium which had a biological effect

in cell culture would not be active in the concentrated medium. This assumes, however, that the growth factor was secreted, while there is a precedent for biologically active TGF-α precursors being expressed on the cell surface and acting in a paracrine manner but not being secreted (Wong *et al.*, 1989; Brachmann *et al.*, 1989). Membrane-bound growth factor would then be biologically active in a cell culture system but not present in the medium from the cultured cells.

The conditioned medium from the HEC-1-A cells was not prepared and separated on the FPLC column. The results of the experiments looking at tyrosine phosphorylation in response to conditioned medium suggested that there was no secretion of an active growth factor for which the cells expressed a protein tyrosine kinase receptor and the decision was made not to proceed any further with this line of investigation.

A.



B.

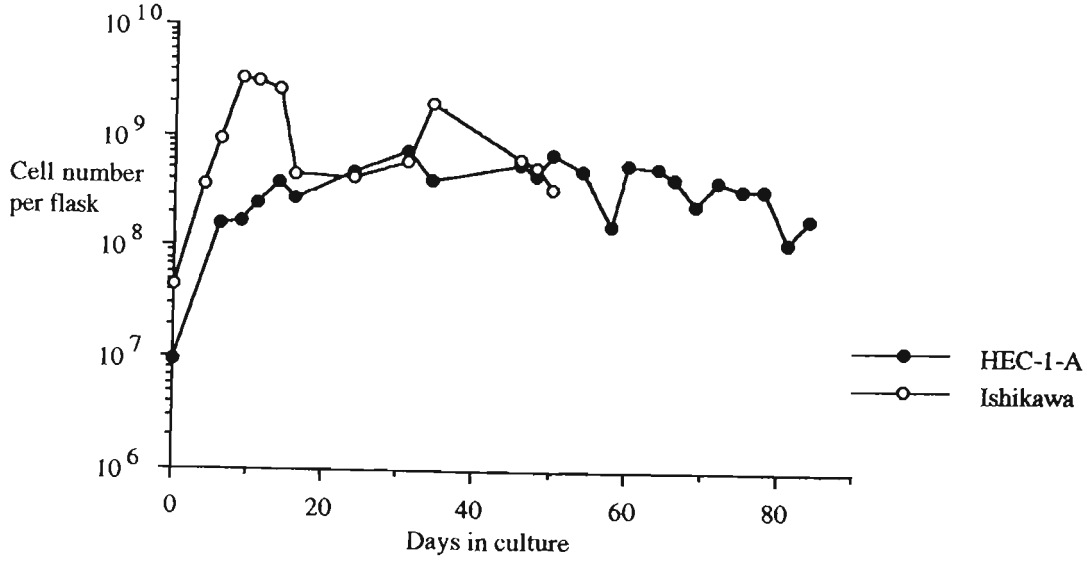
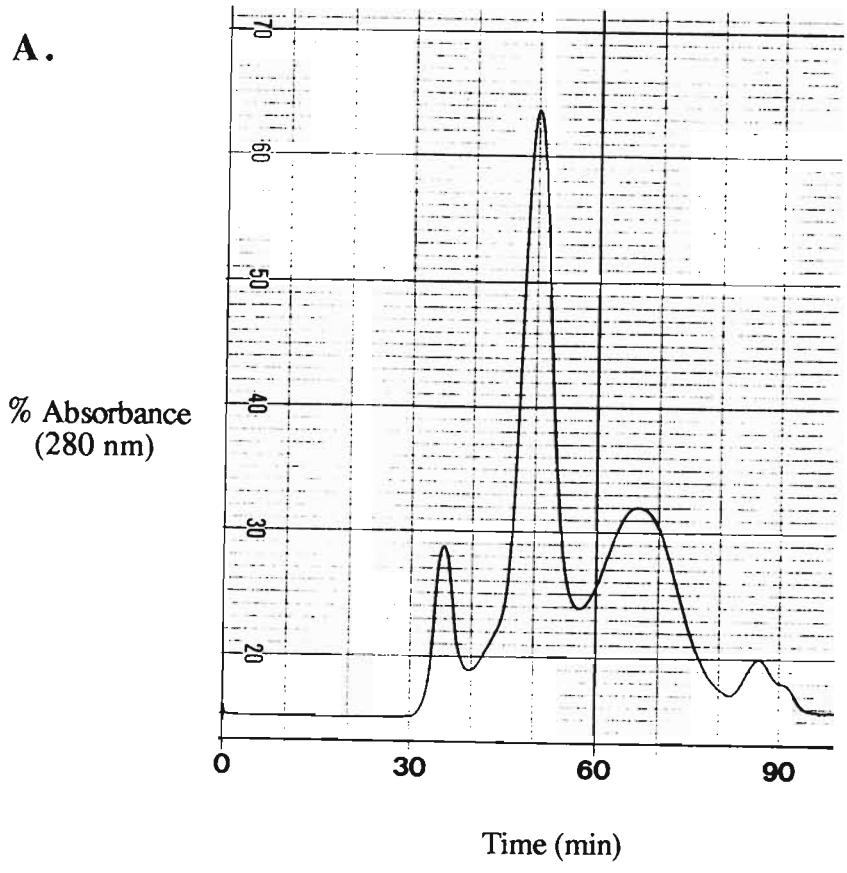


Fig 6.1 Growth of cells on Cytodex beads in Techne stirring flasks. A. HEC-1-A cells on Cytodex 1 (open symbols) and Cytodex 3 (closed symbols). B. HEC-1-A (closed symbols) and Ishikawa (open symbols) cell numbers in long term culture.

A.



B.

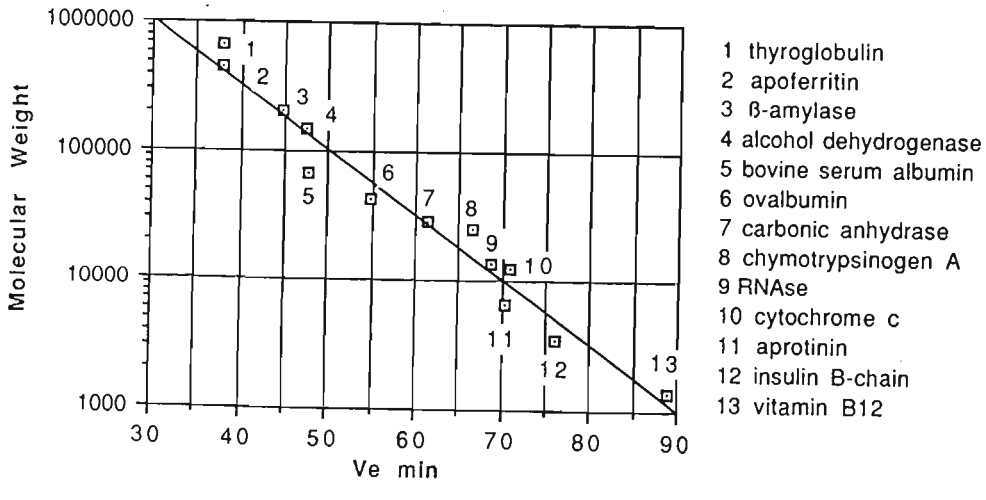
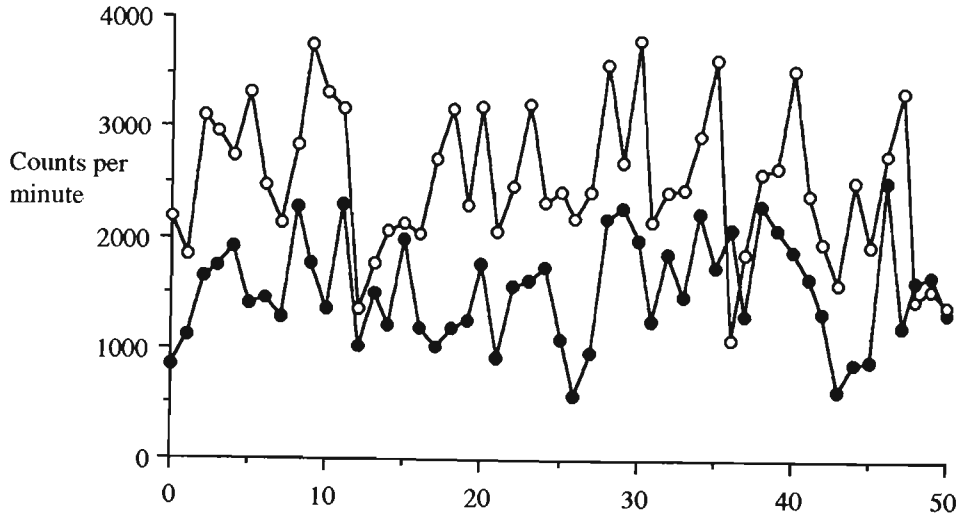


Fig 6.2 Superose 12 column. A. Profile of UV absorbance at 280 nm of run-off from first run of concentrated, conditioned medium from Ishikawa cells.
B. Standardization curve showing run-off times of proteins of known molecular weight.

A. 10 microlitres



Conditioned medium from Ishikawa cells

Conditioned medium from Ishikawa cells plus insulin (1μg/ml)

B. 40 microlitres

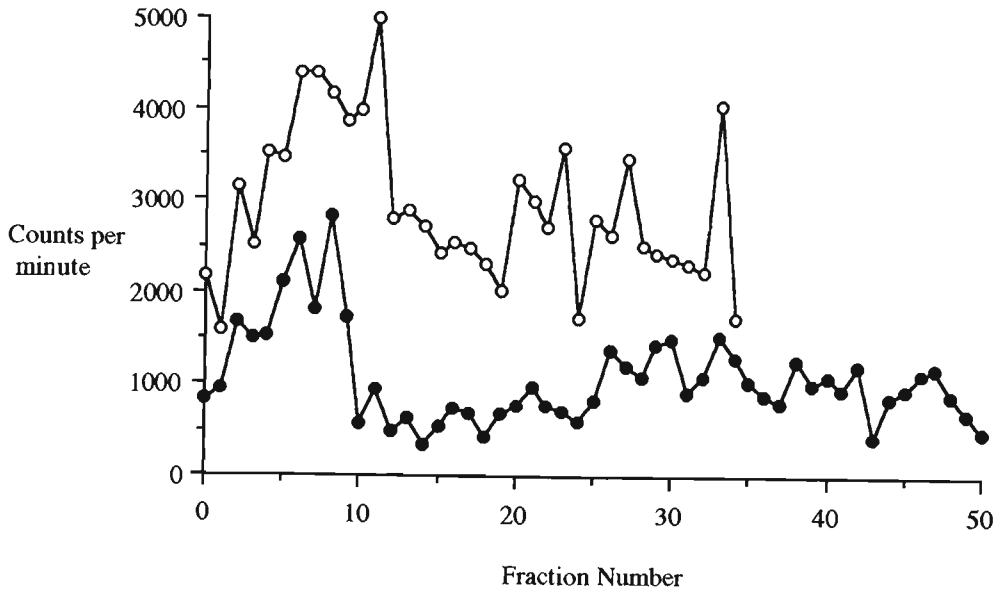


Fig 6.3 Effect of column fractions alone (closed symbols) or with insulin (open symbols) on DNA synthesis using tritiated thymidine uptake into Swiss 3T3 cells. Either 10 (A) or 40 (B) microlitres of each fraction added to duplicate assay wells. Eluate (#s 1-25) and wash through (#s 26-50).

C. Serum control

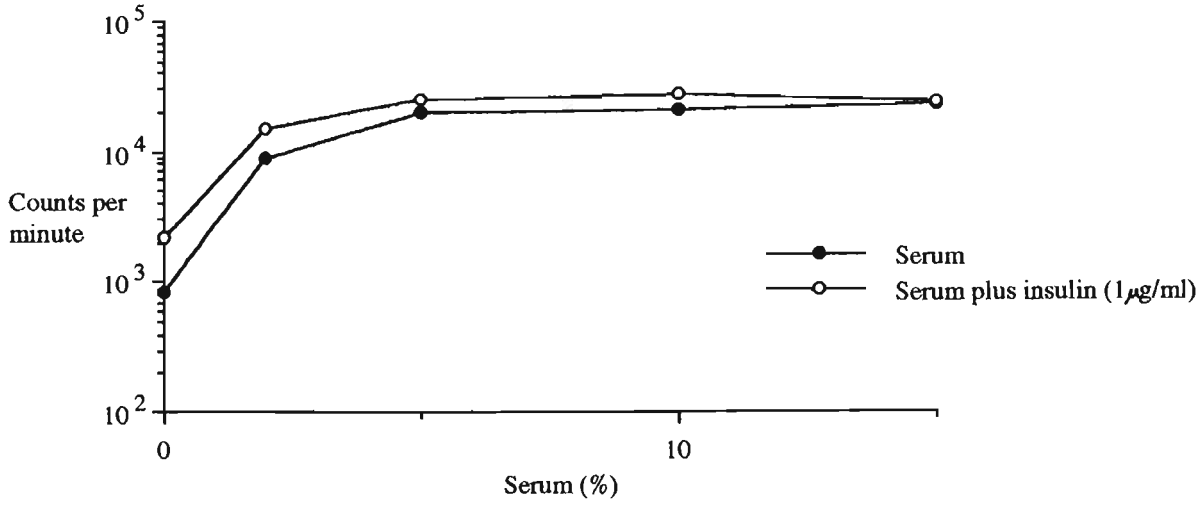


Fig 6.3 C. Effect of increasing serum concentration on DNA synthesis in Swiss 3T3 cells using tritiated thymidine in the presence (open symbols) and absence (closed symbols) of insulin (1 g/ml).

D. EGF binding

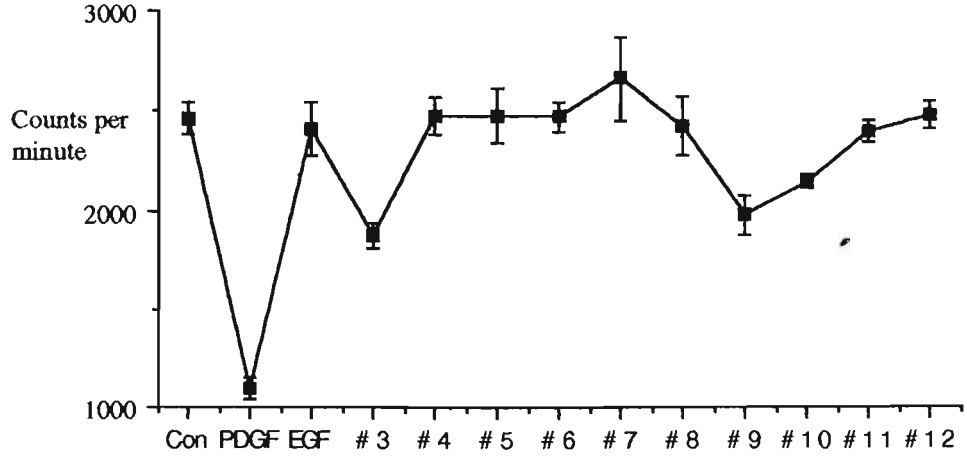


Fig 6.3 D. Competition of column fractions (#3-#12) with 125I-EGF for binding to Swiss 3T3 cells at 37°C. Points are the mean of three values with SEM. Positive controls, PDGF (200 ng/ml) and EGF (100 ng/ml); negative control, PBS alone.

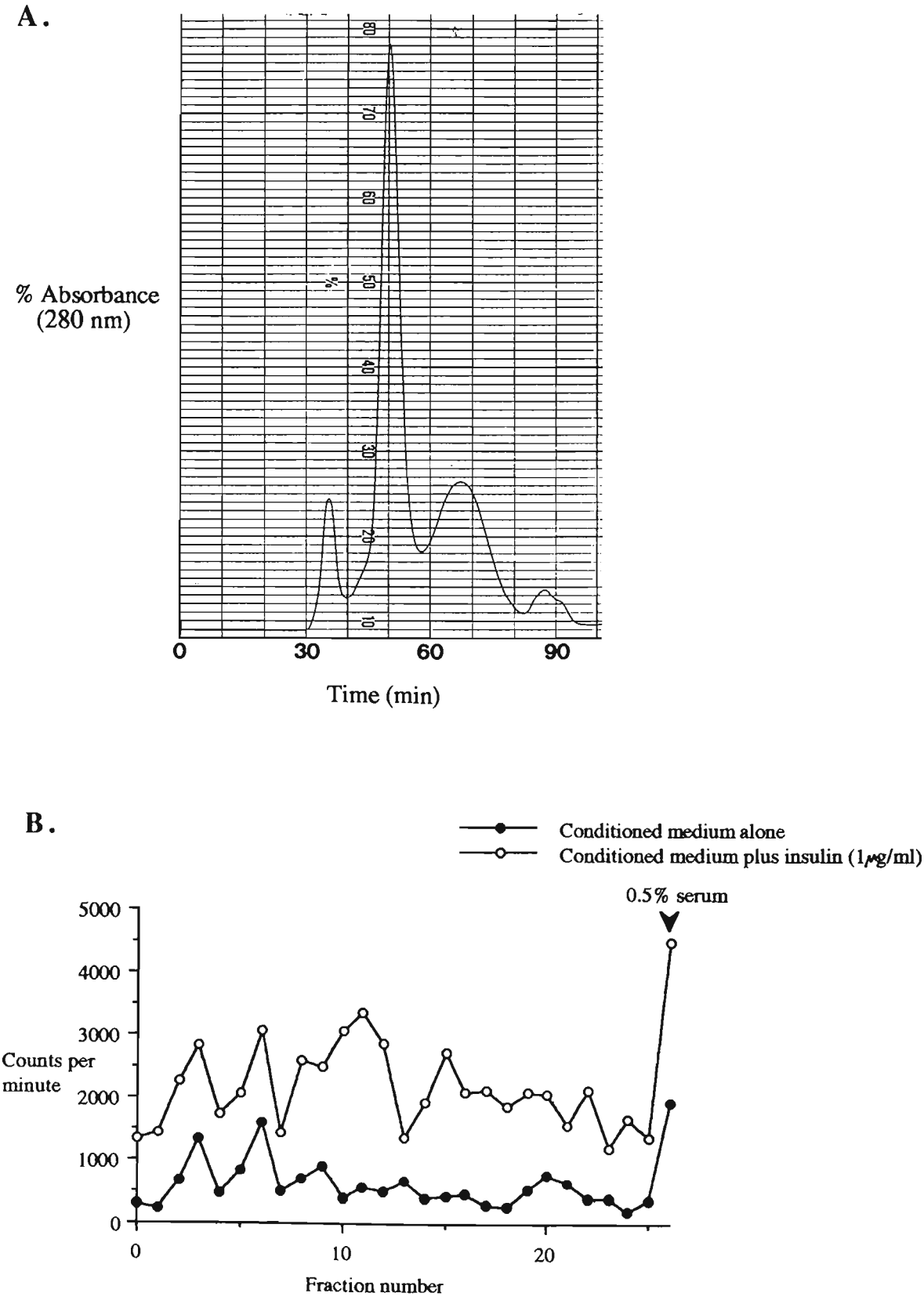
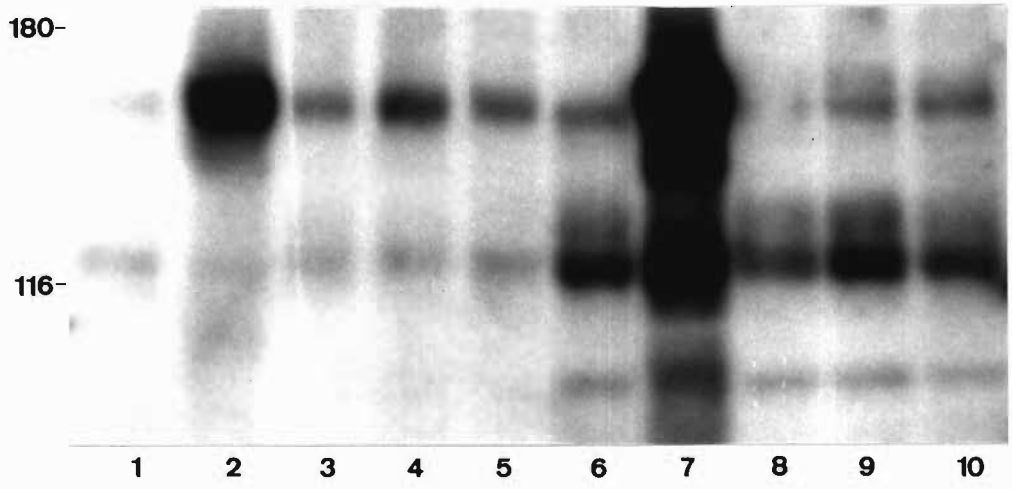


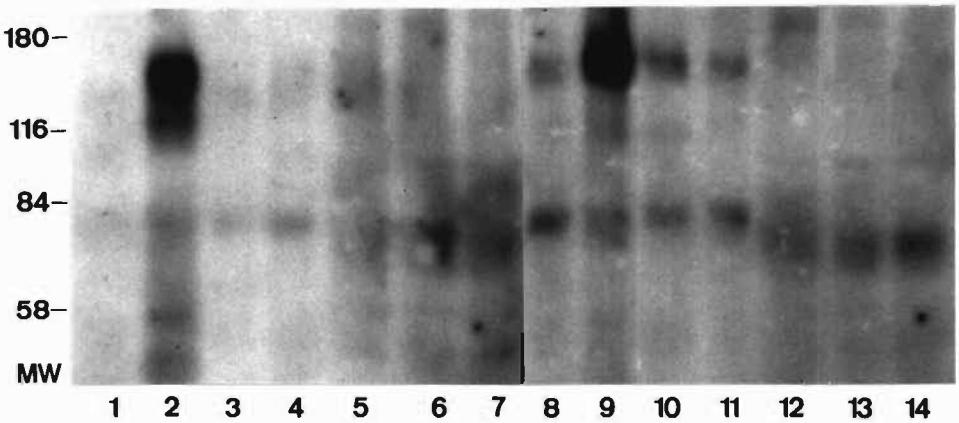
Fig 6.4 Second run of conditioned medium from Ishikawa cells through size exclusion column. Profile of UV absorbance at 280 nm (A) and DNA synthesis assay (B) in the presence (open symbols) and absence (closed symbols) of insulin.

A) Swiss 3T3 and NR6$^+$ cells



Lane	1	Swiss 3T3	6	NR6$^+$
	2	Swiss 3T3 + PDGF (20 ng/ml)	7	NR6$^+$ + EGF (200 ng/ml)
	3	Swiss 3T3 + CM (Ishikawa 1)	8	NR6$^+$ + CM (Ishikawa 1)
	4	Swiss 3T3 + CM (Ishikawa 2)	9	NR6$^+$ + CM (Ishikawa 2)
	5	Swiss 3T3 + CM (HEC-1-A)	10	NR6$^+$ + CM (HEC-1-A)

B) NR6$^+$, HEC-1-A, Swiss 3T3 and Ishikawa cells



Lane	1	NR6$^+$	8	Swiss 3T3
	2	NR6$^+$ + EGF (100 ng/ml)	9	Swiss 3T3 + PDGF-BB (20 ng/ml)
	3	NR6$^+$ + CCM (HEC-1-A)	10	Swiss 3T3 + CCM (HEC-1-A)
	4	NR6$^+$ + CCM (Ishikawa)	11	Swiss 3T3 + CCM (Ishikawa)
	5	HEC-1-A	12	Ishikawa
	6	HEC-1-A + CCM (HEC-1-A)	13	Ishikawa + CCM (HEC-1-A)
	7	HEC-1-A + CCM (Ishikawa)	14	Ishikawa + CCM (Ishikawa)

Fig 6.5 Protein tyrosine kinase activity detected by the use of antiphosphotyrosine antibodies labelled with ^{125}I-Protein-A. A) The effect of conditioned medium (CM) from HEC-1-A, Ishikawa 1 and Ishikawa 2 cells on Swiss 3T3 and NR6$^+$ cells. B) The effect of concentrated conditioned medium (CCM) from HEC-1-A and Ishikawa cells on NR6$^+$, HEC-1-A, Swiss 3T3 and Ishikawa cells.

7 GENERAL DISCUSSION

This thesis has described a series of experiments designed to assess the role of abnormalities in the receptors for EGF and PDGF in the development of endometrial cancer. Until recently, the process of transformation leading to endometrial cancer has been relatively overlooked when compared to the other oestrogen-dependent tumours, breast and ovary. This is probably due to the greater mortality from these latter conditions and the practicalities of collecting suitable endometrial samples for scientific manipulation. While it is relatively easy to obtain a piece of a breast or ovarian tumour with certainty, many early endometrial cancers are of small volume and difficult to distinguish from surrounding normal tissue macroscopically. This leads to failure to remove the appropriate specimen and contamination of tumour sample by normal tissue. These sampling problems are difficult to overcome except in large tumours which are likely to be the more advanced and biologically aggressive tumours. These sampling issues will only be resolved by the use of techniques in which tumour can be visually identified, such as immunohistochemistry or *in situ* hybridization.

While recognizing the problems that exist with sampling endometrial cancers, tumour samples were used to look for abnormalities in the genes for the EGF and PDGF receptors and c-*fms*, the receptor for CSF-1, because of its location on chromosome 5 adjacent to the PDGF-βR (Yarden *et al.*, 1986). In the initial study the integrity of the c-*erb*B-2 gene was also assessed because of the high frequency of amplification of this gene in other adenocarcinomas (see Section 3.2.b).

A major problem encountered in this study was the failure of the histological diagnosis to confirm the presence of carcinoma, the

presumptive diagnosis when the samples were collected. Despite this, 11 of the 28 samples were endometrial cancers and 4 showed varying degrees of hyperplasia. Had any abnormalities been detected, the cases of hyperplasia may have proved useful for comparison. There was no evidence of rearrangement of these genes and only 1 possible example of amplification of c-*erb*B-2. This suggests that neither of these mechanisms are important in the genesis of endometrial cancer.

The lack of amplification of c-*erb*B-2 is at odds with the only report on the c-*erb*B-2 gene in endometrial cancer in which 11 of 16 tumours showed amplification of the gene (Borst *et al.*, 1990). It is consistent however, with the immunohistochemical study of 95 endometrial carcinomas in which increased staining was seen in only 9% of tumours (Berchuck *et al.*, 1991). As the transforming potential of c-*erb*B-2 *in vitro* appears to reside in the increased amount of protein rather than gene amplification per se (e.g. Di Fiore *et al.*, 1987a) and amplification *in vivo* is usually associated with overexpression, at least in breast cancer (Venter *et al.*, 1987), the results of Borst and co-authors (1990) should be viewed with some caution.

In the second part of this work genomic DNA from the endometrial cancer cell lines was included and the PDGFR and c-*fms* genes assessed. Although amplification and/or rearrangement of these genes have not been reported in cancers it was felt to be important to exclude these genetic abnormalities before embarking on the remaining experiments using these cell lines. The lack of amplification or rearrangement of these genes was therefore expected but the presence of RFLPs was not anticipated. Although the additional banding seen on initial examination of the autoradiographs of DNA probed with cDNAs for the PDGF-αR and c-*fms* genes immediately suggested rearrangement, closer inspection failed to

support this conclusion. The presence of similar banding patterns in several tumour samples and identical patterns in normal endometrium suggested the presence of RFLPs as an explanation. This was confirmed by probing 7 samples of normal endometrium for c-*fms* and getting additional banding in 4 of the 7 samples, similar although not identical to the extra bands seen in the tumour samples.

At this point in the study a decision was made to use established epithelial endometrial carcinoma cell lines for the remaining work looking at the receptor from the protein level. An alternative would have been to use fresh tumour explants to look at growth factor binding and receptor activation, growth requirements in culture and the production of growth factors in the conditioned media. However fresh tumour samples were not readily available and their use raises other problems such as the introduction of potentially infected human tissue into the laboratory, whether the cells that grow from explants are representative of the tumour cells, what effect the culturing process has on the properties of the tumour cells and the usually limited time that tumour cells will grow in culutre. For these reasons the cell lines were used and HEC-1-A and Ishikawa cells were chosen as examples of oestrogen receptor negative and positive cell lines, respectively.

Both Ishikawa and HEC-1-A bind EGF with appropriate affinities (K_Ds 1.49 nM and 1.39 nM, respectively) and receptor numbers per cell (69,300 and 48,300, respectively). Binding of EGF leads to activation of the EGFR tyrosine kinase which is of normal molecular weight. Signal transduction was not investigated further than this. Apart from EGF, no effect on either the binding of EGF or activation of the kinase was shown by any of the growth factors or steroids added to the cells. This suggests that there is no transmodulation of the EGFR in these cells.

Neither the A or B isoforms of PDGF activate the PDGFR tyrosine kinase in these cells suggesting that they lack both PDGF-αR and PDGF-βR. Although the binding of PDGF was not formally measured, the absence of kinase activation and the improbability of epithelial cell lines expressing these receptors made this redundant.

It proved to be very difficult to obtain consistent growth patterns for both these cell lines in culture. The major problem was that the cells grew so rapidly, even in the absence of serum, that it was difficult to demonstrate what would have been relatively small differences with different mitogens. In addition there appeared to be inconsistency between experiments in the growth of the individual cell lines. This was most marked in the Ishikawa cells where no reproducible effect of oestrogen was seen. On some occasions there was a suggestion of a dose response to oestrogen while in other experiments there was no effect at all. Despite the use of a new stock of Ishikawa cells and many minor modifications of culturing conditions in different experiments no consistent effect was demonstrated. Even complete replication of all aspects of culturing these cells from the laboratory which had reported a response to oestrogen (Croxtall *et al.*, 1990) again failed to elicit this mitogenic effect. The only sensible explanation for the different results is that there were culturing differences between laboratories however we were unable to discover what these were.

These problems severely hampered the usefulness of Ishikawa cells as a model for a hormone responsive cell line. The only agent which was consistently found to inhibit the growth of the cell line was TGF-β_1. However the laboratory above have recently published data showing that TGF-β_1 stimulated the growth of both Ishikawa and T47-D breast cancer cells in culture (Croxtall *et al.*, 1992). They were unable to detect any

TGF-β in the conditioned medium from the cells so it is unlikely that the TGF-β is acting in an autocrine manner. These workers have also demonstrated that Ishikawa cells in their system are stimulated by 4-hydroxytamoxifen and inhibited by progesterone (Croxtall *et al.*, 1990) while we were unable to influence growth with any of the steroids used.

The growth characteristics of the endometrial cancer cell lines in culture suggested that they were secreting a growth factor or mitogen of some form into the medium. It was therefore logical to proceed to try and identify the potential factor(s) and it was thought that a likely candidate was TGF-α based on the evidence from breast cancer cells. The large scale culture technique chosen was already in use at the laboratory and large quantities of conditioned medium were obtained using this system. The concentration by Mintan-S was quick and effective and the column separation reproducible. However the assays used to look at the column fractions failed to show activity that would have been consistent with a mitogen. There are a number of possible reasons for this: the Swiss 3T3 cells may not have been stimulated by the mitogen present and an alternative cell lineage was necessary, the mitogen was not EGF, TGF-α or PDGF and therefore would not compete with ^{125}I-EGF binding to Swiss 3T3 cells, that the mitogen had been degraded during the preparative stages, or that there was no mitogen there to find. Because of employment constraints it was not possible to explore these alternatives further.

After completing this work the most promising avenues for further research would appear to be continuing the work on growth regulation of endometrial cancer cell lines assuming that the culture problems could be sorted out. Work in many other cell types has shown that growth factors are of crucial importance in the regulation of growth at the cellular level and the interaction with hormones is complex and dependent on many

variables. The other area in endometrial cancer which is going to be of clinical relevance is the expression of proto-oncogenes, tumour suppressor and other cell regulatory genes. In order to make this clinically useful in terms of prognostic information microscopic techniques will have to be employed as mentioned above. Which of these proteins are going to be of most use as prognostic markers will be determined by screening a large number of tumours for a large number of potentiall important proteins such as c-*erb*B-2, p53, and P-glycoprotein (the product of the multidrug resistance gene) and relating their expression to patient disease progression and survival data.

As stated at the beginning of this thesis, one of the major objectives was to obtain experience in, and understanding of, scientific techniques. A broad experience has been gained in a wide range of disciplines including molecular biology, protein chemistry and cell biology thereby achieving this objective. Although this experience is necessarily superficial, it will hopefully enable me to ask the right questions in the future - of my own research and of others.

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