HORMONES AND GROWTH FACTORS IN OVARIAN CANCER CELLS

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FOR MUM AND DAD

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DECLARATION

In accordance with the regulations of the University, I declare that this thesis has been composed by myself entirely, and that the work presented is my own, except where acknowledgement has been indicated in the text.

A. JAYNE CREW

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Abstract

Ovarian cancer is the most common cause of death among gynaecological malignancies in women in the UK. Despite this, the mechanisms which control the growth are unclear. However, the majority of epithelial ovarian cancers express oestrogen receptors (ER), which suggests that oestrogen may be involved with the growth control. In addition a proportion of ovarian tumours express EGF receptors. The majority also contain TGF-α and a smaller number produce EGF. It is therefore possible that EGF and TGF-α are autocrine or paracrine growth regulators in ovarian cancer. Using three serous ovarian adenocarcinoma cell lines, PEO1, PEO4 and PEO14 as model systems, the aim of these studies has been to clarify the role of oestrogen, EGF and TGF-α in ovarian cancer.

The PEO1 and PEO4 cell lines express moderate-high levels of oestrogen receptor, whereas the PEO14 cell line is ER-negative. The growth of the ER-positive cell lines was stimulated by the exogenous addition of 17 β-oestradiol, maximally at concentrations between 10^{-10} and 10^{-8} M, whereas oestrogen produced negligible effects in the ER-negative cell line, PEO14. Thus, the sensitivity to 17 β-oestradiol correlated with the expression of the ER. The 17 β-oestradiol-stimulation of PEO1 and PEO4 cell lines was blocked by incubation with tamoxifen or the "pure" anti-oestrogen ICI 164,384. Incubation with tamoxifen alone produced growth stimulatory effects at concentrations between 10^{-12} and 10^{-8} M in PEO1 and PEO4 cell lines. Similarly incubation with ICI 164,384 produced

growth stimulation in these cells, although at concentrations between 10^{-12} and 10^{-10} M in the PEO1 cell line and 10^{-12} and 10^{-7} M in PEO4 cells. Neither anti-oestrogen produced growth stimulatory effects in the ER-negative cell line, PEO14. In all three cell lines, incubation with 17 β-oestradiol, tamoxifen or ICI 164,384 at concentrations greater than 10^{-6} M produced growth inhibitory effects.

Incubation with EGF and TGF-α produced growth stimulation in the three cell lines. These effects were associated with decreases in the percentages of cells in the G0/G1 phase of the cell cycle and increases in the proportions of cells in the S and G2/M phases. EGF receptors were detected on the three cell lines using immunohistochemical techniques and ligand binding. Using a radioimmunoassay, the presence of EGF-like material was found in the conditioned media of PEO1, PEO4 and PEO14 cells. Preliminary data showed that the basal rate of growth of PEO14 cells can be reduced by incubation with anti-EGF antiserum and the rate of growth of PEO1 cells can be reduced by the presence of anti-TGF-α antiserum suggesting that a growth regulation pathway for EGF/TGF-α may exist in the cell lines.

Factors modulating the expression of EGF receptors have been investigated. EGF produced a rapid decrease in the EGF receptor expression in the three cell lines. In the ER-positive PEO4 cell line, incubation with 17 β-oestradiol also decreased the EGF receptor level, although the response was delayed and of smaller magnitude compared to the effects produced by EGF.

If the results from these studies *in vitro* are reflected *in vivo* it is possible that oestrogens play a role in the growth control of some ovarian tumours, which may therefore be responsive to anti-oestrogen therapy. In addition, agents which could interfere with the effects of EGF and TGF-α on growth may be of therapeutic potential in ovarian cancer patients.

Abbreviations

aa	- amino acid
ABC	- avidin biotin complex
B_{max}	- maximum binding
B_o	- maximum binding in radioimmunoassay
BSA	- bovine serum albumin
°C	- degrees Centigrade
Ci	- Curie
cisplatin	- cis-diamminedichloroplatinum
cm	- centimetre (s)
CO_2	- carbon dioxide
cpm	- counts per minute
dcs-FCS	- double charcoal stripped foetal calf serum
DAB	- diaminobenzidine tetrahydrochloride
DMSO	- dimethyl sulphoxide
DNA	- deoxyribonucleic acid
E_2	- 17 β-oestradiol
EGF	- epidermal growth factor
ER	- oestrogen receptor
ERE	- oestrogen response element
FCS	- foetal calf serum
FITC	- fluorescein isothiocyanate
FSH	- follicle stimulating hormone
g	- gram (s) /centrigugal field
H_2O	- water

HCl	- hydrochloric acid
HITS	- hydrocortisone, insulin, transferrin, sodium selenite
ICRF	- Imperial Cancer Research Fund
IGF-1	- insulin-like growth factor type 1
IU	- international unit (s)
kb	- kilobase (s)
Kd	- dissociation constant
kDa	- kilodalton (s)
LH	- luteinizing hormone
LHRH	- luteinizing hormone releasing hormone
µg	- microgram (s)
µl	- microlitre (s)
µm	- micron (s)
MBq	- mega bequerel
MeOH	- methanol
mg	- milligram (s)
min	- minute (s)
ml	- millilitre (s)
mRNA	- messenger ribonucleic acid
NaCl	- sodium chloride
NaOH	- sodium hydroxide
NGF	- nerve growth factor
OD	- optical density
%	- percentage
PBS	- phosphate buffered saline

PDGF	- platelet-derived growth factor
PEG	- polyethylene glycol
PgR	- progesterone receptor
PKC	- protein kinase C
RIA	- radioimmunoassay
rpm	- revolutions per minute
RPMI 1640	- Roswell Park Memorial Institute 1640
RRA	- radio receptor assay
S.E.	- standard error (s)
sec	- second (s)
TAF	- transcriptional activation function
TB	- tris buffer
TBq	- tera bequerel
TBS	- tris buffered saline
TCA	- trichloroacetic acid
TGF-α	- transforming growth factor-alpha
TGF-β	- transforming growth factor-beta
w	- weight
WHO	- World Health Organisation
v	- volume

CHAPTER 1

INTRODUCTION

1.1 Ovarian cancer

1.11 Incidence and staging

Cancer of the ovary is currently the most common gynaecological malignancy among women in the UK (MRC Gynaecological Cancer Working Party, 1990). Whilst the development of methods of detection for other gynaecological cancers, such as cervical screening, has contributed to a decrease in mortality rates for these malignancies, the death rate for ovarian cancer has doubled over the last 70 years in England and Wales (Beral, 1987). At present, approximately 5000 patients are treated for ovarian cancer every year in the UK, of which 85% die as a result of the disease. Ovarian cancer is therefore the leading cause of death for gynaecologic malignancies (Greene *et al*, 1984). Although ovarian cancer can occur at any age, the majority of ovarian cancers are detected in post-menopausal patients (Stadel, 1975) with a critical period around the age of 45, when the incidence increases dramatically.

The survival of patients is associated with tumour stage, which is determined by the extent of the spread of the disease reported at laporotomy (Friedlander and Dembo, 1991). The definitions of the various stages in ovarian cancer are shown in table 1.1. Patients with stage I disease have a 5 year survival rate of 80-90% and patients with stage II disease have a 40-60% 5 year survival rate. Patients with stage III and IV have a poorer outcome, with 5 year survival rates of 10-15% for stage III and <5% for stage IV. In the early stages of ovarian cancer, the absence of clinical symptoms and the lack of adequate screening techniques means that the majority of

patients at the time of diagnosis have advanced disease, and subsequently the success in the treatment of ovarian cancer is limited.

Table 1.1. Cancer committee of FIGO staging for carcinoma of the ovary, taken from Friedlander and Dembo (1991).

Stage I	Growth limited to the ovaries.
Stage Ia	Growth limited to one ovary; no ascites. No tumour on the external surface; capsule intact.
Stage Ib	Growth limited to both ovaries; no ascites. No tumour on the external surfaces; capsules intact.
Stage Ic	Tumour either Stage Ia or Ib, but with tumour on surface of one or both ovaries; or with capsule ruptured; or with ascites present containing malignant cells or with positive peritoneal washings.
Stage II	Growth involving one or both ovaries with pelvic extension.
Stage IIa	Extension and/or metastases to the uterus and/or tubes.
Stage IIb	Extension to other pelvic tissues.
Stage IIc	Tumour either Stage IIa or IIb, but with tumour on surface of one or both ovaries; or with capsule (s) ruptured; or with ascites present containing malignant cells or with positive peritoneal washings.
Stage III	Tumour involving one or both ovaries with peritoneal implants outside the pelvis and/or positive retroperitoneal or inguinal nodes. Superficial liver metastasis equals Stage III. Tumour is limited to the true pelvis but with histologically proven malignant extension to small bowel or omentum.
Stage IIIa	Tumour grossly limited to the true pelvis with negative nodes but with histologically confirmed microscopic seeding of the abdominal peritoneal surfaces.
Stage IIIb	Tumour involving one or both ovaries with histologically confirmed implants of abdominal peritoneal surfaces none exceeding 2 cm in diameter. Nodes are negative.
Stage IIIc	Abdominal implants greater than 2 cm in diameter and/or positive retroperitoneal or inguinal nodes.
Stage IV	Growth involving one or both ovaries with distant metastases. If pleural effusion is present there must be positive cytology to allocate a case to Stage IV. Parenchymal liver metastasis equals Stage IV.

1.12 Classification

Many forms of ovarian cancer exist. In 1973 the World Health Organisation (WHO) published a classification of tumours, based on their histology (Serov and Scully, 1973). A summary of this classification is shown in table 1.2.

Table 1.2. Summary of the classification of malignant ovarian tumours, according to WHO (Serov and Scully, 1973).

I	Common "epithelial" tumours
	A. Serous tumours
	B. Mucinous tumours
	C.Endometrioid tumours
	D. Clear cell tumours
	E. Brenner tumours
	F. Mixed epithelial tumours
	G. Undifferentiated carcinoma
	H. Unclassified tumours
II	Sex cord stromal tumours
III	Lipoid cell tumours
IV	Germ cell tumours
V	Gonadoblastoma
VI	Soft tissue tumours not specific to the ovary
VII	Unclassified tumours
VIII	Secondary (metastatic) tumours
IX	Tumour-like conditions

In general, ovarian neoplasms originate from the surface epithelium, the germ cells or the stroma of the ovary (Serov and Scully, 1973). Approximately 90% are common epithelial carcinomas which are derived from the surface epithelium (Weiss *et al*, 1977). This is the adult equivalent of the mesothelium of the embryonic ovary. The histological appearances of the various types reflects the close relationship with the Mullerian epithelium. Therefore, the differentiated epithelium of serous tumours resembles that of the Fallopian tube; mucinous tumours the endocervical epithelium and endometrioid and clear cell tumours the endometrium. Serous cystadenocarcinomas are the most common form of ovarian epithelial tumours representing 40 to 68% (Slotman and Rao, 1988) while the mucinous type of tumours account for 3 to 21% and endometrioid tumours for 5 to 20%. Clear cell type tumours constitute 5 to 10% of epithelial ovarian neoplasms. Other forms of epithelial tumours are rare.

1.13 Epidemiology

An understanding of the epidemiology and aetiology of ovarian cancer may lead to the development of earlier diagnosis, better treatments or prevention of the disease. Several studies suggest that environmental, genetic and hormonal factors may be of importance.

(i) Environmental factors

No single environmental factor has been proven to contribute to an increased risk for the development of ovarian cancer. Evidence supporting the role of environmental factors in the development of ovarian malignancies

is based primarily on population studies. The incidence of ovarian cancer is higher in industrialised countries with the exception of Japan. However, Japanese women who have emigrated to countries associated with a higher risk, show an increased incidence of disease (Lingeman, 1974). It has been suggested that dietary factors and chemical carcinogens contribute to this increased risk, although some controversy exists over the results.

An increased risk of ovarian cancer has been reported to be associated with an increased intake of animal fat (Cramer *et al*, 1984; Rose and Bayar, 1986) although this is controversial (Byers *et al*, 1983). More recently, it has been suggested that the increased intake of galactose from lactose in milk products, and not fat intake, contributes to the increased risk (Cramer, 1989; Cramer *et al*, 1989). Specific dietary factors have also been proposed to decrease the risk of ovarian cancer. Among these, consumption of carrots (Engle *et al*, 1991) and a general high intake of β-carotene (Slattery *et al*, 1989) were found to decrease risk.

No link has been established between the incidence of cancer of the ovary and smoking (Cramer *et al*, 1984), or the consumption of coffee (Cramer and Welch, 1983) or alcohol (Byers *et al*, 1983).

Asbestos and talc are the two main industrial chemicals which have been proposed to be involved in the causation of ovarian cancer. Until recently, most talc powders contained asbestos and as a result, have been implicated in ovarian carcinogenesis (Longo and Young, 1979). A modest risk of 1.9

has been reported with the use of talc (Cramer *et al*, 1982), although others have been unable to confirm this (Whitemore *et al*, 1988).

There is no evidence that viral infections of the ovary contribute to the development of cancer. Attempts to culture viruses from ovarian tumour cells of humans and other animals have failed (Lingeman, 1974). However, there are reports which show an association between mumps (Cramer *et al*, 1983) and rubella virus infections (McGowan *et al*, 1979) and an increased risk of ovarian cancer.

(ii) Genetic factors

The existence of familial ovarian cancer has been recognised (Lynch *et al,* 1985; 1986; Piver *et al*, 1984). Two conditions have been identified in association with familial ovarian cancer. These are site-specific ovarian cancer, which is the more common form, and breast-ovarian cancer syndrome which shows clustering of ovarian and breast cancer cases in families (Lynch *et al*, 1978). Site-specific ovarian cancer is thought to be inherited as an autosomal, dominant pattern with high penetrance. Therefore, up to 50% of first degree relatives may inherit the gene and have a high risk of developing ovarian cancer.

In addition, several genetic syndromes have been associated with an increased risk of non-epithelial ovarian tumours. These include gonadal dysgenesis which shows an increased risk of dysgerminoma (Troche and Hernandez, 1986), Peutz Jeghers syndrome, which increases the patients

risk of developing granulosa cell tumour and cystadenoma (Dozois *et al*, 1970) and basal cell nevus syndrome, with an increased incidence of fibroma (Berlin *et al*, 1966).

The specific genetic abnormality that is involved in the development of ovarian cancer has not been identified. However, activation of several proto-oncogenes, such as K-*ras*, H-*ras*, c-*myc* and HER-2/*neu*, has been reported in ovarian cancer, (Piver *et al*, 1991). The biological significance of this finding and the role which it plays in ovarian cancer is not known. In this respect, it is of interest that overexpression of the HER-2/*erb*B-2 gene in ovarian cancer has been associated with a poor prognosis (Slamon *et al*, 1989; Berchuck *et al*, 1990a).

An increased incidence of ovarian cancer has been reported by one group to be associated with blood group A (Bjorkholm, 1984) but the actual difference in blood group frequency although significant, was very low, and therefore may not be meaningful.

(iii) Hormonal factors

Epidemiologic studies suggest that hormonal factors play a role in ovarian cancer. An increased incidence of the disease has been noted in nulliparous women (Cramer *et al*; 1983, Joly *et al*, 1974) and in women with an early menarche or late menopause. A degree of protection against ovarian cancer has been noted with use of oral contraceptives (Cramer *et al*, 1982), early age at first pregnancy and increasing parity (Kvale *et al*, 1989).

Two theories have been put forward to explain these associations (reviewed in Piver *et al*, 1991).

Firstly, the "excess gonadotrophin secretion theory" postulates that ovarian cancer is due to continuous high levels of gonadotrophin acting on the ovaries, caused by ovarian failure or a block in the regulatory ovarian/pituitary feedback pathway which limits the secretion of gonadotrophin (Gardner, 1958). The dramatic increase in the incidence of ovarian cancer around the age of menopause correlates with the period of life at which the level of gonadotrophins increases (Stadel, 1975) which therefore lends support to the theory. During the post-menopausal years, levels of androstenedione, a precursor of oestrogen, increase and may therefore also contribute to the increased incidence of ovarian cancer around the age of 45.

Secondly, the "incessant ovulation theory" suggests that trauma of the ovary caused by continuous ovulation, leads to the development of ovarian malignancies. Therefore, risk is correlated with the number of ovulations (Fathala 1971). It is of interest that the factors which are associated with a decreased risk of ovarian cancer (pregnancy and oral contraceptive use) suppress ovulation.

Early animal studies showed that chronic administration of oestrogens, progestins and androgens resulted in ovarian cancer (Jabara, 1962; Gardner; 1958; Horning, 1958 and Biskind and Bisking, 1944). It has also

been reported that a higher incidence of endometrioid cancer is noted in women who used oestrogens during menopause (Weiss *et al*, 1982), though other studies have failed to detect an effect on the incidence of ovarian cancer following oestrogen replacement (Cramer and Welch, 1983; Kaufman *et al*, 1989).

1.14 Treatment

A combination of therapies including surgery, chemotherapy, radiotherapy and endocrine therapy all contribute to the attempted treatment and control of ovarian cancer.

(i) Surgery

Surgery performs a variety of functions. Initially, it establishes the diagnosis of ovarian cancer and the extent of the disease which allows accurate staging. For stage I disease, surgery is often the only recommended treatment, and consists of total abdominal hysterectomy, bilateral salpingo-oophorectomy and omenectomy (reviewed by MRC Cancer Working Party,1990). Also, in more advanced stages of the disease, cytoreduction of the tumour is performed in the 30 to 50% of cases where it is achievable (reviewed in Hoskins and Rubin, 1991), which may increase the patients' comfort and decrease the adverse effects of the tumour on her metabolism. Reduction of the tumour size improves the chance that the tumour will respond to adjunctive therapy. There is a survival advantage for patients where maximum removal of tumour bulk is possible, as tumour remaining after operation has been shown to be an important prognostic

factor (Griffith, 1975).

As ovarian cancer progresses, there is often increasing tumour growth in the peritoneal cavity, and most patients will develop intestinal obstructions. Although surgery will offer no therapeutic advantage to these patients, removal of the obstructions may provide palliation.

Surgery may also play a supportive role for other forms of therapy by reducing tumour bulk, and therefore providing easier access for intravenous or intraperitoneal therapy. Since patients with ovarian cancer often do not have measurable disease following initial treatments, the assessment of the response may only be achieved by repeat surgery. In a group of studies reviewed by Slotman and Rao, (1988), 29 to 87% of patients with advanced ovarian cancer who were clinically free of disease, were found to have persistent disease, diagnosed by second-look laporotomy. Thus, surgery will remain an important method of evaluating the efficacy of chemotherapy.

(ii) Chemotherapy

Ovarian cancer was one of the first solid tumours to be treated with systemic cytotoxic chemotherapy, which is now widely accepted as the treatment of choice for patients with advanced disease. However, in early stages of the disease, the role of adjuvant therapy is less clear. For patients with stage Ia or Ib disease the 5-year survival rate is good, and adjuvant therapy with alkylating agents does not significantly improve it (Mackintosh *et al*, 1989). Single alkylating agents such as melphalan, chlorambucil,

cyclophosphamide and thiotepa, which have comparable response rates (Young *et al*, 1974; Tobias and Griffiths, 1975), have been used for over 30 years in the treatment of advanced ovarian cancer. The most active non-alkylating drug in ovarian cancer is cis-diamminedichloroplatinum (cisplatin), with response rates greater than 60% being reported (Hall *et al*, 1981; Wiltshaw *et al*, 1986). Considerable interest is also being shown in an analogue of cisplatin, carboplatin, which is less toxic, but as effective as cisplatin (Adams *et al*, 1989; Mangioni *et al*, 1981).

Recently, there has been a tendency to replace single agent chemotherapy with combinations of cytotoxic agents, often including cisplatin. There is evidence to suggest that combination chemotherapy achieves higher response rates than single agent chemotherapy (Young *et al*, 1978), but it is not clear whether the higher response rate confers a survival advantage on the patients. In a series of 39 trials (reviewed in MRC Working Cancer Working Party, 1990), only 3 reported a statistically significant survival advantage for combination chemotherapy. Combinations of agents with cisplatin produce better response rates than combinations not including this drug (Bruckner *et al*, 1981; Decker *et al*, 1982; Barker and Wiltshaw, 1981).

While response rates to chemotherapy have improved in the last few years with the use of cisplatin, the overall 5-year survival rates of ovarian cancer patients remains low. Therefore, there is a need for more effective drugs, or combinations of drugs, which not only produce better response rates but increase the survival rate.

(iii) Radiotherapy

At present, radiotherapy has a controversial and limited role in the management of ovarian cancer. In order to be of maximum benefit, it is now known that radiotherapy must cover the entire abdominal cavity, due to the pattern of spread of ovarian cancer (Slotman and Rao, 1988). However, the dose must be limited due to the sensitivity of the liver, kidneys and bowels to radiation.

The therapeutic benefit seems to be restricted to patients without or with small residual disease. An improved survival rate has been reported in patients with stage Ib, II and III with small or no macroscopic tumour after surgery, who received abdominopelvic irradiation and chemotherapy compared with chemotherapy alone (Dembo, 1985). In patients with large residual mass of tumour after surgery, cures were rarely obtained with radiotherapy. Thus, in patients with more advanced disease, irradiation provides temporary palliation only.

(iv) Endocrine therapy

The major interest in the role of cytotoxic chemotherapy in the potential control of ovarian cancer may have obscured some of the possible benefits of endocrine therapy. Endocrine therapy has mostly consisted of progestins and/or the synthetic anti-oestrogen, tamoxifen.

In most studies using progestins, such as 17-α-hydroxyprogesterone-17-n-caproate, 17-α-hydroxy-19-norprogesterone-17-n-caproate, 6,17-α-dimethyl-

6-dehydroprogesterone, medroxyprogesterone acetate and megestrol acetate, true objective responses were recorded in only 10-15% of patients (reviewed in Slotman and Rao, 1988), although responses have been as low as zero (Mangioni *et al*, 1981) and as high as 55% (Rendina *et al*, 1982). Progestins have also been used in combination with other agents, such as alkylating agents, with good response rates of 85% (Bergqvist, 1981) and 76% (Guthrie, 1979), but these have not been repeated in prospective, controlled trials in which treatment with chemotherapy plus progestins was compared with chemotherapy alone.

A number of studies have been performed with tamoxifen which have been summarised by Slotman and Rao (1988), but low response rates of less than 10% have been achieved. However, some authors have reported the stabilisation of disease (Schwartz *et al*, 1982, Shirey *et al*, 1985, Weiner *et al*, 1987) in up to 80% of patients. One group has reported 10 complete responses (and 7 partial responses) out of 105 patients who previously failed platinum chemotherapy (Hatch *et al*, 1991). Patients who were entered into other trials with tamoxifen had also generally failed chemotherapy previously. This may contribute to the low responses observed, by selecting patients with more aggressive tumours and with a more advanced disease. The mechanism of action of tamoxifen will be discussed in detail in section 1.31.

Fewer studies have investigated the efficacy of anti-androgen therapy. One study in which the androgen fluoxymesterone was used, reported no

responses in 16 patients (Kavanagh *et al*, 1987). In an attempt to down-regulate the production of gonadotrophins, thus reducing any steroid production by the ovary, the luteinizing hormone releasing hormone (LHRH) agonist, D-Trp-6-LHRH, has also been used in the treatment of ovarian cancer. Stabilisation or reduction of tumour size was reported in 50% of 10 patients (Kullander, 1986).

1.2 Steroidal hormones

1.21 Biosynthesis

The steroidal hormones are a large class of lipids which are synthesised mainly in the adrenal gland, testis and ovary. They are cyclic hydrocarbons containing a common ring structure, which consists of three six-membered rings and one five-membered ring. All steroids are derived from the precursor sterol, cholesterol (Stryer, 1981).

The ovary produces three main classes of steroid, oestrogens (C18), androgens (C19) and progesterone (C21). The secretion of these steroids is under the control of the pituitary gonadotrophic hormones, follicle stimulating hormone (FSH) and LH. In turn, the production of FSH and LH is under the control of LHRH produced by the hypothalamus (Eckert and Randall, 1983). The ovarian steroids also have a regulatory role by exerting positive and negative feedback to control the secretion of the pituitary gonadotrophins. Oestrogens, androgens and gonadotrophins influence ovarian cell differentiation, and control cell senescence and death, as demonstrated in the processes of follical atresia and lutenization. It is therefore possible that

loss of control of these processes may contribute to the development of ovarian cancer. The experiments in this thesis are concerned with the action of oestrogens and anti-oestrogens on the growth of ovarian adenocarcinoma cell lines. Therefore, the action of oestrogen will be discussed in more detail. The structure of 17 β-oestradiol is shown in figure 1.1

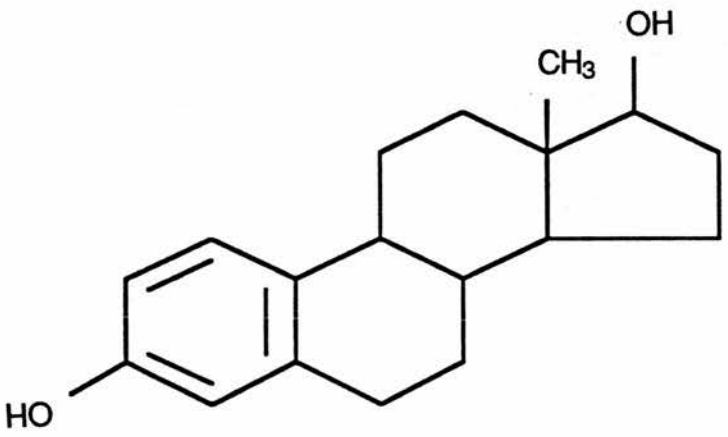

Figure 1.1. The chemical structure of 17 β-oestradiol.

1.22 Mechanism of action of oestrogen

The major effects of oestrogen appear to be mediated through a specific oestrogen receptor (ER) which is localised mainly in the nucleus. The human ER which has been cloned from cDNA libraries prepared from the MCF-7 breast cancer cell line encodes for a large protein of 595 amino acids (aa) with a molecular weight of approximately 66 Kd (Green *et al,* 1986). From the DNA sequencing data, three distinct functional domains can be identified in the protein (reviewed in King, 1987). The receptor consists of a

hydrophobic steroid binding domain which is situated at the carboxy terminal end of the protein, and a hydrophilic DNA-binding region which is nearer to the amino terminus. This region contains a high concentration of cysteine residues. These two regions are separated by a hinge region, the physiological relevance of which is not completely understood. The third functional domain of the receptor is situated at the amino terminus and is involved in transcriptional activation.

The unoccupied ER migrates as an 8s form on low salt gradients. It exists as an oligomeric complex made up of one ligand binding unit and other proteins including the heat shock protein, Hsp90. Following the interaction of oestrogen with its receptor, the complex is transformed to a 4s form. Dimerisation occurs which increases the affinity of the DNA binding domain for DNA. The change in form appears to be due to the loss of the associated Hsp90 (Chaumbraud *et al*, 1990). Therefore, the Hsp90 protein probably performs a role in preventing the unoccupied receptor binding to its target genes, either by preventing dimerisation or by masking the DNA binding region.

It is thought that attachment to the DNA is via two projections from the DNA binding region of the protein (Giguere *et al*, 1986; Green and Chambon, 1987). These loops have been referred to as "zinc fingers" as it has been shown that they are generated by the arrangement of four cysteine residues and a zinc ion to form a finger-like projection (Freedman *et al*, 1988). The ligand-activated receptor modulates gene expression by interaction with

specific regulatory regions termed oestrogen response elements (EREs) which are located in the vicinity of the oestrogen-regulated genes. The ERE is an enhancer as its action is not dependent on orientation or position (Seiler-Tuyns *et al*, 1986).

The ER contains two distinct transcriptional activation functions (TAF), TAF-1 which is in the N-terminal region of the receptor and is oestrogen independent (Tora *et al*,1989), and the hormone-inducible independent activation function,TAF-2, which is located in the hormone binding domain (Webster *et al*, 1988). The activities of TAF-1 and TAF-2 are functionally distinct and depend on the target cell.

Many studies have been performed on breast cancer cells *in vitro* to determine processes regulated by oestrogen. Amongst the more interesting processes which are induced by oestrogen is the synthesis and secretion of peptides which influence cellular proliferation. For example, in oestrogen-responsive MCF-7 cells, several growth factors have been identified in conditioned media (Salomon *et al*, 1984; Dickson *et al*, 1986; Huff *et al*, 1986; Knabbe *et al*, 1987). Incubation with oestrogen increases the levels of growth stimulating growth factors such as TGF-α and the insulin-like growth factors (IGFs) and downregulates the growth inhibitor, TGF-β (Dickson *et al*, 1986; Dickson and Lippman, 1987; Huff *et al*, 1988). This has led to a hypothesis that in breast cancer cells, at least some of the effects of oestrogen are mediated by the release of growth factors.

Growth factors which are produced constitutively from ER-negative MDA MB 231 breast cancer cells do not support the growth of ER-positive MCF-7 breast cancer cells in nude mice (Osborne *et al*, 1988), suggesting that the presence of growth factors may not be sufficient for cell growth to occur.

1.3 Anti-oestrogens

1.31 Mechanism of action of tamoxifen

The structure of the synthetic non-steroidal anti-oestrogen tamoxifen is shown in figure 1.2. Although the antitumour activity of tamoxifen is not fully understood, its major effects are believed to be mediated through the ER. The effects of oestrogen are blocked by tamoxifen competing with the natural ligand for binding to the ER, followed by subsequent inhibition of the expression of induced mRNA associated with oestrogen stimulation. However, some of the effects of tamoxifen cannot be explained by the interaction with the ER, for example, there is evidence of growth inhibition in 10-15% of ER-negative breast tumours (Jackson and Lowery, 1987).

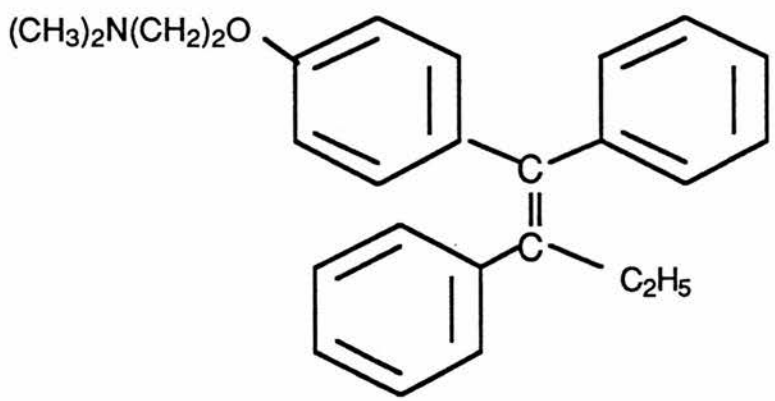

Figure 1.2. The chemical structure of tamoxifen.

Studies with human breast cancer cells *in vitro,* show that tamoxifen inhibits the proliferation by delaying or blocking the transition of cells in the early to

mid-G1 phase of the cell cycle (Osborne *et al,* 1983; Sutherland *et al*, 1983). Therefore, cells accumulate in early G1 phase, while the proportion of cells in the S and G2/M phases decreases. In addition, tamoxifen has been reported to increase the level of TGF-β which is secreted into the media by MCF-7 breast cancer cells in culture and to decrease the levels of TGF-α and IGF-1 (Dickson and Lippman, 1987; Knabbe *et al,* 1987). In T47-D breast cancer cells in culture, effects of anti-oestrogens can be partially blocked by incubation with EGF (Koga and Sutherland, 1987). Reversal of growth inhibition is observed at concentrations of EGF which do not produce growth stimulatory effects when added to the culture media alone. This provides further evidence that tamoxifen may alter the production of growth factors.

Although tamoxifen is usually described as an anti-oestrogen, it is known to be capable of acting as a full or partial oestrogen agonist or antagonist. Its effects depend on the species and target tissue which is being studied (Jordan and Robinson, 1987). The agonistic properties of tamoxifen may be responsible for the phenomenon of "tumour flare". This is characterised by an increase in skeletal pain following initial treatment with tamoxifen which may be accompanied by an increase in size of existing lesions or the appearance of new lesions (McIntosh and Thynne, 1977; Plotkin *et al*, 1978). Several studies on MCF-7 and T47-D breast cancer cells *in vitro* have shown that addition of tamoxifen at concentrations of 10^{-8} or 10^{-9} M to the culture media can stimulate the growth of the cells (Reddel and Sutherland, 1984; Cormier and Jordan, 1989). Tamoxifen also acted as a

partial agonist by increasing the levels of RNAs in MCF-7 cells associated with oestrogen growth stimulation (Johnson *et al*, 1989). In immature rats, tamoxifen stimulates uterine growth, but even when tested at high doses, the maximum stimulatory effect is below that achieved with 17 β-oestradiol (Wakeling and Bowler, 1987), which is characteristic of a partial agonist. When animals were treated with 17 β-oestradiol and tamoxifen, the uterotrophic action of 17 β-oestradiol was only partially blocked. Therefore, the anti-oestrogenic effects are limited by the oestrogenic activity of the compound.

1.32 Steroidal anti-oestrogens

The development of pure anti-oestrogens lacking oestrogen agonist activity might represent an improvement in the treatment of patients on tamoxifen. The steroidal anti-oestrogen ICI 164,384 is one of a group of pure anti-oestrogens which have been developed. The compound is a 7α-alkyl amide analogue of 17 β-oestradiol and is illustrated in figure 1.3.

ICI 164,384 lacks oestrogen agonist activity as monitored by uterine growth of immature rats, and is capable of blocking the uterotrophic action of 17 β-oestradiol or tamoxifen (Wakeling and Bowler, 1987). In addition, ICI 164,384 does not induce the expression of any of six oestrogen stimulated RNAs which have been identified in MCF-7 cells *in vitro* (May and Westley, 1986; 1987); Westley and May, 1987) whereas tamoxifen induces their expression to varying degrees (Wiseman *et al*, 1989). ICI 164,384 has

the same effects on the cell cycle distribution as noted for tamoxifen, but produces an effect on a larger proportion of the cells (Wakeling *et al*, 1989). Proliferation is blocked in the G1 phase on the cycle.

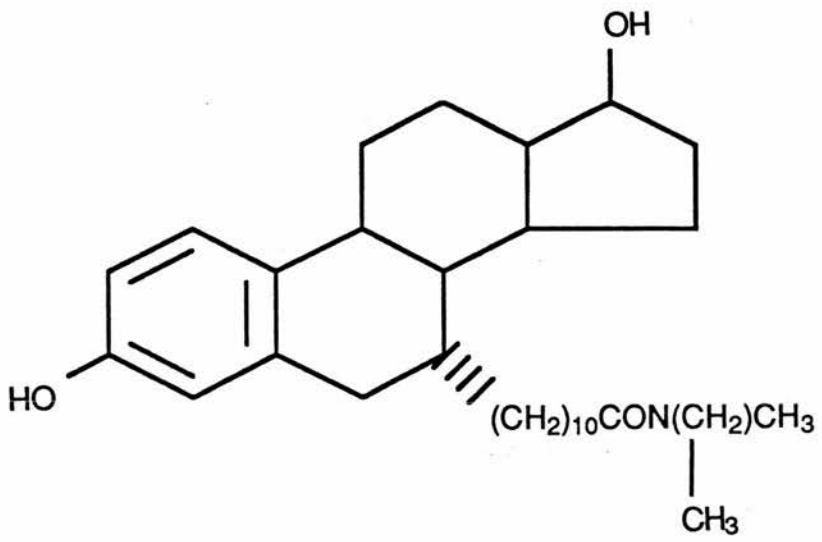

Figure 1.3. The chemical structure of the "pure" anti-oestrogen ICI, 164, 384.

1.4 Growth Factors

1.41 Autocrine, paracrine and endocrine growth pathways

Cell growth and division involves the coordination of a large number of metabolic pathways. Escape from the normal cell control systems, leading to tumour growth, may be achieved by overproduction or suppression of growth factors, their receptors or the second messengers which are induced by their interaction. Growth factors may act in three different ways, which are

summarised in figure 1.4. A growth factor may act as an autocrine factor, inducing an effect on the same cell from which it was secreted, or it may act on neighbouring cells in a paracrine manner. Alternatively, target cells of growth factors may be at a distance from the site of production. Therefore, the growth factor will have to enter the blood circulation and act as an endocrine factor. The effect of the growth factor on the cell is dependent on the type of growth factor secreted, concentration of the factor and the type of target cell.

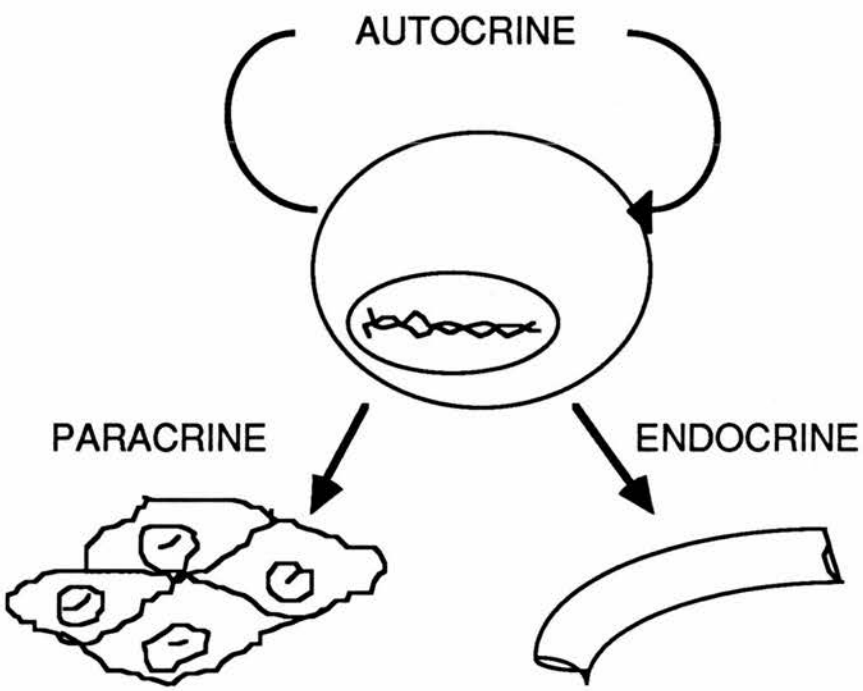

Figure 1.4. Schematic diagram showing autocrine, paracrine and endocrine pathways of growth regulation.

In this study, the effects of EGF and TGF-α were investigated, and it is therefore pertinent to discuss these in more detail.

1.42 Production of EGF

The existence of EGF was first recognised in 1962 (Cohen, 1962). It was discovered as a contaminant of Nerve Growth Factor (NGF) in mouse submaxillary glands, and was capable of inducing precocious eyelid opening and incisor eruption in newborn mice. The aa sequence of murine EGF was determined following purification of the growth factor from the mouse salivary glands. At a similar time, β-urogastrone, a human urinary protein which inhibits gastric acid secretion, was discovered and purified (Starkey *et al,* 1972, Gregory, 1975). The two peptides share 37 common aa residues out of a possible 53, and both contain 3 disulphide bonds in the same relative positions.

EGF is synthesised as a 1217 aa precursor, which includes seven repeating aa sequences homologous to the mature 53 aa peptide (Gray *et al,* 1983, Scott *et al*, 1983), which has a molecular weight of 6045. The function of the repeat sequences is not understood. EGF is found in almost all body fluids under normal physiological conditions but the exact sites of synthesis are unknown. The salivary gland is the only source of concentrated EGF in the male mouse (Byyny *et al*, 1972) and is more concentrated than that found in any other organ in other species. In the mouse submaxillary gland the precursor is broken down into the 53 aa mitogen, whereas in other tissues the EGF precursor is suggested to be expressed as a transmembrane protein.

1.43 Structure of TGF-α

Transforming growth factors were first discovered in the conditioned media of murine 3T3 fibroblasts transformed with Moloney murine sarcoma virus (De Larco and Todaro, 1978). This transforming activity was known as sarcoma growth factor. This was purified and found to contain two separate factors which are now known as TGF-α and TGF-β. TGF-α could be characterised by its capacity to compete with ^{125}I EGF in radioreceptor assays and its ability to transform cells. The addition of TGF-α to normal rat kidney (NRK) cells in soft agar causes anchorage independent colony formation of the cells (Dart *et al*, 1985), although full transformation is achieved only in the presence of TGF-β. This effect is reversed if TGF-α is removed from the medium. TGF-β does not compete with ^{125}I EGF binding. TGF-α is also synthesised by many human tumour cell lines (Derynck *et al*, 1987) and from normal tissues (Roberts *et al,* 1981).

The cDNA of TGF-α has been cloned and the sequence has been characterised (Derynck *et al,* 1984). The gene for TGF-α is 70 to 100 Kb and the mRNA is approximately 4.5 Kb. The biologically active 50 aa form of TGF-α is derived from a 160 aa precursor which is anchored to the cell surface via its C-terminus (Bringman *et al*, 1987). The portion of the TGF-α anchored to the membrane is palmitoylated. It has been suggested that this may play a role in slowing the passage of the TGF-α precursor through the rough endoplasmic reticulum and golgi, thus enabling more efficient processing of the precursor to produce more mature TGF-α. Alternatively, it

has been proposed that the membrane bound precursor may function as a receptor for an unknown ligand or play a role in cell to cell signalling. This was originally proposed to be a possible function of the larger EGF precursor (Pfeffer and Ullrich, 1985). In addition to the 50 aa form of TGF-α, higher molecular weight forms are also released, which represent N-glycosylated forms of TGF-α produced after proteolytic cleavage from the precursor (Bringman *et al*, 1987). TGF-α possesses 30-40% homology with EGF (Yeh and Yeh, 1989).

1.44 Structure of the EGF receptor

Both EGF and TGF-α exert their biological activity by binding to specific receptors. The EGF receptor was cloned and sequenced in 1984 (Ullrich *et al*, 1984). It is a glycoprotein which spans the plasma membrane once and consists of an amino-terminal 621 aa extracellular portion, a 27 aa transmembrane component and a carboxy-terminal 542 aa cytoplasmic intracellular portion.

The linear sequence predicts that the extracellular portion is folded into 4 domains termed L1, L2, S1 and S2. The L1 and L2 domains form a β-barrel superstructure thought to be involved with the binding of the ligand, whereas S1 and S2 may play a structural role (Bajaj *et al*, 1987). The extracellular domain contains a high proportion of cysteine residues (51 out of 621 aa) which are clustered in the 2 S domains and are probably involved in the formation of disulphide bonds. Several ligands are capable of binding to the EGF receptor including EGF and TGF-α. The growth factors released by

cells infected with members of the Pox virus family, such as Vaccinia (Brown *et al*, 1985 ; Stroobant *et al*, 1985), are known to bind to the EGF receptor. In addition, a recently identified growth factor named amphiregulin, which was first identified in conditioned media of a breast cancer cell line *in vitro,* (Shoyab *et al,* 1988) has been shown to bind to the EGF receptor (Shoyab *et al*, 1989).

The transmembrane domain consists of uncharged, hydrophobic aa residues which probably form an α-helix. It is anchored to the membrane by a highly-basic stop sequence. It is not known whether the transmembrane domain has a functional role in signal transduction. However, it has been shown that if the lipid environment of cell lysates is altered , the affinity of binding of the ligand and the basal kinase activity may be modified (Downward *et al*, 1985).

The intracellular portion of the receptor consists of a 50 aa juxta-membrane sequence followed by 250 residues which possess tyrosine kinase activity. This is linked to the autophosphorylation site domain of approximately 150-200 aa (Downward *et al*, 1984b).

Some of the protein sequences of the human EGF receptor are closely related to those of the transforming protein encoded by the v-*erb-B* oncogene of avian erythroblastosis virus (AEV) (Downward *et al*, 1984a). The v-*erb-B* oncogene encodes only the transmembrane region of the EGF receptor and the tyrosine kinase domain. It is therefore possible that

transformation results from the acquisition of a truncated receptor, which is constitutively active due to loss of the ligand-binding regulatory domain. Thus, altered expression or activation of the human EGF receptor may lead to uncontrolled growth.

The EGF receptor is stucturally related to 2 other genes which have been recently identified. These have been called c-*erb*B-2 (Schechter *et al*, 1984; 1985) and c-*erb*B-3 (Plowman *et al*, 1990). The c-*erb*B-2 gene is also known as *neu*, HER-2 or NGL. Due to the closely related structure of these 3 genes, they are now known as the type 1 growth factor receptor family. Amphiregulin binds most strongly to the c-*erb*B-3 receptor, but also binds weakly to the EGF receptor (Shoyab *et al*, 1989). In addition, EGF binds weakly to the c-*erb*B-3 receptor. Recently, a 30 kDa growth factor which is secreted by some breast cancer cells (Lupu *et al*, 1990) has been identified to activate the c-*erb*B-2 receptor inducing phosphorylation. This factor is also able to bind to the EGF receptor with low affinity. A second ligand specific for the c-*erb*B-2 receptor has been identified which has a molecular weight of 75 kDa (Lippman and Lupu, 1991). Neither EGF or TGF-α are capable of inducing phosphorylation of the c-*erb*B-2 receptor.

The majority of studies on the EGF receptor have been performed on a vulval carcinoma cell line A431, which was first described in 1973 (Giard *et al*, 1973). The cell line overexpresses the EGF receptor to a degree of approximately 50 fold more compared with most other cell lines (Fabricant *et al*, 1977; Wrann and Fox, 1979) thus facilitating studies on the receptor. In

some of the clones of the cell line, overexpression is asscociated with gene amplification and a translocation event (Merlino *et al*, 1984). The biosynthesis of the EGF receptor has been studied in A431 cells using antibodies against the EGF receptor (Mayes and Waterfield, 1984). These studies show that approximately 11 N-linked oligosaccharide chains are co-translationally added to a 135 kDa core polypeptide to form a 160 kDa precursor. The majority of these oligosaccharide chains are then modified further by addition of terminal sugars to produce the mature 170 kDa receptor.

1.45 Binding of EGF to the EGF receptor

EGF receptors have been measured in tissues and cell lines by radioligand binding assays using ^{125}I EGF. Scatchard analysis of the binding data has shown that in many tissues two components of binding can be identified, varying in their dissociation coefficients (Hawkins *et al*, 1991). It is not known how the sub-domains of the extracellular region of the EGF receptor interact to form high and low affinity binding sites, but recent experiments have shown that the L2 domain may play an important role in ligand binding (Lax *et al*, 1988). Cleavage of EGF receptors, which had been previously crosslinked to ^{125}I EGF, showed that it was the L2 domain which contained the ^{125}I EGF.

1.46 Events following the binding of EGF/TGF-α to the receptor

The majority of studies which have examined the events following ligand binding to the EGF receptor have been performed using EGF. However, it is

probable that TGF-α induces similar reactions. The exact pathway of events which follow the binding of EGF and TGF-α to the EGF receptor and which ultimately leads to DNA synthesis is unknown. However, it is known that after the binding of EGF to the receptor, the first biochemical events to occur are the phosphorylation of serine, threonine and tyrosine residues on the EGF receptor (Hunter and Cooper, 1981). Tyrosine phosphorylation of the EGF receptor is due to self-phosphorylation by the activation of the tyrosine kinase activity (Cohen *et al*, 1982), whereas the phosphorylation at serine and threonine residues is probably mediated by protein kinase C (Iwashita and Fox, 1984). TGF-α also induces tyrosine phosphorylation of the EGF receptor (Reynolds *et al*, 1981). The exact role of the tyrosine phosphorylation in the transfer of the mitogenic signal to the DNA is not known, but the presence of the activity in a number of growth factor receptors and oncogene products suggests that it may be one of the first necessary intracellular signals triggered by some mitogens.

The method by which the kinase domain is activated has not been completely elucidated, but it is believed to involve receptor-receptor interactions. Evidence for this comes from studies by Yarden and Schlessinger (1987a). It was shown that self-phosphorylation had a parabolic dependence on the concentration of EGF receptors, cross-linking of the EGF receptors induced self-phosphorylation, and immobilisation of the receptors prevented the activation of the receptor kinase. In addition, two forms of the EGF receptor have been isolated, the monomeric and dimeric form (Boni-Schnetzler and Pilch, 1987; Yarden and Schlessinger, 1987b).

In the absence of EGF, the majority of the receptors are in the monomeric form. However, following addition of EGF, the equilibrium between monomers and dimers is shifted towards dimers. The dimeric form of the receptor has a higher basal autophosphorylation rate than the monomers. On addition of EGF, both monomers and dimers are phosphorylated, but the monomers to a greater degree. The studies show also that the receptor monomers and dimers differ in their affinity of binding for EGF. Dimers show a higher binding affinity compared with the monomeric receptors which were isolated. Therefore, EGF probably stimulates the receptor autophosphorylation by inducing kinase-inactive receptor monomers to associate and form receptor dimers, in which the autophosphorylation activity is enhanced. The higher affinity of the dimeric form may induce increased associations between receptors.

Although the EGF-induced activation of the receptor kinase involves intermolecular associations, the autophosphorylation of the tyrosine residues on the receptor is an intramolecular event, as phosphorylation of tyrosine residues can be demonstrated in membranes which have been immobilised following incubation with EGF (Yarden and Schlessinger, 1987a).

Many other studies have been performed on the oral epidermal carcinoma KB cell line which express 2 x 10^5 binding sites/cell. EGF receptors have been visualised on the cell surface by immunofluorescence (Bequinot *et al,*

1984) using the monoclonal antibody EGFR1 (Waterfield *et al*, 1982). In the absence of ligand, the EGF receptors were distributed randomly over the plasma membrane of the cells. This is in contrast with the distribution of other receptors such as low density lipoprotein, which are found to be clustered in "coated pits" in the absence of ligand (Goldstein *et al*, 1979). Coated pits are specialised structures which are covered in a lattice like protein, clathrin, on their cytoplasmic face (Pearse, 1980). Following 10 min incubation with EGF, EGF receptors were visualised to be redistributed into concentrated patches, the coated pits. After 30 min incubation with EGF, the immunofluorescence was located in structures around the nucleus. No EGFR1 could be localised after 120 min. Vesicles are thought to be pinched off from the coated pits containing the ligand/receptor complex to form receptosomes or endosomes (Helenius *et al*, 1980). The endosome then transfers the complex to the Golgi. EGF is first seen in the reticular portion of the Golgi and later in the lysosomes (Willingham and Pastan, 1982). EGF receptors differ from other systems by being "down-regulated" following the addition of ligand. Other receptors such as those for transferrin (Dautry-Varsat *et al*, 1983) or α2-macroglobulin (Dickson *et al*, 1981) are recycled to the cell surface. Other studies have shown that the EGF receptor is proteolytically processed by lysosomes following binding of EGF (Das and Fox, 1978) and not recycled.

The exact role that clustering of the receptors plays in conveying the mitogenic signal to the nucleus is unknown. However, in studies where analogues of EGF have been constructed which retain their binding ability,

but are unable to induce receptor clustering, the analogues are unable to induce DNA synthesis (Schechter *et al*, 1979). The receptor clustering pattern and biological activity can be restored by bivalent anti-EGF antibodies. These results suggest that receptor clustering and internalisation of receptor/ligand complexes may have a functional role in the mitogenic pathway. Furthermore, the EGF receptor/ligand complex still possesses active kinase activity following internalisation (Cohen and Fava, 1985) which may suggest that the endocytosis of the receptor is of physiological importance. It has been suggested that its function may be to translocate the receptor kinase activity from the plasma membrane to the interior of the cell, to phosphorylate substrates which the complex would not be accessible if the complex was held at the plasma membrane.

Although the initial biochemical events occur rapidly after the binding of EGF to the EGF receptor, these are not adequate to induce the mitogenic effects which are associated with EGF. Experiments which have been performed *in vitro* have shown that EGF must be present in the culture media for at least 6-8 hours to trigger mitogenesis (Carpenter and Cohen,1976). It is possible that the cell needs a continuous signal to divide for a certain period of time. The finding that even in the presence of saturating concentrations of EGF, not all the EGF receptors are removed from the cell surface by down-regulation (Das and Fox, 1978) provides further evidence for this hypothesis. New complexes of EGF/EGF receptor would form continually and produce further signals to instruct the cell to divide. A proportion of EGF receptors may remain at the cell surface due to an inherrant unknown

difference or may be due to the synthesis of the EGF receptor being stimulated in the presence of EGF (Clark *et al*, 1985; Kudlow *et al*, 1986).

1.5 Role of oestrogen in ovarian cancer

As the ovary is sensitive to oestrogen and it is a site of its synthesis, it is possible that oestrogens may have a role in the control of growth of ovarian cancer. Oestrogens are implicated in this control by the presence of ER.

1.51 Steroid hormone receptors in ovarian cancer

Over the last 10-15 years many studies have investigated ovarian malignant tissue for the presence of receptors for oestrogen and progesterone. However, differences in the levels of receptors have been reported between groups. This may be explained by several factors. Relatively small sample numbers were included in many studies, different proportions of various types of ovarian tumours were present within the sample groups, heterogeneity within tissues exists and different assays for receptor measurement were used. In addition, the level of expression which was considered positive varied between the studies.

The combined data of 52 published reports are summarised by Slotman and Rao (1988). Overall, receptors for oestrogen and progesterone were present on 63% and 48% of ovarian tumours, respectively. Both receptors were present on 36% of the tumours, whereas neither receptor could be detected on 25%. In comparison with ER and progesterone receptors (PgR), fewer groups have investigated the presence of androgen receptors in

ovarian tumours. Combining the available data, Slotman and Rao (1988) reported that androgen receptors were present of 69% of tumours.

1.52 Prognostic value of steroid receptors

The relationship between expression of steroid receptors and prognosis of patients is still not clear, which is probably a consequence of the large range of receptor levels reported. However, there have been some reports that the presence of ER and PgR confers a prognostic advantage (Kauppila *et al*; 1983; Iversen *et al*, 1986; Bizzi *et al*, 1988; Leake and Owens, 1990). Caution must be taken in interpretation of the results, as small patient numbers were investigated in each of the studies. A relationship between histological type and expression of ER has been reported by some authors. ER were found to be present in serous tumours more often than in mucinous or clear cell (Quinn *et al*, 1982, Ford *et al*, 1983, Kauppila *et al*, 1983). Endometrioid tumours have been reported to contain more PgR often in association with ER (Freidman *et al*, 1979; Ford *et al*, 1983; Sutton, *et al*, 1986).

1.6 Role of EGF/TGF-α in ovarian cancer

1.61 Stimulation of ovarian primary tumour cells

Both EGF and TGF-α induce the *in vitro* growth of a wide variety of primary tumour cells, including ovarian carcinoma (Hamburger *et al*, 1981; Singletary *et al*, 1987). In addition, EGF has been shown to stimulate the growth of ovarian carcinoma cells lines in culture as measured by thymidine

incorporation (Berchuck *et al*, 1990b). In general, the potency of EGF and TGF-α are equivalent, although TGF-α is more potent in other systems. For example, TGF-α is more potent in promoting calcium release from rat foetal rat long bones (Stern *et al*, 1985; Ibbotson *et al*, 1985).

1.62 EGF receptors

Abnormal expression of the EGF receptor has been associated with various types of malignancies. In breast cancer, it has been shown that one third of tumours express EGF receptors, and that the expression is inversely correlated with the presence of ER (Sainsbury *et al*, 1985). This is associated with a poor prognosis (Sainsbury *et al,* 1987). Recently, several studies have examined the expression of EGF receptors in ovarian cancer. Various percentages of ovarian tumours have been reported to express EGF receptors. Owens *et al* (1991) reported that 40% of ovarian tumours expressed the receptor and Bauknecht *et al* (1989b) detected EGF receptors in 45%. Others have found EGF receptors in as many as 75% (Battaglia *et al,* 1989) and 77% (Berchuck *et al*, 1991) of ovarian tumours.

The majority of studies have found no correlation between expression of EGF receptors and histologic grade or stage (Bauknecht *et al*, 1988; Berchuck *et al*, 1991; Owens *et al*, 1991), although a higher EGF receptor content has been noted in poorly differentiated tumours compared with moderate and well-differentiated (Battaglia *et al,* 1989). The presence of EGF receptors on ovarian cancers has been suggested to be associated with a better response to chemotherapy (Bauknecht *et al*, 1989a). However,

the presence of EGF receptors on tumours confers a poor prognosis on the patients (Kohler *et al*, 1989; Berchuck *et al*, 1991; Rodenburg *et al*, 1990) which is represented by the patients having an overall shorter survival time.

1.63 Presence of EGF/TGF-α

EGF and TGF-α have been detected in the tissue extracts of a number of ovarian tumours. TGF-α has been detected more frequently in 85 to 91 % of tumours compared with EGF which has been detected in only 26 to 38 % (Owens and Leake, 1989; Leake and Owens,1990). The presence of EGF-like factors in tumour tissue extracts has been suggested to be of prognostic significance (Bauknecht *et al*, 1989, Kohler *et al*, 1989). Patients with increased levels (greater than 3.55 ng/ml) of EGF-like activity have an overall survival time less than patients with low levels of the factors present in the tissue extracts.

Ascitic fluid which is often produced in association with ovarian tumours contains growth promoting activity (Mills *et al*, 1988). Various studies have been performed to identify the factors which are responsible for the growth stimulatory potential of these ascitic fluids. TGF-α has been reported to be present in 42% of effusions from ovarian cancer patients at levels exceeding those found in non-malignant tissues (Arteaga *et al*, 1988), although other investigators have been unable to identify the presence of EGF or TGF-α activity in the fluids (Wilson *et al*, 1991). Differences in results may be due to different antibodies being used in the detection of the factors.

The combination of a large number of studies which have shown that EGF receptors are present on a proportion of ovarian tumours, and that tumours may secrete EGF or TGF-α suggests that these growth factors may function in an autocrine or paracrine growth regulation pathway. In addition, the observation that primary ovarian cancer cells in culture can be stimulated by the exogenous addition of EGF/TGF-α lends support to the possibility that EGF and TGF-α may be important factors in the growth control of ovarian tumours *in vivo*.

1.7 Interaction of oestrogen and EGF

Studies on breast cancer cells *in vitro* have suggested that some of the oestrogen-induced growth stimulation may be mediated by altering the levels of growth factors, such as EGF, which are secreted by the cells. It is therefore possible that oestrogen may also have effects on the level of expression of EGF receptors. Oestrogen administration to female rats has been shown to increase the level of EGF receptors 3-fold with no change in affinity of the receptors in the uterus (Mukku and Stancel, 1985). Culture of MCF-7 breast cancer cells in the presence of 17 β-oestradiol intially reduces the EGF receptor level, but following 5 days in culture, the EGF receptor level is increased by 200% (Berthois *et al*, 1989). This suggests that an interaction between the ER and EGF receptor growth promoting systems exists.

1.8 Aims of study

The development of cell lines has enabled controlled, systematic analysis of growth control pathways to be initiated. The use of cell lines avoids the difficulties encountered when working with primary ovarian tumours such as heterogeneity within tumours, which produces uncertainty as to which type of cells are producing the effects which are under investigation. In addition, reproducibility between experiments is often a problem when using primary material, due to the heterogeneity between tumours. A limited supply of material may also prevent adequate studies being performed.

Using three human ovarian adenocarcinoma cell lines, PEO1, PEO4 and PEO14, as model systems, the aims of this study were two-fold ie to clarify the role of (1) hormones and (2) growth factors in the growth regulation of ovarian cancer.

(1) Since the PEO1 and PEO4 cell lines expressed ER and the PEO14 cell line was ER-negative, these were used to test the hypothesis that ovarian carcinoma cells are sensitive to oestrogen and antioestrogens, and to test whether sensitivity correlated with the presence of the receptor.

(2) A proportion of ovarian tumours express EGF receptors and/or secrete EGF or TGF-α. Therefore, the growth of the tumours may be influenced by these factors in an autocrine or paracrine manner. Using the same three ovarian cell lines, the presence of these growth regulation pathways for EGF and TGF-α was investigated.

The elements necessary to form an autocrine or paracrine loop are illustrated in fig 1.5.

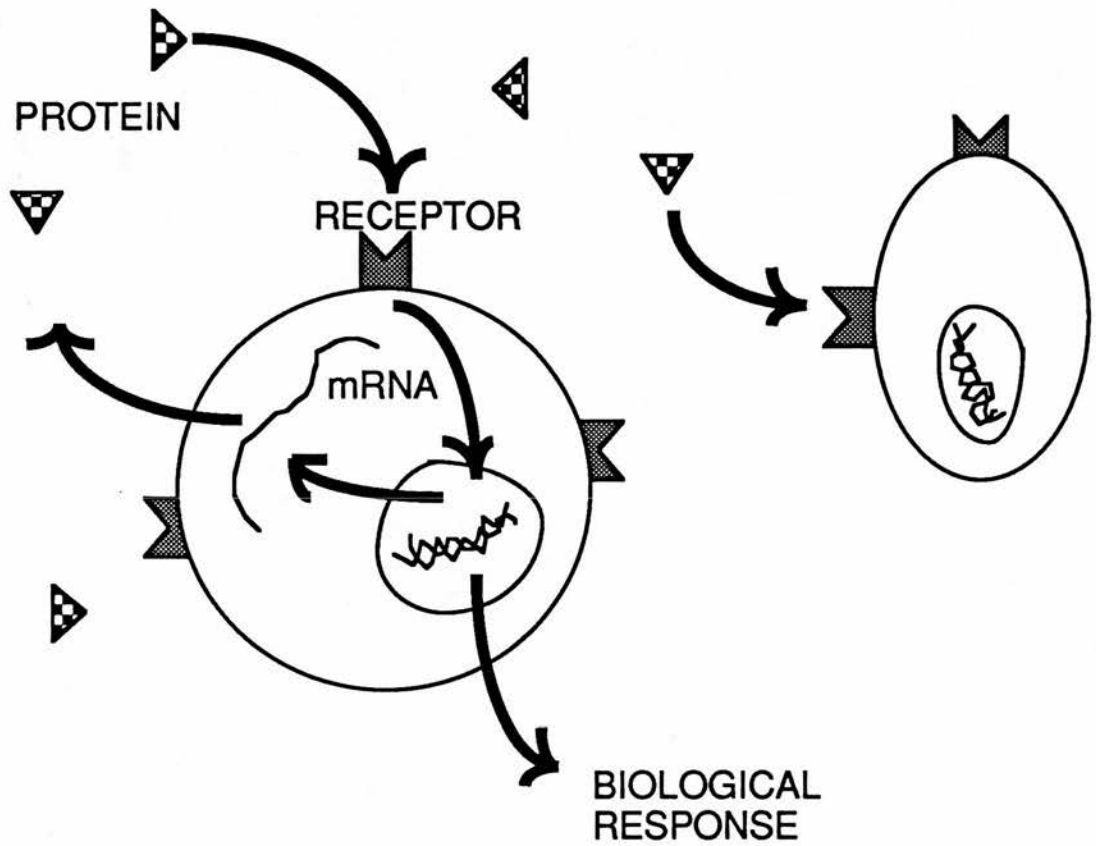

Figure 1.5. Schematic diagram showing the components of an autocrine and paracrine growth regulation pathway.

Thus, the aim of the second part of this study was to determine whether the cells responded to EGF and TGF-α, if this correlated with the presence of EGF receptors and whether the cells produced the growth factors. In addition the possibility that these growth factor actions may be under hormonal regulation was also investigated.

CHAPTER 2

MATERIALS AND METHODS

2.11 MATERIALS

(Suppliers are listed by techniques)

2.11 Immunocytochemistry

All antibodies were obtained from ICRF, London

(i) Static cytometry

All materials were supplied by Sigma except where stated.

Avidin Biotin Complex-Dakopatts

Biotlnylated rabbit anti-mouse immunoglobulin-Dakopatts

DPX mountant-BDH Analar

Lab-Tek 8 well chamber slides-Gibco

Lithium carbonate-BDH Analar

Multispot slides-Hendley Essex

Normal rabbit serum-Dakopatts

Tris Buffer-BDH Analar

Xylene-May and Baker

(ii) Flow cytometry

All materials were obtained from Sigma except where stated.

Propidium iodide - Fluka Chemicals Ltd

2.12 Ligand binding assay

All materials were supplied by Sigma except where stated.

Bio-Rad protein assay dye reagent concentrate - Bio-Rad

Murine ^{125}I EGF, specific activity 5.6-7.4 MBq/μg -Du Pont (UK) Ltd

Polystyrene LP3 tubes-Luckam Ltd

2.13 Radioimmunoassay

Anti-EGF antiserum (raised in sheep to purified human EGF)-Dr H Gregory, ICI, which was kindly supplied by Dr F Habib, Department of Surgery, Western General Hospital, Edinburgh.

Donkey anti-sheep IgG-Scottish Antibody Production Unit

Human ^{125}I EGF, specific activity >27.7 TBq/mMol-Amersham

2.14 Tissue culture

All materials were supplied by Sigma except where stated.

Cell scrapers - Costar Corporation

Dextran T70 - Pharmacia

Dimethyl sulphoxide (DMSO) - BDH Analar

Foetal calf serum - Gibco

Liquid scintillator Unisolve 1- Koch-Light Ltd

Penicillin/streptomycin - Gibco

Phenol red - Flow Laboratories

Phosphate Buffered Saline - Oxoid

RPMI 1640 with/ without phenol red - Gibco

[methyl-^{3}H]- thymidine, specific activity 45.5 Ci/mMol -Amersham

Tissue culture flasks and multi-well plates-Corning.

Trypsin - Gibco

(i) Cell lines

The three ovarian serous adenocarcinoma cell lines, PEO1, PEO4 and PEO14 were derived from ascitic fluids as previously described by Langdon *et al*, (1988). PEO1 and PEO4 were derived from the same patient whose tumour was described as a poorly differentiated serous adenocarcinoma. The ascitic fluid from which PEO1 was derived was collected after the patient had received cis-platinum, 5-fluorouracil, and chlorambucil chemotherapy before the tumour had developed drug resistance, whereas PEO4 was collected when the patient was in clinical relapse and the tumour had developed drug resistance. PEO14 was derived from a patient with a well-differentiated serous adenocarcinoma who had not received prior drug treatment. Photographs illustrating the three ovarian carcinoma cell lines are shown in figures 2.1-2.3.

The oestrogen receptor (ER) content of the cell lines was determined by Dr R A Hawkins of Department of Surgery (Royal Infirmary), University of Edinburgh, using a dextran-coated charcoal adsorption assay (Hawkins *et al*, 1975, Hawkins *et al,* 1981). Assays were performed on cells which were in early plateau phase of their growth. PEO1 and PEO4 cells were ER-positive, expressing levels of 96 and 112 fmol/mg protein respectively whereas the PEO14 cell line was ER-negative (Langdon *et al*, 1990).

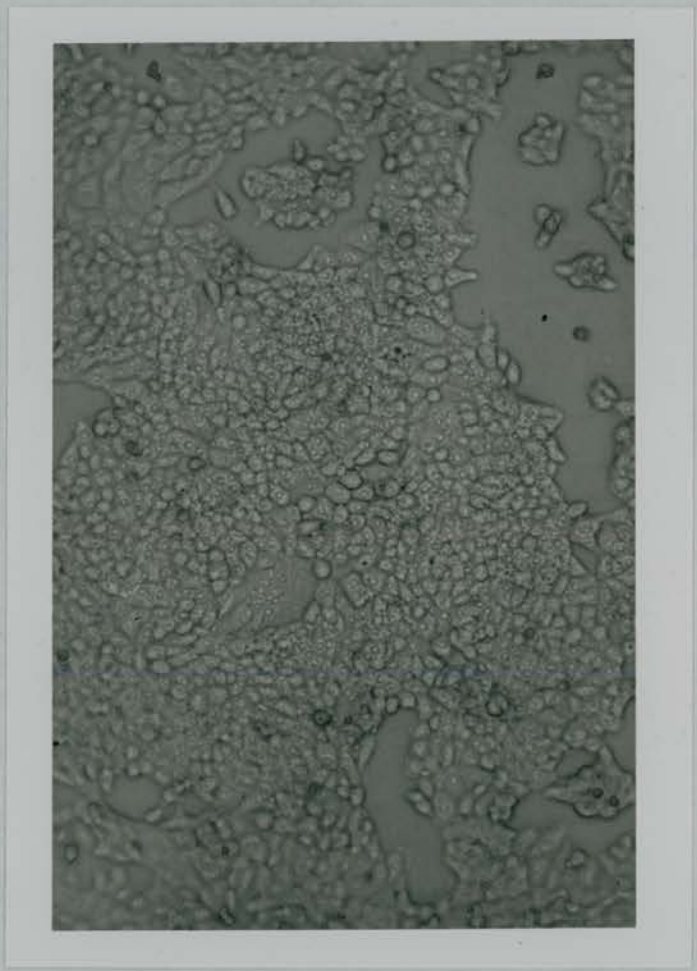

Figure 2.1. A photograph of the PEO1 cell line in mid-logarithmic phase of growth, shown at a magnification of x 100.

Figure 2.2. A photograph of the PEO4 cell line in early-logarithmic phase of growth, shown at a magnification of x 100 .

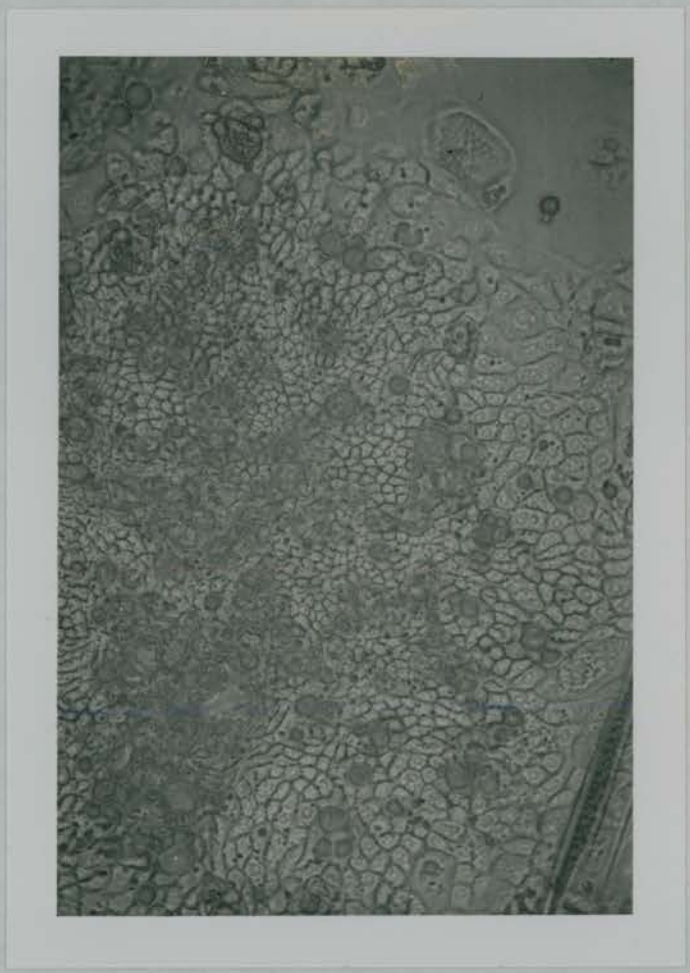

Figure 2.3. A photograph of the PEO14 cell line in late-logarithmic phase of growth, shown at a magnification of x 100.

The vulval carcinoma cell line A431 was obtained from the European Collection of Animal Cell Cultures (Giard *et al*, 1973).

The small cell lung cancer cell line NIH-H69 was obtained from Professor A Harris with the permission of Dr D Carney, Mater Hospital (Gazdar *et al,* 1980).

(ii) Growth factors/hormones

Human recombinant EGF-Sigma

Human recombinant TGF-α-Boehringer Mannheim

ICI 164,384-ICI

17 β-oestradiol-Sigma

Tamoxifen-ICI

Growth factors were reconstituted in sterile PBS and stored at -40°C in aliquots of 20 μg/20 μl. The 17 β-oestradiol, tamoxifen and ICI 164,384 were dissolved in absolute alcohol to give stock solutions of 10^{-2} M, which were stored in glass universal containers at 4°C in the dark.

(iii) Antisera

Antisera raised against either EGF or TGF-α were kindly supplied by ICI.

2.2 METHODS

2.21 Routine culture of cell lines

(i) Growth of cell lines

The ovarian adenocarcinoma cell lines PEO1, PEO4 and PEO14, the vulval carcinoma cell line A431 and the small cell lung cancer cell line NIH-H69 were all routinely cultured at 37°C in an atmosphere of 5% CO_2, 95% air (90% humidity) in Roswell Park Memorial Institute (RPMI) 1640 media containing phenol red indicator. This was supplemented with 10% heat-inactivated (by incubation at 56°C for 20 min with occasional stirring) foetal calf serum (FCS), L-glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (100 μg/ml) .

(ii) Cell harvesting

PEO1, PEO4, PEO14 and A431 cells were grown to confluence in 75 or 125 cm^2 flasks. Cells were harvested by detachment from the plastic by washing cell monolayers with phosphate buffered saline (PBS), pH 7.3 and then incubating with trypsin (0.25% [w/v] in Gibco solution A)/versene (1 mM EDTA in PBS, 0.5% [v/v] phenol red) solution (1:1) for approximately 5 min at 37°C. The trypsin was inactivated by the addition of RPMI 1640 with 10% FCS. Single cell suspensions were prepared by passing the cells through a 21.5 (PEO1, PEO4) or a 19.5 (PEO14, A431) gauge needle, before seeding into new flasks. Cells were passaged each week and fed with fresh media every 3-4 days. The viability of cells following trypsinisation was confirmed to be greater than 95% using a vital dye exclusion test with trypan blue.

The small cell lung cancer cell line NIH-H69 grows in suspension. Cells were routinely fed once a week. When the requirement for media exceeded this, or clumps were formed, cells were split 1:4 by transferring aliquots to new flasks. Clumps were disaggregated by gentle passage through a pastette.

(iii) Freezing down and recovery of cells from liquid nitrogen

Cells which had reached 80-90% confluence were trypsinsed as described above. A single cell suspension was made and an aliquot was removed for counting using a haemocytometer. The suspension was centrifuged and the pellet resuspended in ice-cold 10% (v/v) dimethyl sulfoxide (DMSO) in newborn calf serum (NBCS) at a concentration of $5x10^6$ cells/ml. Aliquots (1 ml) were transferred to cryotubes which were kept at -40°C overnight before being placed in liquid nitrogen for long term storage. When cells were to be recovered, cryotubes were removed from the liquid nitrogen tanks and thawed rapidly by immersing in warm water. The DMSO was removed by washing with RPMI 1640 containing 10% FCS, and the mixture centrifuged at 1000 g for 5 min. The cell pellet was resuspended in an appropriate volume of RPMI 1640 containing 10 % FCS and transferred to a 25 cm^2 flask. Cells were incubated for 24 hours to allow for attachment, washed with PBS, pH 7.3 to remove dead cells, and fresh media added.

(iv) Preparation of cells for ligand binding assay

Cells were grown to 80-90% confluence in RPMI 1640 supplemented with 10% FCS in 175 cm^2 flasks. The cell monolayer was washed twice with

ice-cold PBS , and the cells were then removed by scraping into 25 ml of 10 mM tris buffer in saline (0.9% NaCl) (TBS), pH 7.2. A cell pellet was prepared by centrifugation at 1000 g for 5 min. The pellet was resuspended in 1-2 ml of TBS and transferred to a cryotube. The supernatant was removed, and the cryotubes spun briefly in a microcentrifuge. Cells were stored at -80°C until assayed.

2.22 Cell growth assays

(i) Measurement of plating efficiency

Cells in mid-log phase were harvested by trypsinisation as described. The number of viable cells in single cell suspensions was determined by using a haemocytometer and a vital dye exclusion test. Suspensions of cells were made in RPMI 1640 plus 10% FCS (PEO1 and PEO4 at $5x10^4$ viable cells/ml and PEO14 at 10^5 viable cells/ml). These cell densities were used for all growth assays. Cells were plated out in 24-well plates (0.5 ml of cell suspension/well). These were incubated for 24 hours to allow cells to adhere to the plastic. The wells were then gently washed with pre-warmed PBS to remove unattached cells. Attached cells were counted in a haemocytometer after trypsinisation. Plating efficiencies were expressed as a percentage of the number of attached cells compared to number of cells which were seeded.

(ii) Removal of steroids from FCS

The method used was adapted from Stanley *et al* (1977). To strip 100 ml of heat-inactivated FCS, a dextran-charcoal suspension was prepared containing 1g of charcoal and 5 mg of dextran T70 in distilled H_2O (10 ml) and stored at -4°C until use. The heat-inactivated serum was incubated with sulphatase (250 units) at 37°C for two hours in a shaking water bath. The pH of the serum was then adjusted to 4.2 with 2 M HCl before the addition of charcoal-dextran solution (5 ml). The suspension was stirred overnight at 4°C and the dextran-charcoal removed by centrifugation at 18,000 g (10,500 rpm) for 20 min at 4°C in a Sorval RC-5B centrifuge with a SS34 rotor. Dextran-coated charcoal suspension (5 ml) was added and the process was repeated. The pH of the FCS was adjusted to 7.2 with 2 M NaOH and sterilised by passing through 0.22 μm filters. The double-charcoal-stripped (dcs) FCS was stored in aliquots at -20°C until required.

(iii) Measurement of growth response to hormones and growth factors

All measurements of growth based on cell numbers were performed in 24-well plates. Responses of the ovarian cell lines to the exogenous addition of growth factors or hormones were investigated in one of three different conditions: RPMI 1640 alone or with 0.5 or 5 % dcs-FCS. Serum free media was supplemented with hydrocortisone (50 nM), insulin (20 mg/ml), transferrin (10 mg/ml), and sodium selenite (25 nM), (HITS) (Langdon *et al,* 1990). In all three conditions RPMI 1640 without the pH indicator phenol red was used, as phenol red is known to possess weak

oestrogenic activity (Berthois *et al*, 1986).

The doubling times of the cell lines were determined in these three conditions and for growth in RPMI 1640 containing phenol red and 10% FCS. Cells were harvested in mid-logarithmic phase by trypsinisation. Single cell suspensions were made in RPMI 1640 with phenol red and 10% FCS. Aliquots were added to 24-well plates using an Eppendorf multidispenser. Cells were plated at an initial number of 2.5×10^4 for the PEO1 and PEO4 cell lines and 5×10^4 for the PEO14 cell line and incubated for 24 hours to allow the cells to adhere to the plastic. In experiments which involved the measurement of growth responses to the exogenous addition of growth factors or hormones, media was removed and the cells were washed twice gently with pre-warmed PBS to remove unattached cells and FCS. In each well the media was replaced with RPMI 1640 (0.5 ml) without phenol red containing either 0.5 or 5% dcs-FCS or HITS. Cells were incubated in this media for a further 24 hours to minimise the potential effects of phenol red and residual steroids contained in the FCS. Media was then replaced with fresh phenol red-free RPMI 1640 containing 0.5 or 5 % dcs-FCS or HITS with or without the addition of growth factor or hormone. This time point was designated day 0. Each condition was studied in quadruplicate. Cells were cultured for 7 days and media was replenished on days 2 and 5. In some experiments cells were counted on days 0, 2, 5, and 7, whilst in studies which involved determining the effects on growth in a large number of test systems, cells were counted on only day 0 and 7. Cell counts were performed on a ZF Coulter Counter.

(iv) Coulter counting

The media was removed from each well and the cells were gently washed in PBS. Cells were removed from the plates by incubation with a trypsin/versene solution (250 µl). PEO1 and PEO4 cells were passed through a 21.5 gauge needle and PEO14 cells through a 19.5 gauge needle to obtain single cell suspensions. Aliquots of the cells (200 µl) were added to 0.9% NaCl (9.8 ml) and mixed. Triplicate counts from the same suspension were performed using a ZF Coulter Counter fitted with a 200 µm diameter probe. Cell numbers/well were calculated by taking the mean of the triplicate counts and multiplying by the dilution factor (x 25).

(v) Antibody blocking experiments

The effect of the addition of antibodies directed against EGF and TGF-α on the growth rates, as assessed by cell numbers, was investigated in PEO1, PEO4 and PEO14 cells. Cells were cultured in phenol red-free RPMI 1640 containing 5% dcs-FCS or HITS. Experiments previously performed by ICI showed that the antisera against EGF and TGF-α blocked the stimulation of [^{3}H]-thymidine incorporation into NIH 3T3 cells produced by EGF/TGF-α. The blocking abilities of the antisera were tested at the concentrations recommended by ICI (1:10,000 for anti-EGF and 1:2000 for anti-TGF-α antiserum) in the ovarian cell line PEO4 in the presence of 5% dcs-FCS. Cells were plated out at 3-4 x 10^4 cells / well, slightly higher than densities used in other cell growth assays. Cells were incubated for 24 hours and media changed as described before. On day 0, media were prepared containing varying concentrations of EGF or TGF-α (0.01-1 nM) and a fixed

concentration of antiserum against EGF (1:1000 or 1:10,000) or TGF-α (1:2000). These were mixed and left for 15 min to equilibrate before addition to the cells. Cells were cultured in the presence of growth factor and antiserum for 4 days, and then counted using the Coulter Counter. Due to the very limited supply of antisera media was not replenished during experiments. The effects of the antisera on the basal rate of growth of the three ovarian cell lines in 5% dcs-FCS and HITS were also investigated. Cells were cultured for 4 days in the presence of the antisera at the concentrations stated above and then counted. Bovine serum albumin (BSA) at the same concentrations was used as a control to check that effects were not due to non-specific addition of protein.

(vi) Thymidine incorporation

Incorporation of [methyl-^{3}H]-thymidine into DNA was used as an additional method of measuring responses of the ovarian cell lines to 17 β-oestradiol and tamoxifen. The method used was a modification of that described by Langdon *et al* (1990). Cells were plated in 6-well plates (at comparable concentrations to those used for 24-well plates) in RPMI 1640 containing 10% FCS (2 ml). The same procedure for replacement of media was followed for growth curves as described above. On the day of harvesting, media were removed from the cells and replaced with RPMI 1640 containing [methyl-^{3}H thymidine] (1 μci/ml). Cells were incubated for 2 hours at 37°C. Media were removed, cells washed twice with PBS and detached from the plastic by trypsinisation. To each well 5% (w/v) trichloroacetic acid (TCA) (2 ml) was added and incubated for 15 min at room temperature. DNA was

separated from TCA -soluble material by passing through 2.5 cm Whatman glass microfibre filters under suction. The nucleic acid was retained by the filter. Wells were washed twice with TCA and material passed through filters. The filters were semi-dried by passing methylated spirit through them, transferred to scintillation vials and dried by overnight incubation at 37°C. Unisolve 1 Scintillation fluid (10 ml) was added. Vials were placed in the dark for 1 hour and counted for 4 min on a Packard Tri-Carb 1900CA analyser. The efficiency of counting was at least 60%.

(vii) Statistics

The data obtained from the growth experiments was analysed statistically where the growth effects produced in control groups and groups treated with hormones or growth factors were not markedly different. The Students' t-test was used to analyse the data to determine whether the changes in the growth rate were significant at a value of $P < 0.05$.

2.23 Cell cycle analysis

Cells were cultured in 6-well plates and treated in an identical way to that described for growth assays. The cell cycle distributions of PEO1, PEO4 and PEO14 were investigated using a modification of the method described by Vindelov *et al* (1983). Samples of approximately 10^6 cells were prepared from quadruplicate wells at times 0, 12, 24, 34, 48, and 72 hours. Cells were removed by trypsinisation, and washed twice in PBS by centrifugation at 1000 g for 5 min. The pellets were resuspended in citrate buffer (100 µl), pH 7.6 and were stored at -20°C until analysed. At analysis, stored cells

were allowed to reach room temperature and 0.003% (w/v) trypsin in stock solution (450 µl), pH 7.6 was added, and incubated for 10 min at room temperature with gentle inversion. Trypsin inhibitor (0.5 mg/ml) and RNAase A (0.1 mg/ml) dissolved in stock solution (325 µl) were added to each tube, mixed and incubated for 10 min at room temperature. DNA was then stained with propidium iodide (0.416 mg/ml) and spermine tetrahydrochloride (1 mg/ml) dissolved in stock solution (250 µl), by incubation for 10 min on ice. Analysis was performed using a FACScan flow cytometer equipped for doublet discrimination using Cellfit Software. All data was gated on forward and side scatter signals to exclude fragmented and clumped material, and on a fluorescence width versus fluorescence area signal to exclude doublets. The solutions were made up as described below.

Citrate buffer

Sucrose (85.5 g) and trisodium citrate (11.76 g) were dissolved in distilled H_2O (800 ml). DMSO (50 ml) was added and the volume made up to 1000 ml. The pH was adjusted to 7.6.

Stock solution

Trisodium citrate (2000 mg), Tris (121 mg), spermine tetrahydrochloride (1044 mg) and Nonidet 40 (2 ml) were dissolved in distilled H_2O, made up to 2000 ml and adjusted to pH 7.

2.24 Measurement of EGF receptors by ligand binding assay

The method used was that described by Hawkins *et al* (1991), and was

based on the binding of ^{125}I EGF to "membrane" fractions.

(i) "Membrane" preparation

Tissue was weighed, cut into small pieces and homogenised at maximum speed with a Silverson laboratory mixer emulsifier in ice-cold TBS (2 ml) for 20 sec, surrounded by a jacket of ice. The mixture was allowed to cool for 1 min, before a further 15 sec homogenisation. The resulting homogenate was filtered through a coarse metal sieve to remove particulate matter and centrifuged at 105,000 g (45,000 rpm) in a Beckman TL 100 ultracentrifuge for 30 min at 4°C. The supernatant was removed and discarded, and the pellet resuspended in ice-cold TBS using a glass-glass hand homogeniser to obtain a smooth suspension. An aliquot (100 µl) was removed and stored at -20°C for protein estimation at a later date. The method for cell lines was similar except that filtration of the homogenate was not required. Thus, the term "membrane" is equivalent to the total particulate fraction.

(ii) Binding to "membranes"

Multipoint analyses were performed in duplicate by a displacement method, in which varying final concentrations (0.024, 0.049, 0.098, 0.195, 0.39, 0.78, 1.56, 3.125, 6.25, 12.5, 300 nM) of non-radioactive EGF (200 µl) were added to a fixed concentration (approximately 0.02 nM, equivalent to 10,000 cpm) of ^{125}I EGF (100 µl). These were vortex mixed and allowed to stand on ice for 15 min to permit equilibration, before the addition of "membranes" (100 µl) to give a final reaction volume of 400 µl. Tubes were then incubated at 26°C in a shaking water bath for 90 min

which were found to be optimal incubation conditions (see figure 3.35).

(iii) Termination of reaction

All tubes were placed on ice and ice-cold 0.5% (w/v) immunoglobulin G solution (0.5 ml) followed by 25% (w/v) polyethylene glycol (PEG) (1 ml) were added with a mix between additions. The tubes were remixed and the bound and free ^{125}I EGF were separated by centrifugation at 3000 rpm for 15 min at 4°C in a swing out rotor of a Beckman CPR centrifuge. After centrifugation, the supernatant (free ^{125}I EGF) was aspirated and the remaining pellet (bound ^{125}I EGF) was counted twice for 10 min in a Packard Cobra Gamma counter. Three tubes containing only ^{125}I EGF (100 µl) were also counted to calculate the starting cpm/tube.

(iv) Calculation of receptor site concentration

After correction of the counts bound in each tube, by subtraction of the value for the non-specific binding (300 nM tube), the binding data was subjected to Scatchard analysis (Scatchard, 1949). Non-specific binding averaged 3.6 ± 0.8% (n=50) of the total radioligand added. Where two components were present, the plots were examined by the computer analysis method of Hetherington (Sainsbury *et al*, 1985; Nicholson *et al*, 1988) to assign, fit, and calculate both the slopes (Kd) and x-axis intercepts (B_{max}).

(v) Protein concentration estimation

The method used was a modification of that described by Bradford *et al* (1976). Samples of the "membrane" preparations (100 µl) were removed from the freezer and allowed to reach room temperature. At the same time, aliquots (100 µl) of BSA standards (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 mg/ml)

and 2 BSA quality controls (0.4 and 0.8 mg/ml) were also removed and allowed to thaw. "Membrane" preparations were incubated with 2M NaOH (100 µl) for 1 hour at 60°C. These were allowed to cool and 2M HCl (100 µl) was added to neutralise the alkali. Triplicate 12 x 75 mm glass culture tubes received either buffer, a BSA standard, a quality control or a dilution of a "membrane" preparation sample (20 µl). Bio-Rad Protein Dye Assay Reagent Concentrate was diluted in distilled H_20 (1:5), mixed and filtered through Whatman Number One filter paper. The reagent solution (1 ml) was added to the tubes which were mixed briefly with a vortex mixer. Aliquots (200 µl) of each solution were placed in the wells of a 96-flat bottomed well microtitreplate. Optical densities (OD) were read in a Bio-Rad automatic plate reader at 595 nm. Protein concentrations were determined by comparison of sample OD with those of the standard curve. The mean of the three values was calculated. Standard curves were discounted and reconstructed with fresh BSA aliquots if the quality control values varied by more than ± 10%.

2.25 Measurement of EGF receptors by static immunocytochemistry

(i) Preparation of immunohistochemistry slides

Cells were harvested by trypsinisation as previously described. Cell pellets were washed three times in RPMI 1640 by centrifugation at 1000 g for 5 min in order to remove serum. A single cell suspension was prepared by gentle passage through a pastette, and the cells placed onto multispot slides at approximately $2x10^4$ cells/spot. The slides were air-dried and then fixed in

acetone:methanol (1:1) for 5 min. Slides were stored at -20°C and wrapped in foil until use.

ii) Immunoperoxidase Staining

The method used was a modification of that described by Hsu *et al*, (1981). A schematic diagram showing the method of detection of EGF receptors is shown in figure 2.4.

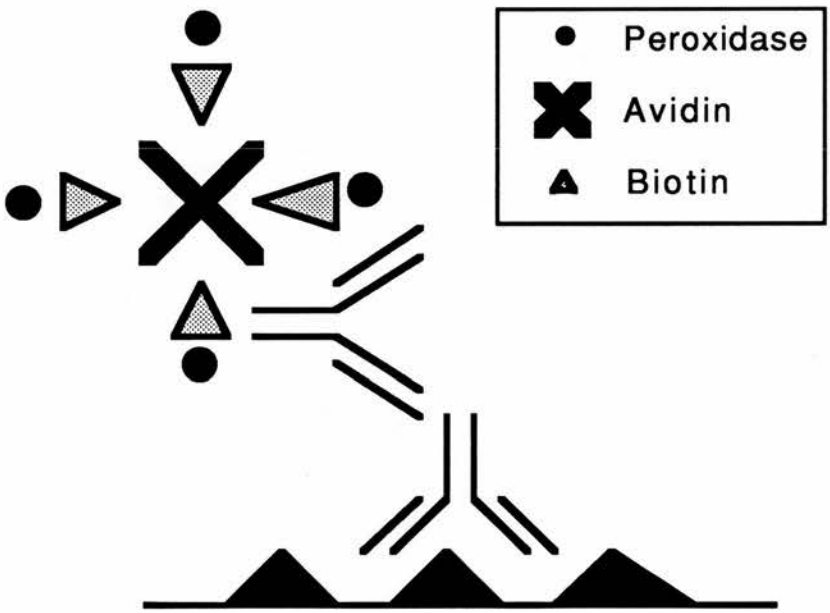

Figure 2.4. Schematic representation of indirect immunoperoxidase staining using avidin-biotin complex with DAB as substrate.

Slides were removed from foil and allowed to reach room temperature. They were then incubated for 10 min at room temperature in 3% (v/v) hydrogen peroxide in methanol to block endogenous peroxidase activity. The slides were washed in tris buffer (TB), pH 7.6 for 5 min and cells incubated with normal rabbit serum (15 µl) diluted in TB (1:5) for 10 min. Excess serum was tapped off gently and cells were then incubated with a murine monoclonal antibody (15 µl) for 30 min. All incubations were carried out in a humidified atmosphere to prevent the slides from drying. The slides were washed in TB for 10 min, and cells incubated for a further 30 min with biotinylated rabbit anti-mouse immunoglobulin (15 µl) diluted in TB (1:200). After washing with TB, freshly prepared Avidin Biotin Complex (ABC) (15 µl) was applied to cells and left for 30 min in the dark. A final TB wash was given before peroxidase was localised using a fresh mixture of 1% (w/v) 3, 3 diaminobenzidine tetrahydrochloride (DAB) and 0.01% (v/v) hydrogen peroxide in Tris imidazole buffer (15 µl), pH 7.6 for 10 min. After washing with tap water, cells were counterstained with hematoxylin. The blue stain was intensified by dipping the slides briefly in a saturated solution of lithium chloride. The cells were dehydrated and cleared with xylene or histoclear, mounted with DPX on coverslips and air-dried overnight. They were then scored for the presence and intensity of positive staining by at least 2 independent readers. In each staining run, the murine monoclonal antibodies were replaced with TB as a negative control, and the monoclonal antibody Cam 5.2 which reacts against cytokeratin (Makin *et al*, 1984) was included as a positive control.

The presence of the EGF receptor was identified using the murine monoclonal antibody EGFR1, which was raised against the A431 cell line (Waterfield *et al*, 1982) and with the F4 antibody, raised against a synthetic peptide from the cytoplasmic domain of the EGF receptor (residues 985-996) (Gullick *et al*, 1986). The optimal dilutions were calculated by titrating the antibodies, and staining the vulval carcinoma cell line A431 which overexpresses EGF receptors (Merlino *et al*, 1984), and the small cell lung cancer cell line NIH-H69 which does not express the EGF receptor (Gamou *et al*,1987; Haeder *et al*, 1988). The dilution at which no positive staining was seen in NIH-H69 cells but was present in A431 cells was used to investigate the expression of EGF receptors in the three ovarian cell lines, PEO1, PEO4, and PEO14. The dilutions routinely used were 1:100 for EGFR1 and 1:50 for F4, both diluted in TB. A431 and NIH-H69 cells were included in each staining run as additional positive and negative controls.

2.26 Measurement of EGF receptor expression by flow cytometry

Cells were plated out in 6-well plates in RPMI 1640 plus 10% FCS, incubated for 24 hours, and media with or without the addition of growth factor or hormone added as described for the growth assays. Cells were cultured for 5 days, media being replenished on day 3. Aliquots of 10^6 cells were removed from the plastic and washed in ice-cold PBS by centrifugation. To each tube EGFR1 antibody (100 µl) diluted in 5% FCS/PBS was added and incubated for 30-60 min on ice. The antibody was omitted and replaced by FCS/PBS (100 µl) as a negative control. The cells were washed in cold FCS/PBS and incubated for 60 min on ice with

sheep anti-mouse immunoglobulin conjugated with FITC (100 µl) diluted 1:20 in FCS/PBS. Cells were then washed twice in cold FCS/PBS and resuspended in PBS (1 ml). Samples were kept on ice until analysed on the flow cytometer. Data was gated on forward and 90° side scatter to exclude dead cells and doublets and the green fluorescence analysed on a logarithmic scale. The intensity of staining was expressed as a ratio to the background fluorescence obtained with the omission of the primary antibody. The vulval carcinoma cell line A431 was analysed as a positive control cell line and the small cell lung carcinoma line NIH-H69 was used as a negative control.

In addition, in some cases, the cell cycle distributions of the cells were examined simultaneously to determine whether EGF receptor expression varied with phases of the cell cycle. Cells which had been stained with EGFR1 were fixed for 30 min in 70% ethanol on ice. They were washed once and then resuspended in 1ml of 5% FCS/PBS. Ribonuclease A (5 mg/ml, 100 µl)) and propidium iodide (100 µg/ml, 100 µl) were added to the cells which were incubated for 15 min at room temperature before being analysed on the flow cytometer.

2.27 Measurement of EGF production in conditioned media

(i) Collection of conditioned media

PEO1, PEO4 and PEO14 cells were grown to 80-90% confluence in phenol red containing RPMI 1640 plus 10% FCS in 175 cm^2 flasks. Cell

monolayers were washed twice in pre-warmed PBS and media were replaced with phenol red-free RPMI 1640 containing 5% dsc-FCS and incubated for 24 hours. Media were discarded and replaced with fresh RPMI 1640 plus 5% dcs-FCS (50 ml). Cells were incubated for a further 48 hours. Media were then removed and centrifuged at 1000 g for 5 min to remove debris, before freezing in a MeOH/CO_2 bath. The frozen media were then freeze-dried using an Edwards freeze dryer. The lyophilised media were reconstituted to 1/10 of the original volume with distilled H_2O and dialysed against PBS for 12 hours. The media were lyophilised for a second time, followed by reconstitution in distilled H_2O to 1/10 of the secondary volume resulting in 100 x concentrates. These were then dialysed twice against PBS for 12 hours and for 48 hours and stored at -80°C.

(ii) Radioimmunoassay (RIA)

The presence of immunoreactive EGF in conditioned media was investigated using a liquid phase competitive radioimmunoassay as described by McDonald *et al* (1990).

Varying concentrations (0.14-10 ng/ml) of human EGF (100 µl) were incubated with 15,000 cpm of ^{125}I EGF (100 µl) and a fixed concentration (1:10,000) of sheep anti-human EGF polyclonal IgG (100 µl). All solutions were made up in 0.01 M PBS. The mixture was vortex-mixed and incubated for 2 hours at 37°C. A cross-linking antibody (donkey anti-sheep polyclonal IgG diluted 1:20 in 1:200 normal sheep serum) (250 µl) was then added and the solutions incubated overnight at 4°C. An aliquot of distilled H_2O (500 µl)

was added to each tube, and bound and free ^{125}I EGF were then separated by centrifugation at 3500 rpm at 4°C and the supernatant (containing free ^{125}I EGF) was removed, and the pellet counted for 1 min in a Multiprias Gamma counter. Each sample was assayed in duplicate. Non-specific binding was estimated by measurement of binding in the absence of unlabellled EGF and sheep anti-human EGF antiserum. Estimation of maximum binding (B_0) was performed by binding ^{125}I EGF to the antiserum in the absence of unradioactive EGF. For estimation of quantity of EGF-like activity in conditioned medium, samples (100 µl) were incubated as above but in the absence of unlabelled EGF. To enable the quantity of EGF to be estimated standard curves were constructed of the ratio of % bound compared with B_0, against the log of the concentration of unlabelled EGF. The quantity of EGF-like activity was estimated by comparison with the standard curve. The method of EGF detection was validated by calculating the quantity of EGF-like activity in samples of known EGF concentrations, and verifying that dilution of the samples revealed concentrations of EGF which decreased in parallel with the standard curve. No cross reactivity was found with murine EGF or human TGF-α.

(iii) Radioreceptor assay (RRA)

A similar method to that described in the ligand binding assay was used to test for the presence of EGF-like material in conditioned media from the ovarian carcinoma cell lines. Briefly, rat liver membrane preparations (100 µl) were incubated with a fixed concentration (10,000 cpm equivalent to approximately 0.01 nM) of ^{125}I EGF (100 µl) and either varying

concentrations (0.024-300 nM) of unlabelled EGF (200 µl) or aliquots of conditioned media (200 µl) at 26°C for 90 min. The reaction was terminated as described previously. A standard curve was constructed to show the relationship between displacement of ^{125}I EGF and concentration of unlabelled EGF. Displacement of ^{125}I EGF produced by conditioned media was compared with the standard curve to give an estimation of the EGF-like activity present.

CHAPTER 3

RESULTS

3.1 Growth characteristics of the ovarian carcinoma cell lines

The plating efficiencies and doubling times of PEO1, PEO4 and PEO14 cells in 24-well plates were investigated to determine the optimal plating concentrations of cells for future experiments, which involved the modulation of their growth rates.

3.11 Plating Efficiencies

Plating efficiencies of the cell lines were investigated in cells which had been harvested at mid-log phase. Results are shown in table 3.1. Whilst some variation in the plating efficiencies was observed between experiments, PEO1 cells showed consistently higher values than PEO4 and PEO14 cell lines, both of which demonstrated similar plating efficiencies.

Table 3.1. Plating efficiencies of PEO1, PEO4 and PEO14 cell lines. A known number of viable cells were plated in 24-well plates in RPMI 1640 containing 10% FCS. Cells were incubated for 24 hours, to allow cells to settle, harvested by trypsinisation, and counted using a haemocytometer. Plating efficiencies are expressed as a percentage of cells attached compared with cell numbers seeded. Mean ± S. E. are shown for 4 separate experiments.

Cell Line	Cell no. plated	Plating Efficiency (%)
PEO1	2.5×10^4	77 ± 16
PEO4	2.5×10^4	51 ± 7
PEO14	5×10^4	56 ± 11

3.12 Doubling Times

The rates of growth of the three ovarian cell lines in RPMI 1640 containing 10% FCS were measured over 13 days by cell counts. Results from representative experiments are shown in figures 3.1 a,b and c. For each of the cell lines, cell numbers decreased on day 1, due to their plating efficiencies, before increases in cell numbers were noted. Cell numbers were converted to logarithmic values to enable the doubling times to be calculated. Logarithmic plots are shown in figures 3.2 a, b and c. Doubling times were calculated from the steepest part of this curve over a minimum period of 4 days. A summary of the doubling times of PEO1, PEO4 and PEO14 cells is shown in table 3.2.

The ER-negative cell line, PEO14 has a longer doubling time than the two ER-positive cell lines.

Table 3.2. Doubling times of PEO1, PEO4 and PEO14 cell lines in RPMI 1640 containing 10% FCS in hours. Table shows the mean ± S.E of 3 experiments.

Cell line	Doubling time (hours)
PEO1	32 ± 7
PEO4	47 ± 4
PEO14	66 ± 11

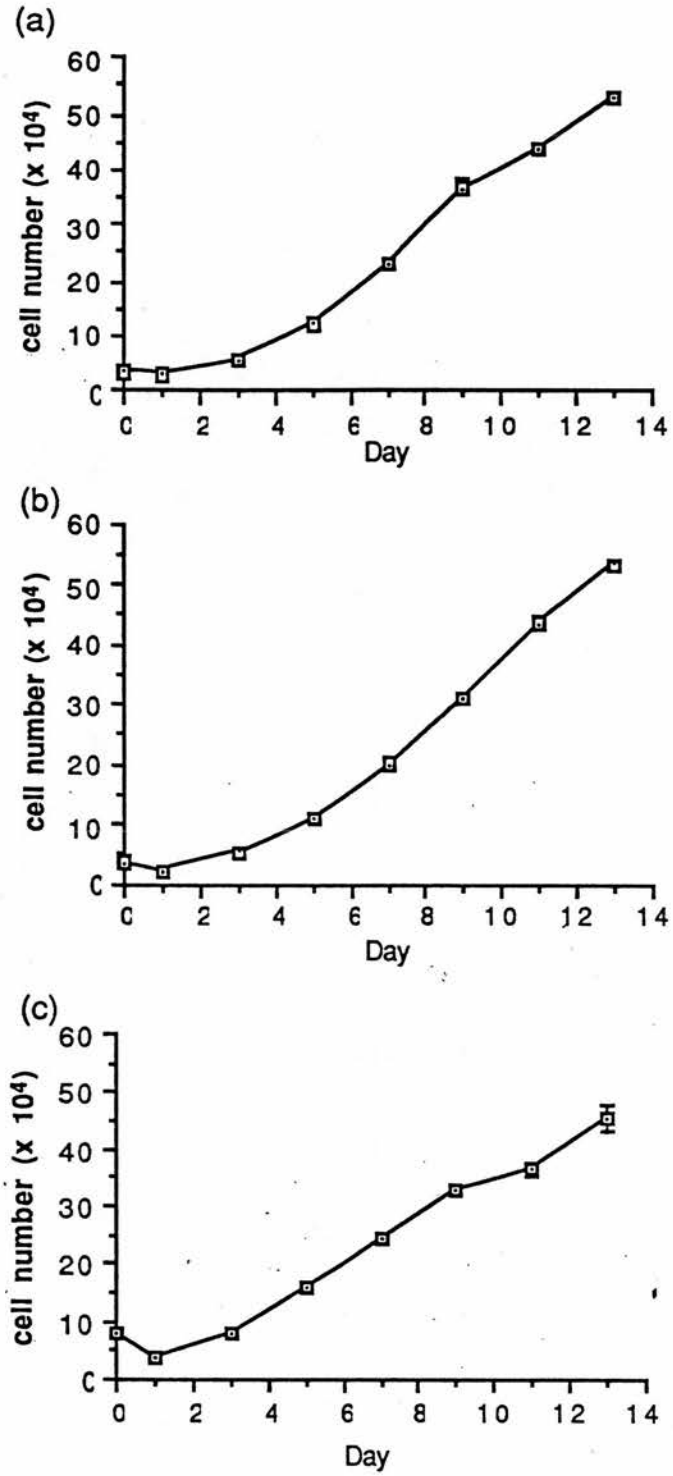

Figure 3.1. Growth of the (a) PEO1, (b) PEO4 and (c) PEO14 cell lines in RPMI 1640 containing 10% FCS. Cells were plated at a density of approximately 2.5 x 10^4/well for PEO1 and PEO4 cells and 5 x 10^4/well for PEO14 cells in 24-well plates. Media were replenished on every second day. Cells were counted on days 0, 1, 3, 5, 7, 9, 11 and 13. Triplicate counts of each sample were taken and each time point was performed in quadruplicate. Figure shows values for mean ± S. E of a representative experiment. Where error bars are not visible, the S.E. was small.

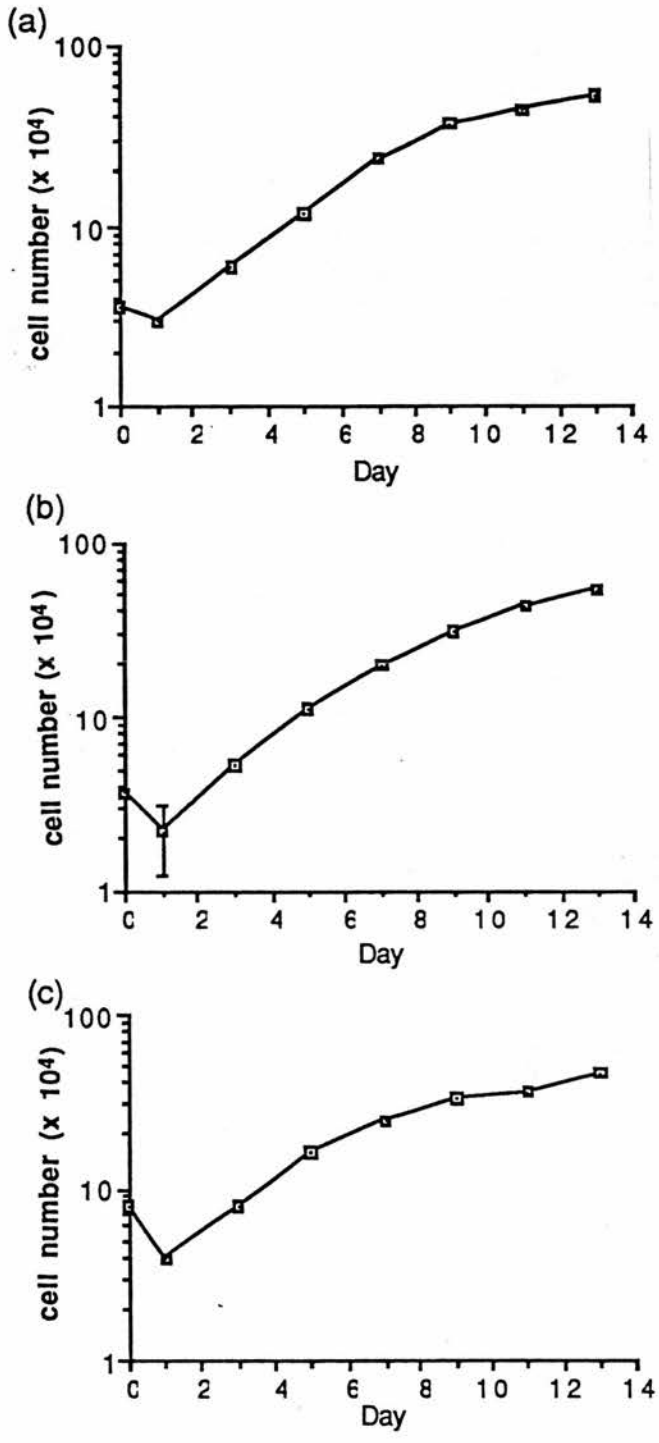

Figure 3.2. Logarithmic plots of growth of (a) PEO1, (b) PEO4 and (c) PEO14 cells grown in RPMI 1640 containing 10% FCS as described in figure 3.1. Data is transformed from figure 3.1. Where error bars are not visible, the S.E is small.

3.13 Effect of dextran-charcoal stripping of serum on growth rate

As the majority of growth experiments were performed to determine the effects of oestrogens and growth factors on cell growth, and since serum may contain similar factors, these studies were performed in phenol red free RPMI 1640 containing 5% dcs-FCS. It was therefore of interest to investigate the effect of dextran-charcoal stripping of serum and the absence of phenol red, on the rates of growth of the ovarian cell lines. Representative experiments for the PEO4 and PEO14 cell lines are shown.

PEO4 cell line

The results from a representative experiment, showing the effect of removal of phenol red and dextran-charcoal stripping of the serum, on the growth of PEO4 cells is illustrated in figure 3.3. Incubation of the cells in phenol red-free medium containing dcs-FCS produced a substantial decrease in the growth of the cells which was observed after both 4 and 7 days in culture.

PEO14 cell line

The effects of dextran-charcoal stripping of serum on the growth of the PEO14 cell line are shown in figure 3.4. After 4 and 7 days in culture, the growth of the PEO14 cells was reduced in the phenol red-free medium containing 10% dcs-FCS compared with control cells maintained inRPMI 1640 containing phenol red, supplemented with 10% FCS. These effects were similar but less marked than those in the PEO4 cell line.

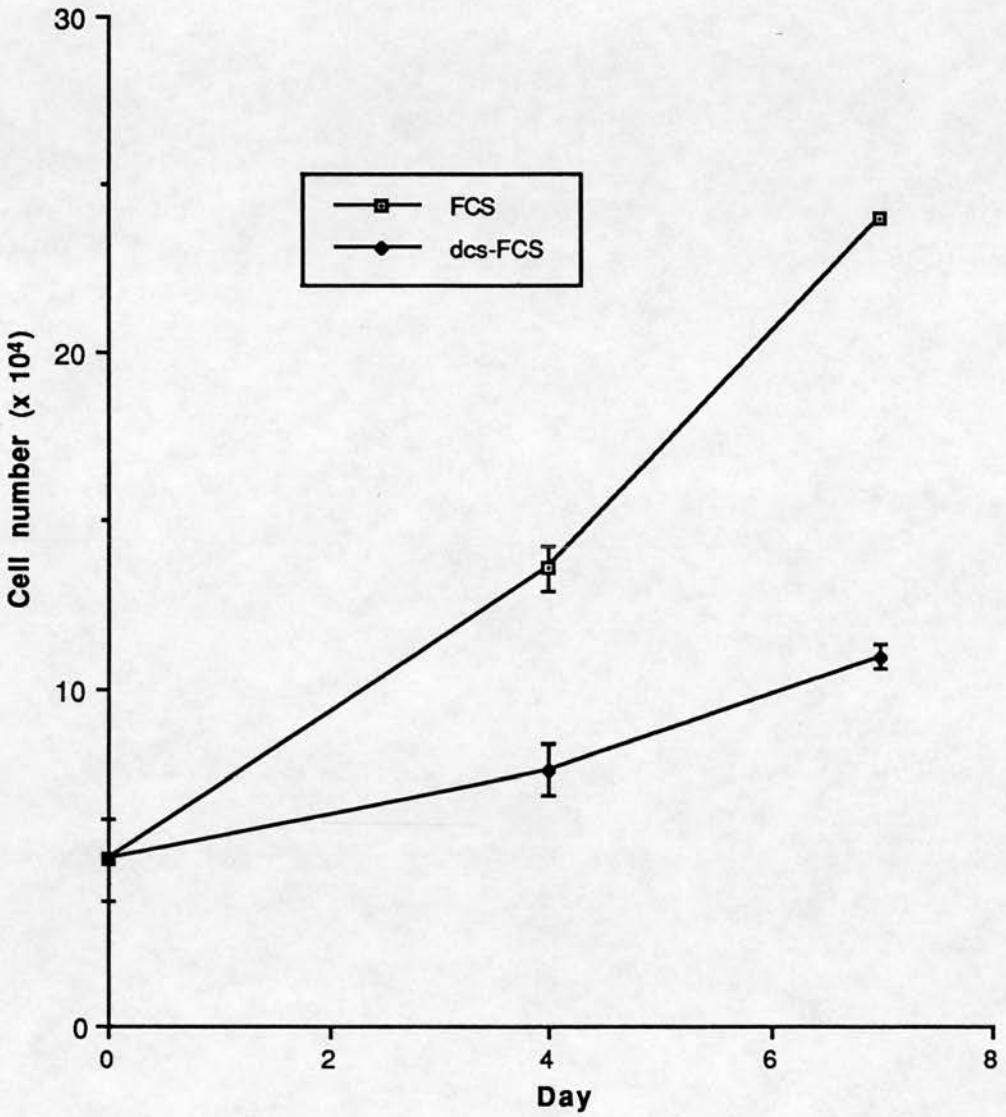

Figure 3.3. The effect of dextran-charcoal stripping (dcs) of serum on the growth of PEO4 cells. Cells were cultured in RPMI 1640 containing phenol red and 10% FCS or without phenol red plus 10% dcs-FCS for 7 days. Cells were harvested by trypsinisation and counted using a Coulter Counter on days 0, 4 and 7. The figure shows the mean ± S. E. of quadruplicate values obtained from a representative experiment. The experiment was performed on three separate occasions with similar results.

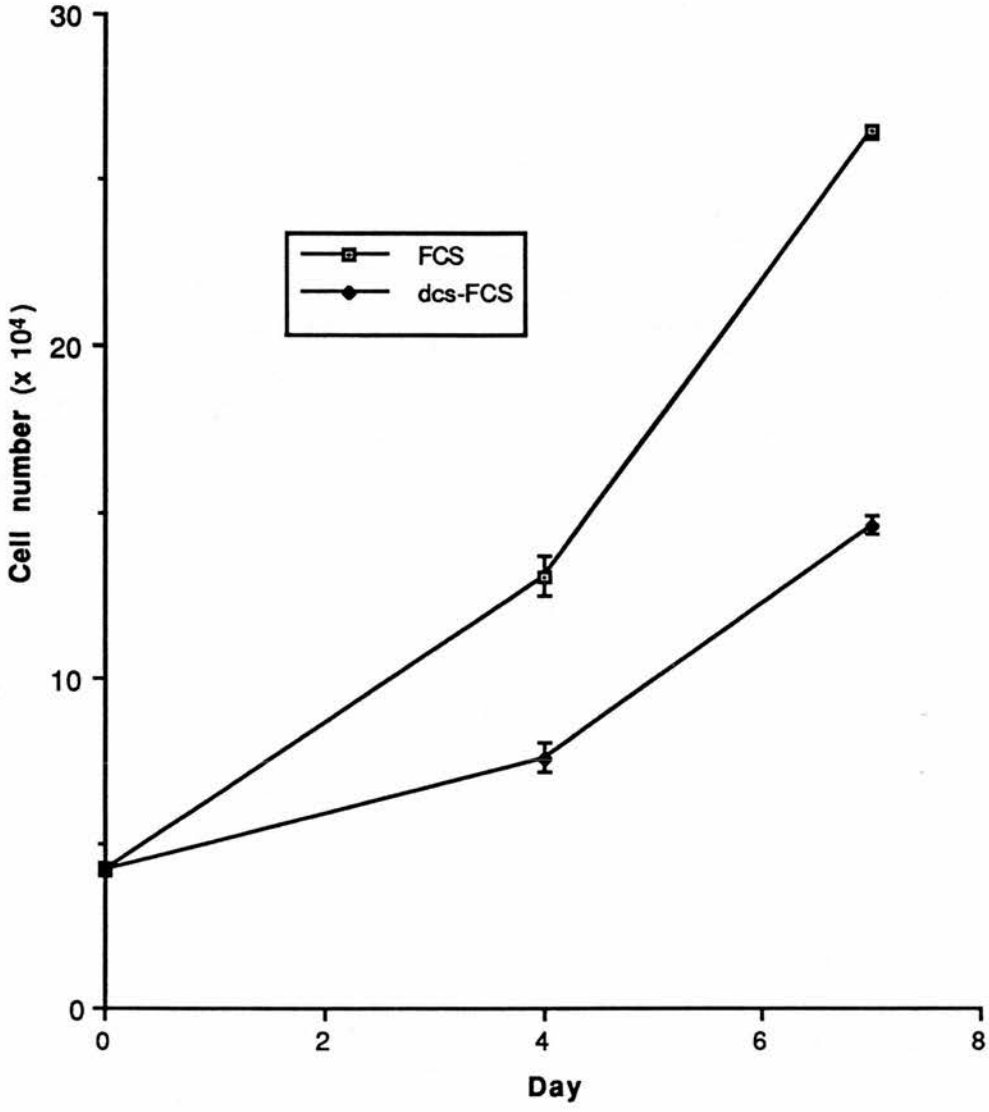

Figure 3.4. The effect of dextran-charcoal stripping (dcs) of serum on the growth of PEO14 cells. Cells were cultured in RPMI 1640 containing phenol red and 10% FCS or without phenol red plus 10% dcs-FCS for 7 days. Cells were harvested by trypsinisation and counted using a Coulter Counter on days 0, 4 and 7. The figure shows the mean ± S. E. of quadruplicate values obtained from a representative experiment. The experiment was performed on three separate occasions with similar results.

3.2 Modulation of cell growth by oestrogen/anti-oestrogens

The oestrogen receptor (ER) content of the three ovarian cell lines had previously been measured by Dr R A Hawkins, Department of Surgery, Royal Infirmary (Edinburgh), using the dextran-coated charcoal adsorption assay (Hawkins *et al*, 1975, Hawkins *et al*, 1981). These results are shown in table 3.3. Moderate to high levels of ER were detected in the PEO1 and PEO4 cell lines whereas ER were not detected in PEO14 cells. Therefore, the effects on the growth of the three ovarian cell lines of the exogenous addition of factors which interact with the ER were investigated, to determine whether growth sensitivities were consistent with the presence of receptors.

Table 3.3. ER content of PEO1, PEO4 and PEO14 cell lines. The ER contents were determined on 3 separate occasions using a dextran-coated charcoal adsorption assay. Assays were performed on cells which were in the early plateau phase of growth. Table shows range and median values (fmol/mg protein) and binding affinities (M).

Cell line	ER (fmol/mg protein)	Range	Kd (M)
PEO1	96	73 - 145	0.75×10^{-10}
PEO4	112	60 - 203	0.66×10^{-10}
PEO14	0	0 - 2	-

3.21 Effects of varying 17 β-oestradiol concentration, as measured by cell number

The effects of the addition of a range of concentrations (10^{-13} to10^{-4} M) of 17 β-oestradiol on the cell lines were examined in the presence of 5% dcs-FCS and in the absence of phenol red in the culture media.

PEO1 cell line

Effects on cell counts after 7 days exposure to 17 β-oestradiol are shown in figure 3.5. Concentrations of 17 β-oestradiol from 10^{-13} to 10^{-9} M produced a progressive increase in cell numbers. At 10^{-9} M a maximal stimulation of approximately 80% above control values was observed. Concentrations between 10^{-8} and 10^{-5} M were also associated with stimulatory effects on proliferation, but were less marked than those at 10^{-9} M. The highest concentration of 17 β-oestradiol tested,10^{-4} M, produced inhibitory effects, cell numbers being substantially less than those plated down.

PEO4 cell line

Results with the PEO4 cell line are shown in figure 3.6. Concentrations of 10^{-13} to 10^{-9} M produced a progressive increase in cell number, with a maximal effect of approximately 90% over control values was observed at 10^{-10} and 10^{-9} M. Concentrations of 17 β-oestradiol between 10^{-8} and 10^{-6} M were also stimulatory. The effects of 17 β-oestradiol were similar to those produced in the PEO1 cell line. However, at 10^{-5} and 10^{-4} M, 17 β-oestradiol produced dramatic growth inhibition.

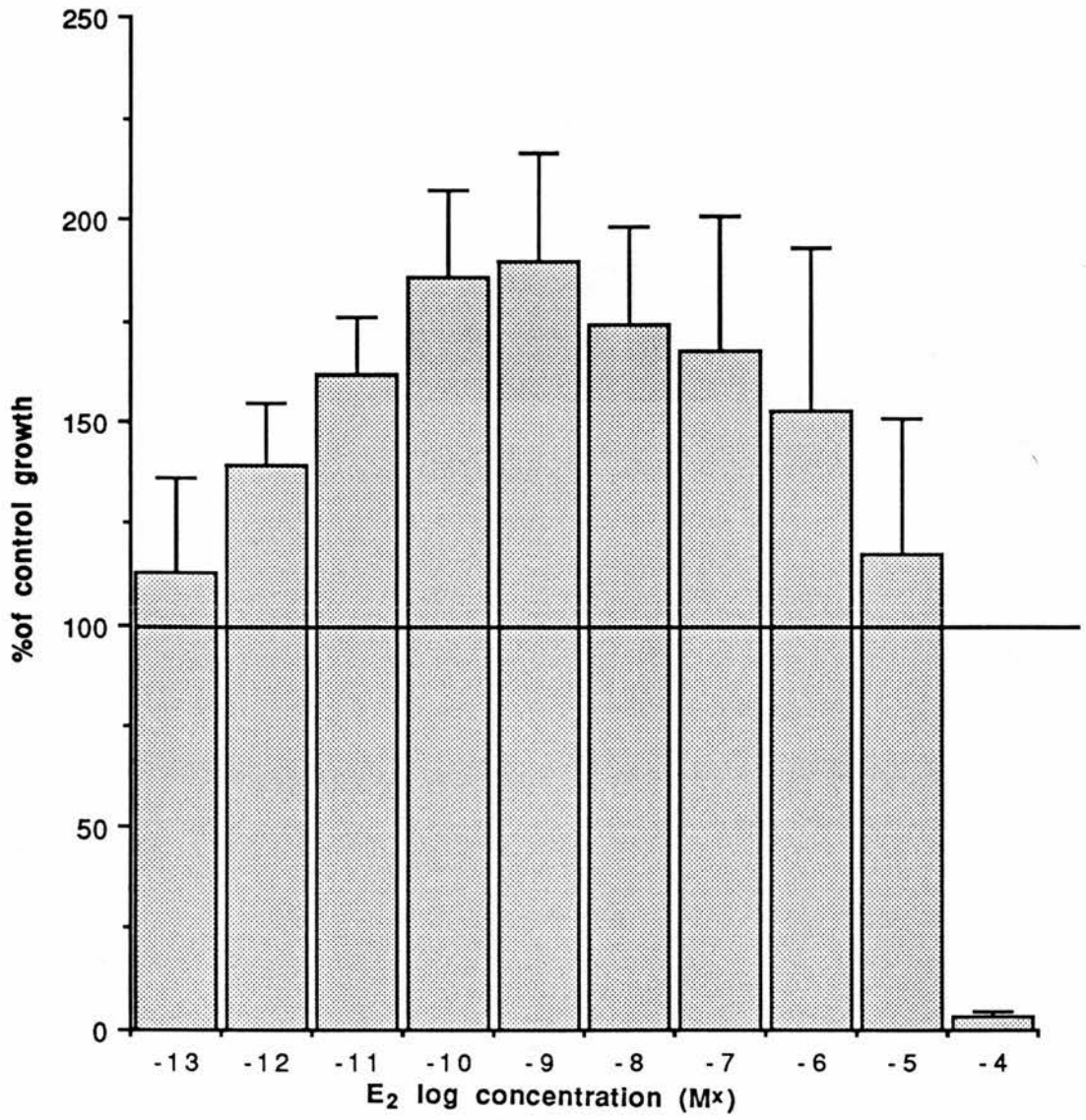

Figure 3.5. Effect of exogenous addition of 17 β-oestradiol (E_2) on the growth of PEO1 cell line. Cells were incubated in phenol red-free RPMI 1640 containing 5% dcs-FCS in the absence or presence of various doses (10^{-13} -10^{-4} M) of E_2 for 7 days. Cells were harvested by trypsinisation and counted using a Coulter Counter. In each experiment triplicate counts for each sample were taken, and each experimental condition was performed in quadruplicate. The figure shows the mean ± S. E. of 3 separate experiments.

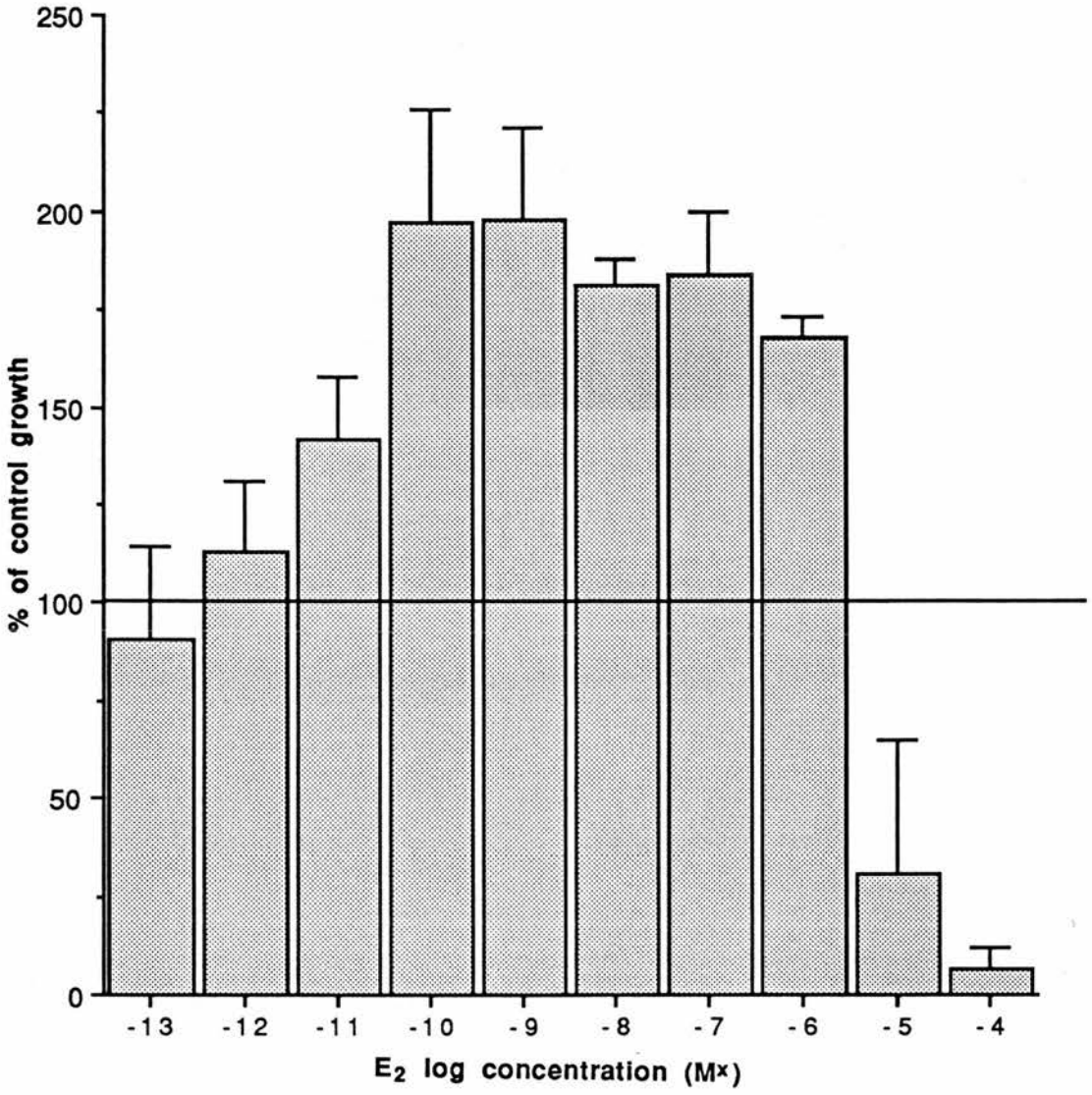

Figure 3.6. Effect of exogenous addition of 17 β-oestradiol (E_2) on the growth of PEO4 cell line. Cells were incubated in phenol red-free RPMI 1640 containing 5% dcs-FCS in the absence or presence of various doses (10^{-13} -10^{-4} M) of E_2 for 7 days. Cells were harvested by trypsinisation and counted using a Coulter Counter. In each experiment triplicate counts for each sample were taken, and each experimental condition was performed in quadruplicate. The figure shows the mean ± S. E. of 3 separate experiments.

PEO14 cell line

These results are shown in figure 3.7. In contrast to PEO1 and PEO4 cells, concentrations of 17 β-oestradiol between 10^{-13} and 10^{-6} M produced negligible effects on cell growth. However, 17 β-oestradiol at 10^{-5} M was inhibitory, and this effect was even more marked at 10^{-4} M.

3.22 Effects of tamoxifen on 17 β-oestradiol-induced growth stimulation

In other cell lines which are ER-positive and sensitive to oestrogen such as the breast cancer cell line, MCF-7, the anti-oestrogen tamoxifen may block the growth stimulatory effect of 17 β-oestradiol (see Introduction). Therefore, the effects of tamoxifen on the growth sensitivities to 17 β-oestradiol were investigated in the ER-positive cell line PEO4, and the ER-negative PEO14 cell line. The antagonistic properties of tamoxifen were tested using concentrations 10,000-fold higher than 17 β-oestradiol. Effects on the cell lines in culture were monitored by measuring cell counts and [^{3}H]-thymidine incorporation.

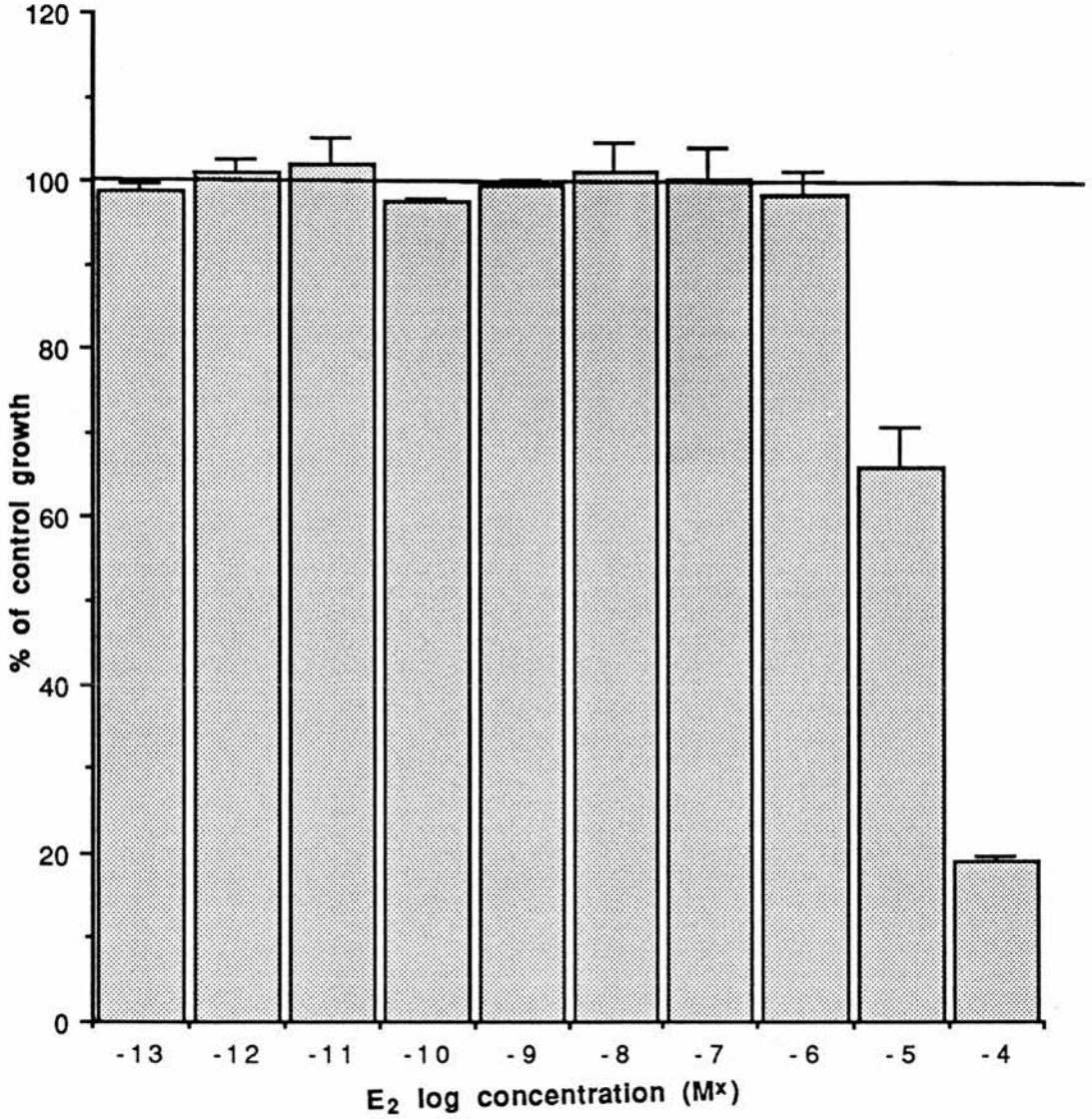

Figure 3.7. Effect of exogenous addition of 17 β-oestradiol (E_2) on the growth of PEO14 cell line. Cells were incubated in phenol red-free RPMI 1640 containing 5% dcs-FCS in the absence or presence of various doses (10^{-13} -10^{-4} M) of E_2 for 7 days. Cells were harvested by trypsinisation and counted using a Coulter Counter. In each experiment triplicate counts for each sample were taken, and each experimental condition was performed in quadruplicate. The figure shows the mean ± S. E. of 3 separate experiments.

(i) Measurement by cell numbers

PEO4 cell line

Cell counts were performed on days 0, 4, and 7 following incubation with 17 β-oestradiol (10^{-10} M), tamoxifen (10^{-6} M) or both in combination. The results are shown in figure 3.8. In comparison with control cells stimulatory effects of 17 β-oestradiol were noted after 4 days, and the difference in cell numbers increased further at 7 days. The presence of 10^{-6} M tamoxifen alone produced a small decrease in cell number compared with controls at day 4, but after 7 days the difference between tamoxifen treated cells and controls was negligible. Cells grown in the combination of 17 β-oestradiol and tamoxifen grew at approximately the same rate as controls.

PEO14 cell line

These results are shown in figure 3.9. The addition of 10^{-10} M 17 β-oestradiol to cultures in phenol red-free RPMI 1640 containing 5% dcs-FCS had no effect on the growth of the PEO14 cell line. This is consistent with previously shown data (figure 3.7). The presence of 10^{-6} M tamoxifen alone or in combination with 17 β-oestradiol had negligible effects on cell numbers after 4 and 7 days in culture.

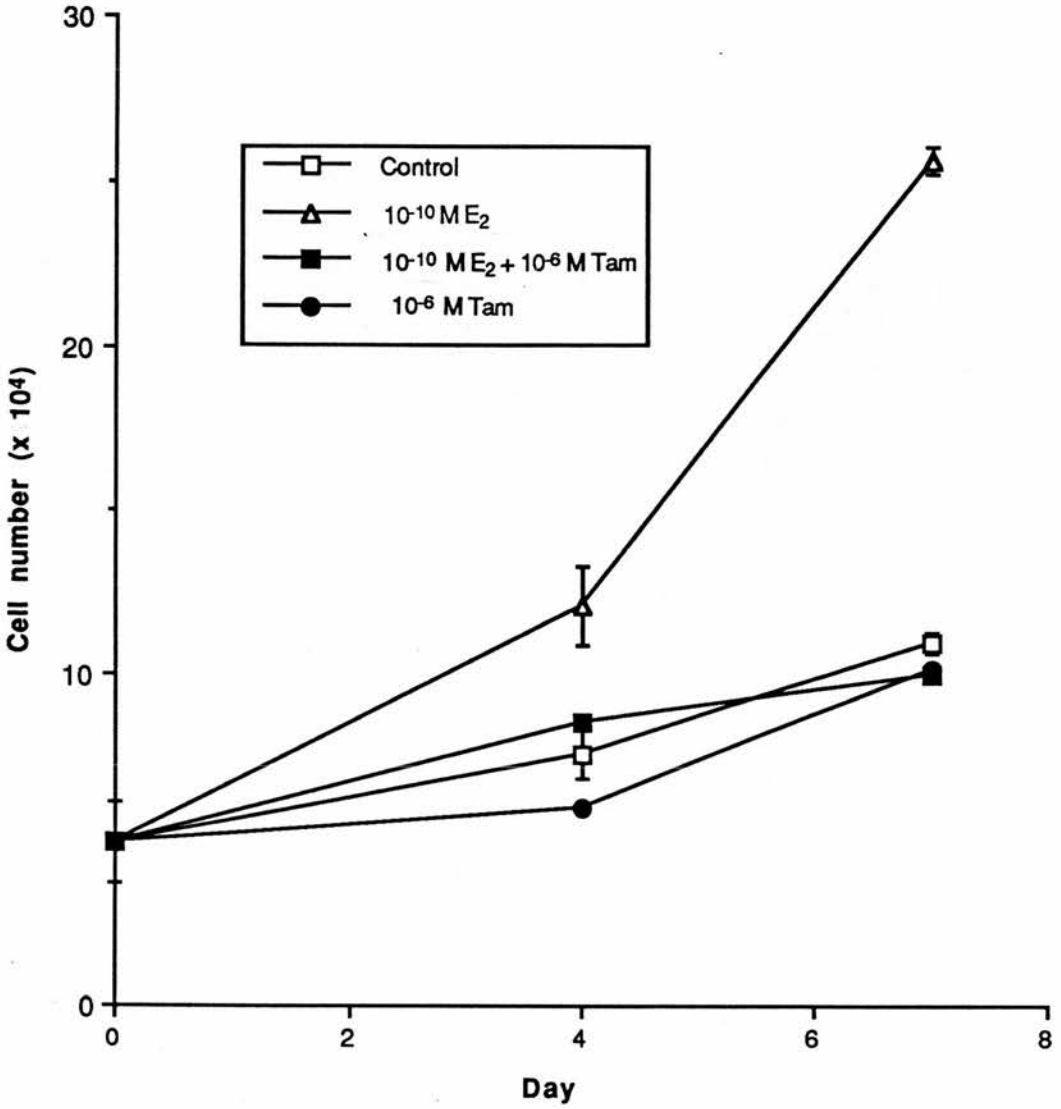

Figure 3.8. Effects of tamoxifen (Tam) on the 17 β-oestradiol (E_2)-induced growth stimulation of PEO4 cells over a 7 day culture period. Cells were incubated in phenol red-free RPMI 1640 containing 5% dcs-FCS. Additions of 10^{-10} M E_2, 10^{-6} M tamoxifen alone or in combination were made to media. Media was replenished on days 2 and 5. Cells were harvested and counted on days 0, 4 and 7 of culture. Triplicate counts for each sample were taken and each experimental condition was performed in quadruplicate. The figure shows the mean ± S. E. obtained from a representative experiment. The experiment was performed on three separate occasions with similar results.

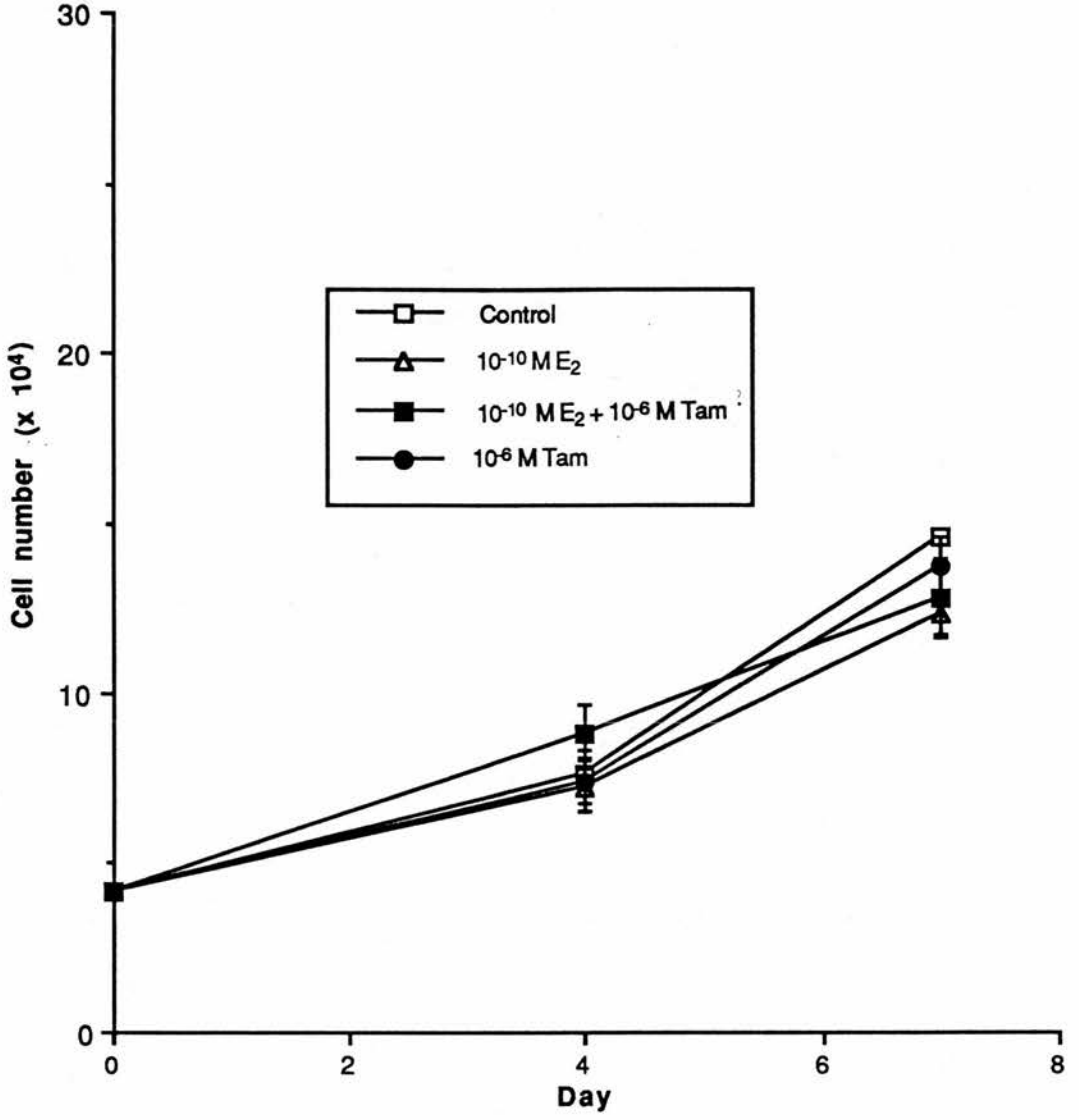

Figure 3.9. Effects of the exogenous addition of 17 β-oestradiol (E_2) and tamoxifen (Tam) on the growth of PEO14 cells over a 7 day culture period. Cells were incubated in phenol red-free RPMI 1640 containing 5% dcs-FCS. Additions of 10^{-10} M E_2 and 10^{-6} M tamoxifen alone or in combination were made to the media. Media was replenished on days 2 and 5. Cells were harvested and counted on days 0, 4 and 7 of culture. Triplicate counts for each sample were taken and each experimental condition was performed in quadruplicate. The figure shows the mean ± S. E. obtained from a representative experiment. The experiment was performed on three separate occasions.

(ii) Measurement by thymidine incorporation

PEO4 cell line

On days 4 and 7 after incubation with or without E_2/tamoxifen, cells were pulsed with [^{3}H]-thymidine (1 μCi/ml) for 2 hours. TCA-precipitatable material was counted in a liquid scintillation counter. Results are shown in figure 3.10. At each time point, in the presence of 10^{-10} M 17 β-oestradiol the incorporation of [^{3}H]-thymidine into DNA was above that observed in control PEO4 cells, which were grown in phenol red free RPMI 1640 plus 5% dcs-FCS. As was noted in the cell growth experiments (figure 3.8), incubation with 10^{-6} M tamoxifen reduced the effects of 17 β-oestradiol so that incorporation of [^{3}H]-thymidine was equivalent to control levels. Incubation with 10^{-6} M tamoxifen alone produced negligible effects on the amount of [^{3}H]-thymidine which was incorporated compared with controls.

PEO14 cell line

These results are shown in figure 3.11. The level of [^{3}H]-thymidine incorporation into PEO14 cells was unaffected by incubation with 17 β-oestradiol, 10^{-6} M tamoxifen or in combination at either day 4 or 7.

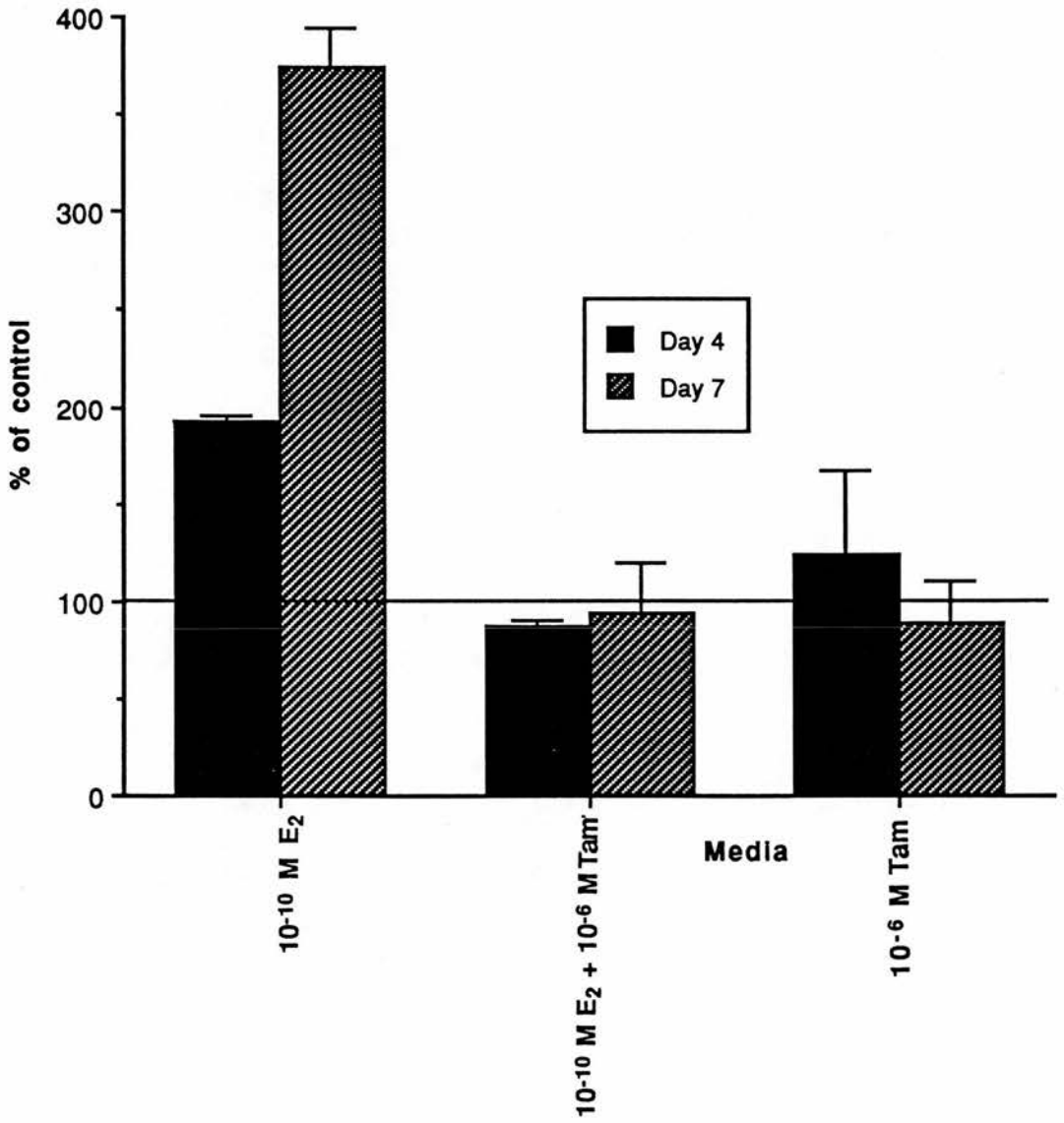

Figure 3.10. The effects of 17 β-oestradiol (E_2) and tamoxifen (Tam) alone or in combination on [^{3}H]-thymidine incorporation by PEO4 cells after 4 and 7 days in culture. Cells were incubated in phenol red-free in RPMI 1640 containing 5% dcs-FCS. Additions of 10^{-10} M E_2, 10^{-6} M tamoxifen or 10^{-10} M E_2 and 10^{-6} M tamoxifen together were made to media. Media was replenished on days 0 and 2. Cells were pulsed with [^{3}H]-thymidine (1 μCi / ml) for 2 hours before harvesting. Incorporated radioactivity was counted for 4 min in a liquid scintillation counter. The figure shows the mean ± S.E of triplicate counts, expressed as a % of control (untreated) cells, from a representative experiment. The experiment was performed on 2 occasions with similar results.

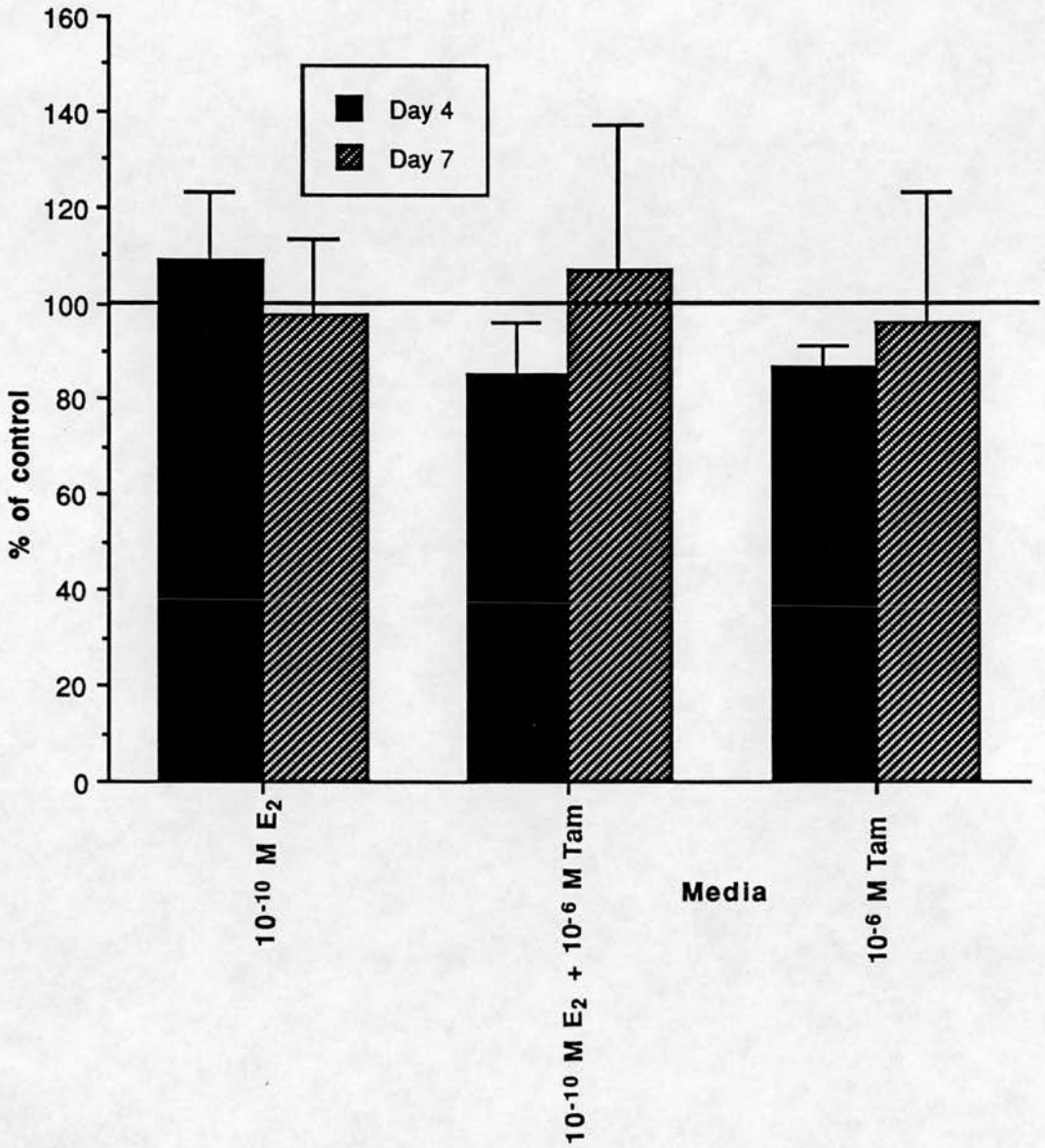

Figure 3.11. Effects of the exogenous addition of 17 β-oestradiol (E_2) and tamoxifen (Tam) alone or in combination on the incorporation of ^{3}H-thymidine into PEO14 cells after 4 and 7 days in culture. Cells were incubated in phenol red-free in RPMI 1640 containing 5% dcs-FCS. Additions of 10^{-10} M E_2, 10^{-6} M tamoxifen or 10^{-10} M E_2 and 10^{-6} M tamoxifen together were made to media. Media was replenished on days 0 and 2. Cells were pulsed with [^{3}H]-thymidine (1 μCi / ml) for 2 hours before harvesting. Incorporated radioactivity was counted for 4 min in a liquid scintillation counter. The figure shows the mean ± S.E of triplicate counts, expressed as a % of control (untreated) cells, from a representative experiment. The experiment was performed on 2 occasions with similar results.

3.23 Effects of ICI 164,384 on 17 β-oestradiol-induced growth stimulation

Recently, a new class of "pure" antioestrogens, which are steroidal in structure, have been developed. One of these compounds, ICI 164,384 was investigated. The effects of 10^{-8}, 10^{-7} and 10^{-6} M ICI 164,384 on the growth stimulation induced by 10^{-10} M 17 β-oestradiol in PEO4 cells was measured by cell counts, performed following 6 days in culture.

PEO4 cell line

Cell counts relative to control cells following 6 days culture with 17 β-oestradiol in the presence and absence of ICI 164,384 are shown in figure 3.12. Incubation with 10^{-10} M 17 β-oestradiol stimulated growth by approximately 120% above controls. In the presence of 10^{-8} M ICI 164,384 this stimulation was reduced so that growth was approximately 50% over controls. Increasing concentrations produced further decreases in the stimulation. The highest concentration of 10^{-6} M ICI 164,384 essentially blocked the growth stimulation of 10^{-10} M 17 β-oestradiol.

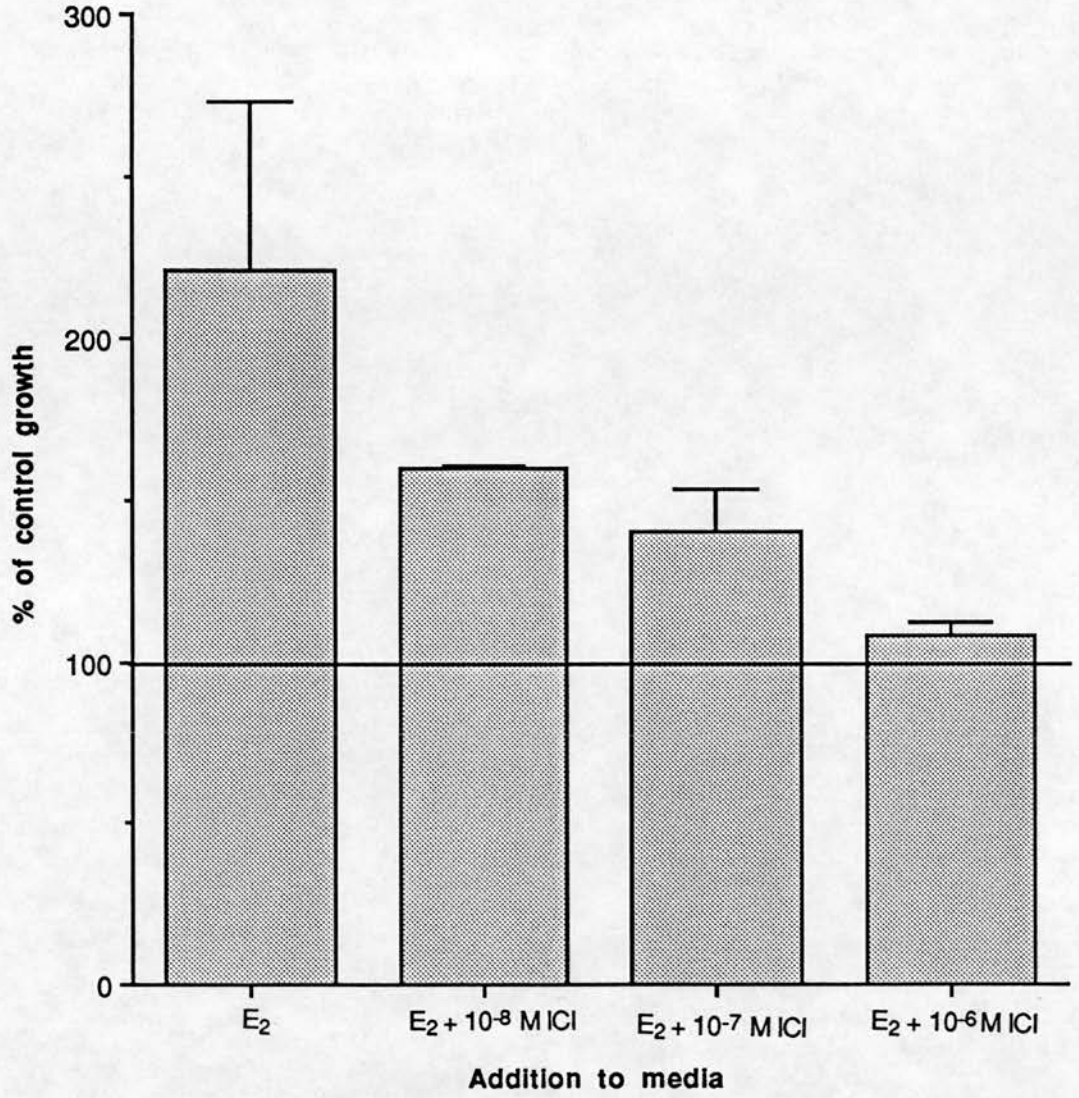

Figure 3.12 Antagonism of the 17 β-oestradiol (E_2)-induced growth stimulation of PEO4 cells by the "pure" antioestrogen, ICI 164,384. Cells were incubated in phenol red-free RPMI 1640 containing 5% dcs-FCS in the absence or presence of 10^{-10} M E_2 for 6 days. The ability of the anti-oestrogen to block the growth stimulation was tested at 10^{-8}, 10^{-7} and 10^{-6} M. Cells were harvested by trypsinisation and counted using a Coulter Counter. Results are expressed relative to growth of untreated cells. The figure represents the mean ± S.E. of two experiments.

The effects of incubation with concentrations of ICI 164,384 alone are shown in figure 3.17.

3.24 Effects of varying tamoxifen concentration, as measured by cell counts

The effects of incubating the cell lines with a range of concentrations (10^{-12} to 10^{-5} M) of tamoxifen were investigated in phenol red-free RPMI 1640 containing 5% dcs-FCS. Studies were performed using the ER-positive cell lines PEO1 and PEO4, and the ER-negative cell line PEO14.

PEO1 cell line

Effects on cell growth after 6 days exposure to tamoxifen are shown in figure 3.13. Concentrations between 10^{-12} and 10^{-7} M stimulated cell numbers up to approximately 10-20% above control cells cultured in RPMI 1640 containing 5% dcs-FCS. The stimulation was statistically significant by the Student's t-test at all concentrations except 10^{-12} M. Maximal stimulation was noted at 10^{-9} M. Cell numbers were unaffected following incubation with 10^{-6} M tamoxifen. The highest concentration of tamoxifen which was 10^{-5} M produced significant, dramatic inhibitory effects on growth.

PEO4 cell line

Results for the PEO4 cell line are shown in figure 3.14. The effects of tamoxifen on PEO4 cells were similar to those produced in the PEO1 cell line, although all concentrations between 10^{-12} and 10^{-6} M stimulated cell growth. The stimulation was significant by the Students t-test for all concentrations with the exception of the lowest concentration of 10^{-12} M. Maximal stimulation of approximately 20% was observed at 10^{-7} M. As for the PEO1 cell line, 10^{-5} M produced a significant inhibition of cell growth.

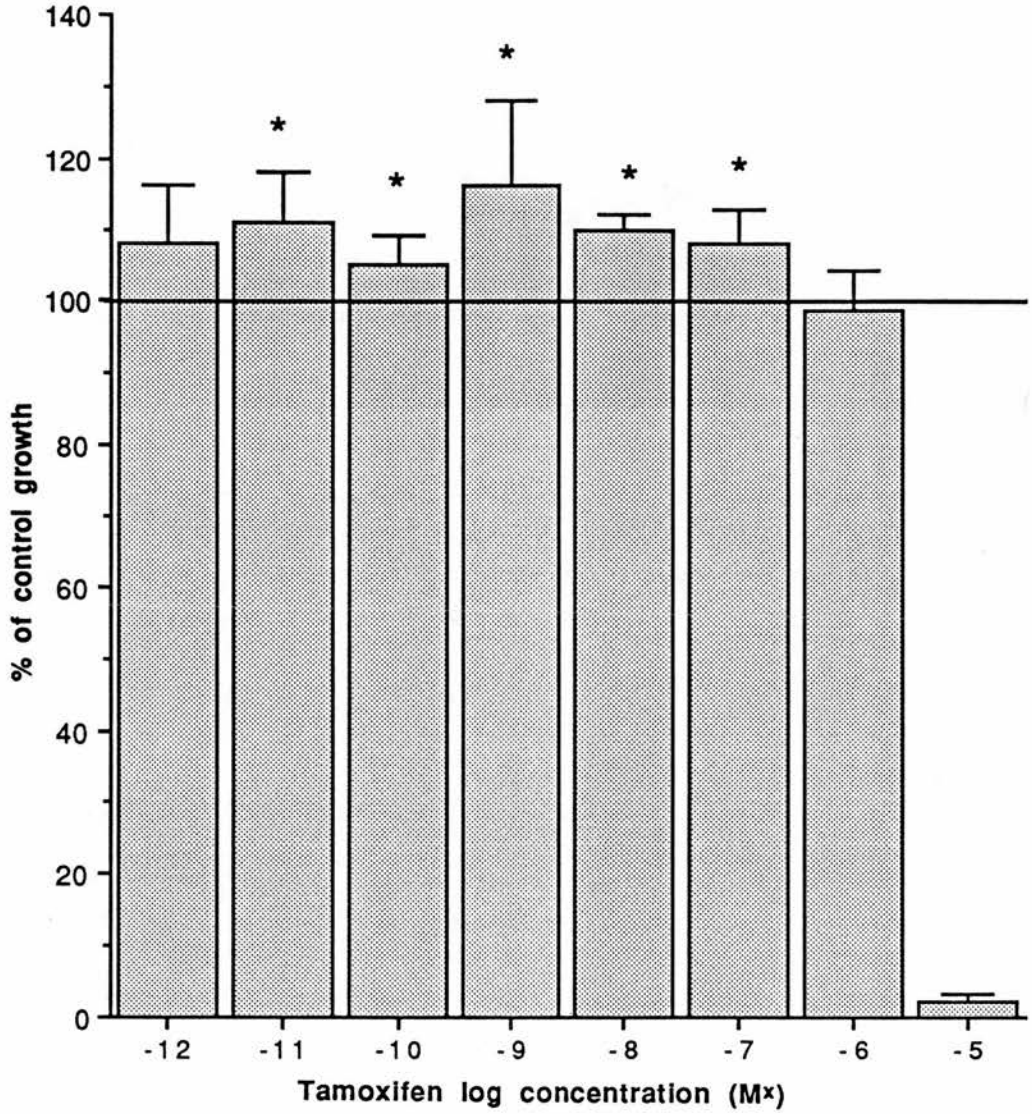

Figure 3.13. Effects of the exogenous addition of tamoxifen on the growth of PEO1 cell line. Cells were incubated in phenol red-free RPMI 1640 containing 5% dcs-FCS in the absence and presence of various concentrations (10^{-12}-10^{-5} M) of tamoxifen for 6 days. Cells were harvested by trypsinisation and counted using a Coulter Counter. In each experiment triplicate counts for each sample were taken, and each experimental condition was performed in quadruplicate. Results are expressed relative to growth of untreated cells. The figure shows the mean ± S.E. of 3 separate experiments. * $P < 0.05$, according to Students' t-test.

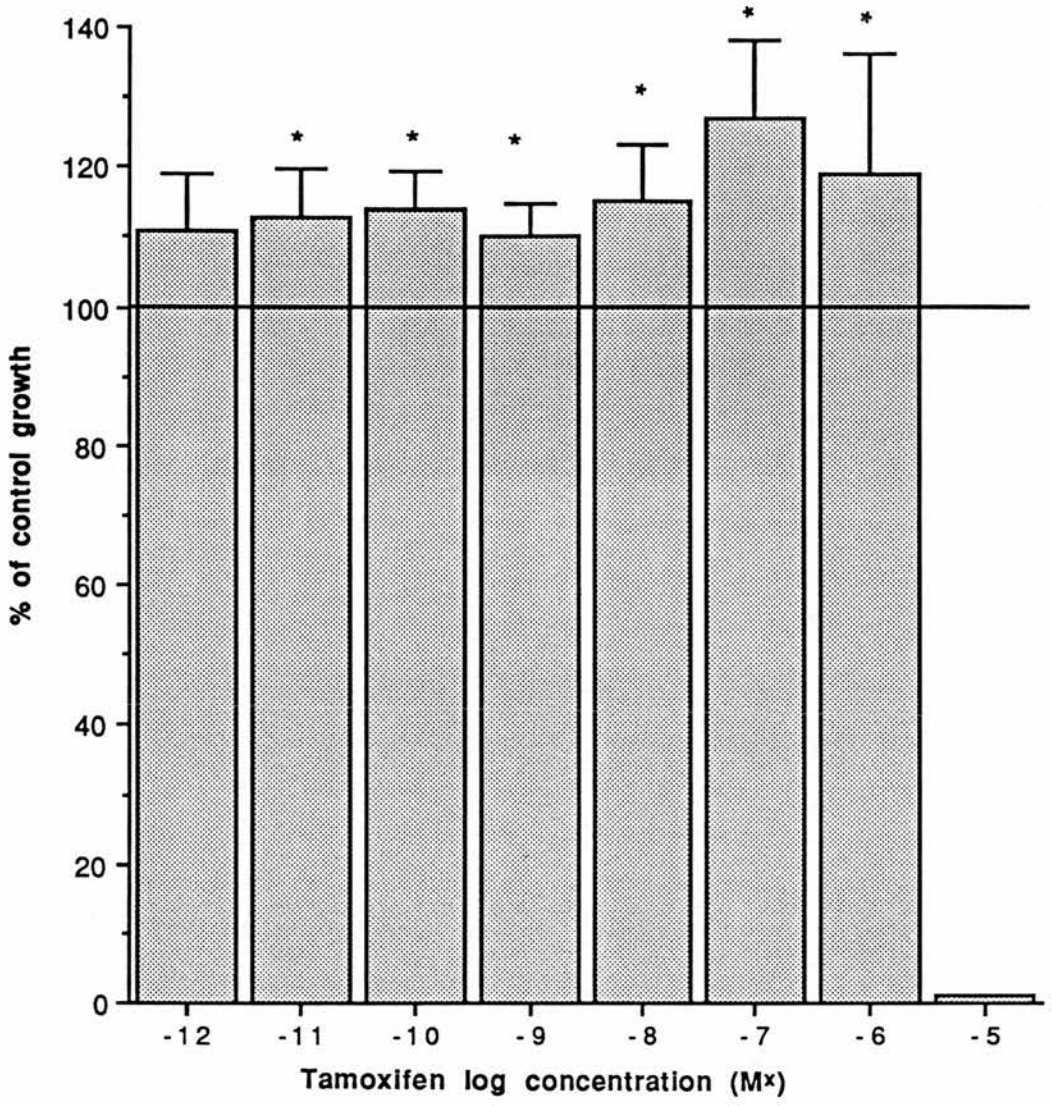

Figure 3.14. Effects of the exogenous addition of tamoxifen on the growth of PEO4 cell line. Cells were incubated in phenol red-free RPMI 1640 containing 5% dcs-FCS in the absence and presence of various concentrations (10^{-12}-10^{-5} M) of tamoxifen for 6 days. Cells were harvested by trypsinisation and counted using a Coulter Counter. In each experiment triplicate counts for each sample were taken, and each experimental condition was performed in quadruplicate. Results are expressed relative to growth of untreated cells. The figure shows the mean ± S.E. of 5 separate experiments. * $P < 0.05$, according to Students' t-test.

PEO14 cell line

These results are shown in figure 3.15. In contrast to PEO1 and PEO4 cells, all concentrations of tamoxifen between 10^{-12} and 10^{-6} M produced negligible effects on cell growth. The highest concentration tested which was 10^{-5} M, produced a significant inhibition of the growth of PEO14 cells.

3.25 Effects of varying ICI 164,384 concentration, as measured by cell counts

The effects of incubation with a range of concentrations (10^{-12}-10^{-5} M) of the "pure" antioestrogen ICI 164,384 were investigated in the three cell lines PEO1, PEO4 and PEO14.

PEO1 cell line

Effects on cell counts following 6 days exposure to ICI 164,384 are shown in figure 3.16. A progressive increase in cell number was produced by concentrations between 10^{-12} and 10^{-10} M compared with control growth. The stimulation above control was statistically significant by Student's t-test. Maximal stimulation of approximately 30% was seen at 10^{-10} M. The 10^{-9} M concentration of ICI 164, 384 had a negligible effect on growth, whereas concentrations between 10^{-8} and 10^{-5} M produced significant growth inhibition. Maximal growth inhibition was produced by the highest concentration tested, 10^{-5} M, although this was not as dramatic as effects produced by tamoxifen at this concentration (figure 3.13).

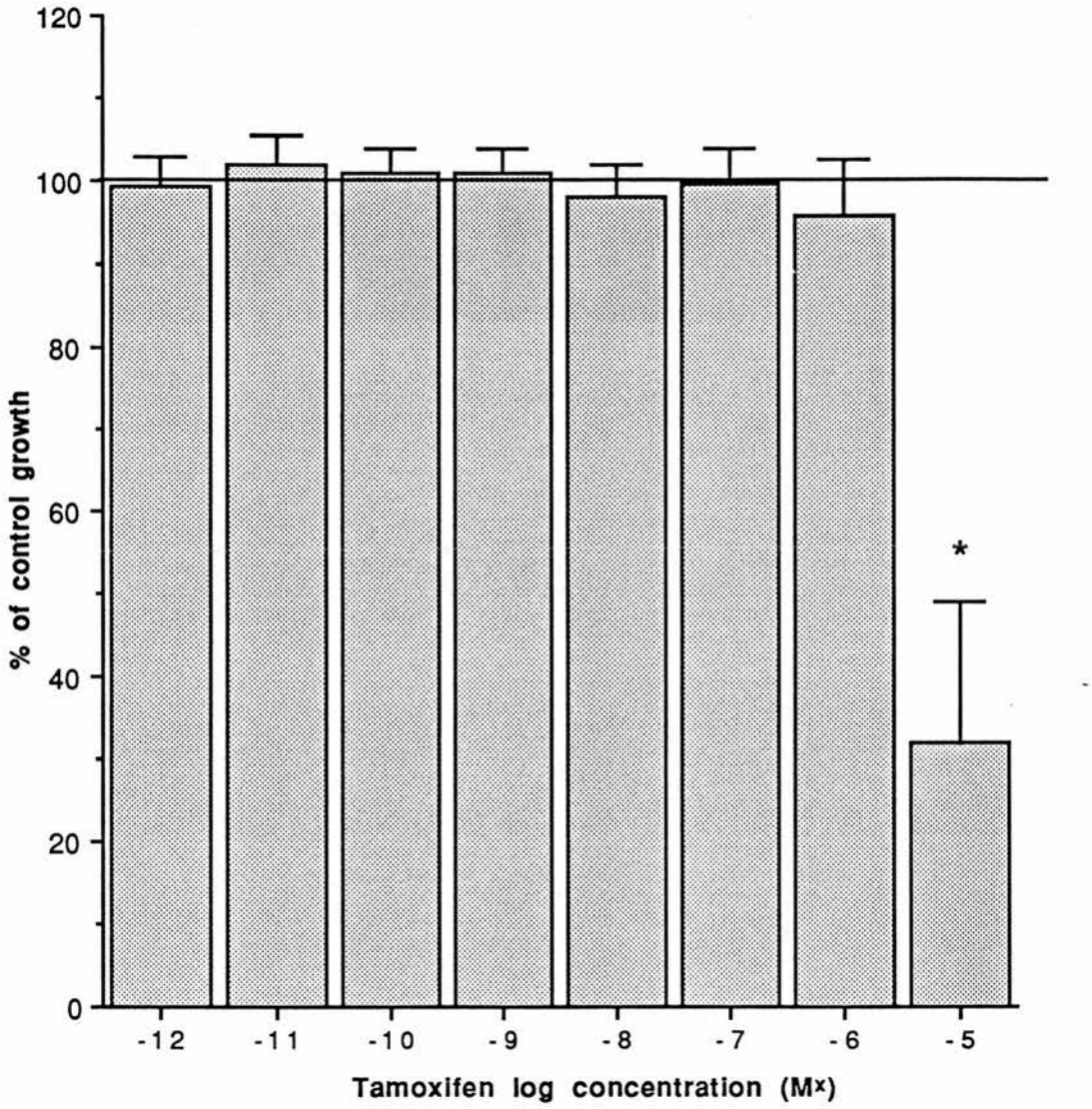

Figure 3.15. Effects of the exogenous addition of tamoxifen on the growth of PEO14 cell line. Cells were incubated in phenol red-free RPMI 1640 containing 5% dcs-FCS in the absence and presence of various concentrations (10^{-12}-10^{-5} M) of tamoxifen for 6 days. Cells were harvested by trypsinisation and counted using a Coulter Counter. In each experiment triplicate counts for each sample were taken, and each experimental condition was performed in quadruplicate. Results are expressed relative to growth of untreated cells. The figure shows the mean ± S.E. of 3 separate experiments. * $P < 0.05$, according to Students' t-test

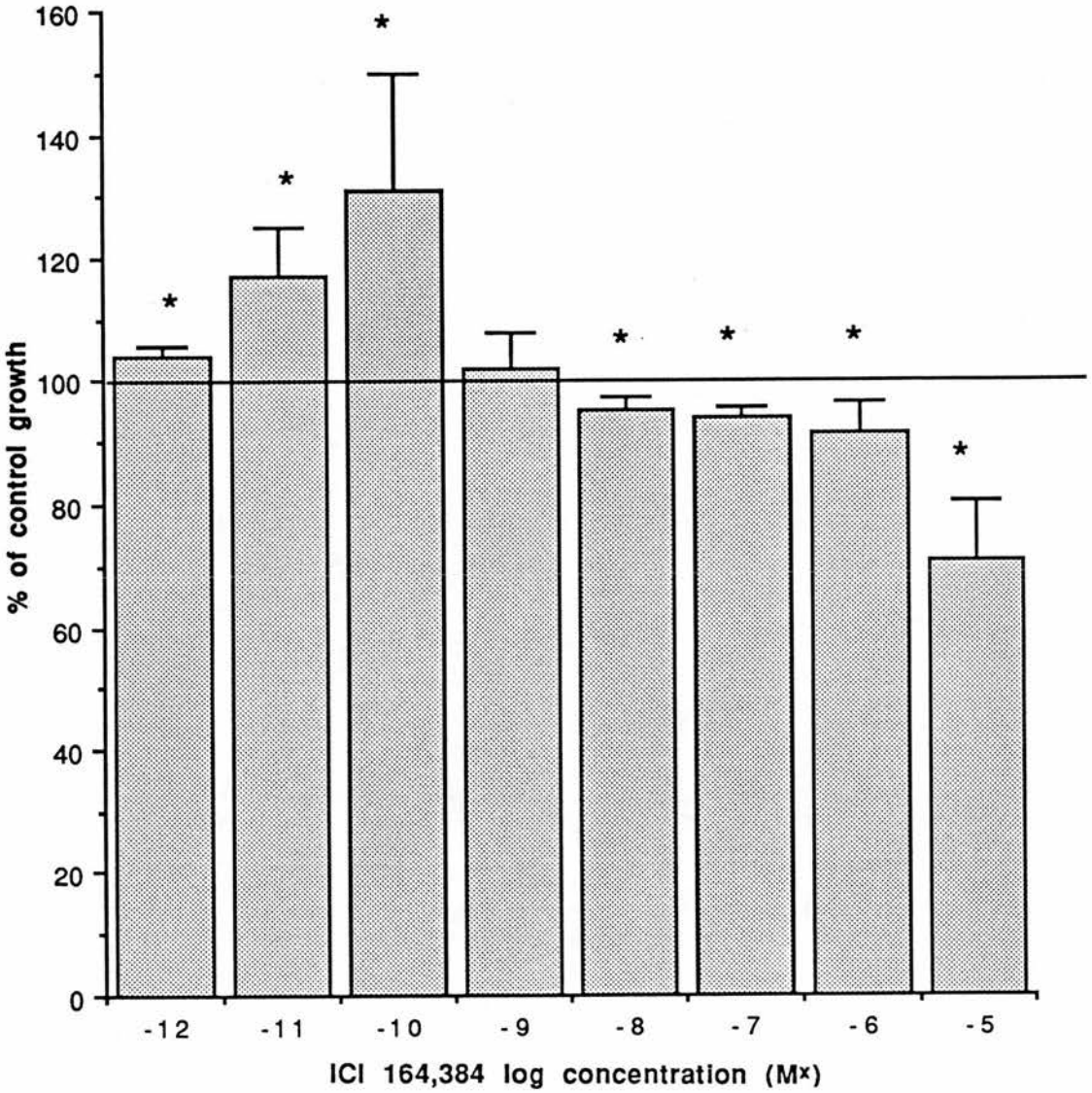

Figure 3.16. Effects of the exogenous addition of ICI 164,384 on the growth of PEO1 cell line. Cells were incubated in phenol red-free RPMI 1640 containing 5% dcs-FCS in the absence and presence of various concentrations (10^{-12}-10^{-5} M) of ICI 164,384 for 6 days. Cells were harvested by trypsinisation and counted using a Coulter Counter. In each experiment triplicate counts for each sample were taken, and each experimental condition was performed in quadruplicate. Results are expressed relative to growth of untreated cells. The figure shows the mean ± S.E. of 2 separate experiments. * $P < 0.05$, according to Students' t-test.

PEO4 cell line

Results with the PEO4 cell line are shown in figure 3.17. Incubation with ICI 164,384 at concentrations between 10^{-12} and 10^{-10} M produced statistically significant, progressive increases in cell numbers, with a maximal effect of around 30% stimulation above control at 10^{-10} M. A reduced growth stimulation was also produced by 10^{-9}, M ICI 164,384, and lesser effects for 10^{-8} M. Negligible effects on growth were produced at 10^{-7} and 10^{-6} M. The highest concentration of 10^{-5} M significantly inhibited cell growth. Thus, ICI 164,384 produced similar effects in PEO4 cells as those in the PEO1 cell line.

PEO14 cell line

These results are shown in figure 3.18. In contrast to PEO1 and PEO4 cells, culture in the presence of ICI 164,384 between 10^{-12} and 10^{-8} M produced negligible effects on cell growth in the ER-negative PEO14 cell line. The higher concentrations of 10^{-7} and10^{-6} M reduced cell growth by a small but significant extent. Marked inhibition of cell growth was produced by 10^{-5} M ICI 164,384.

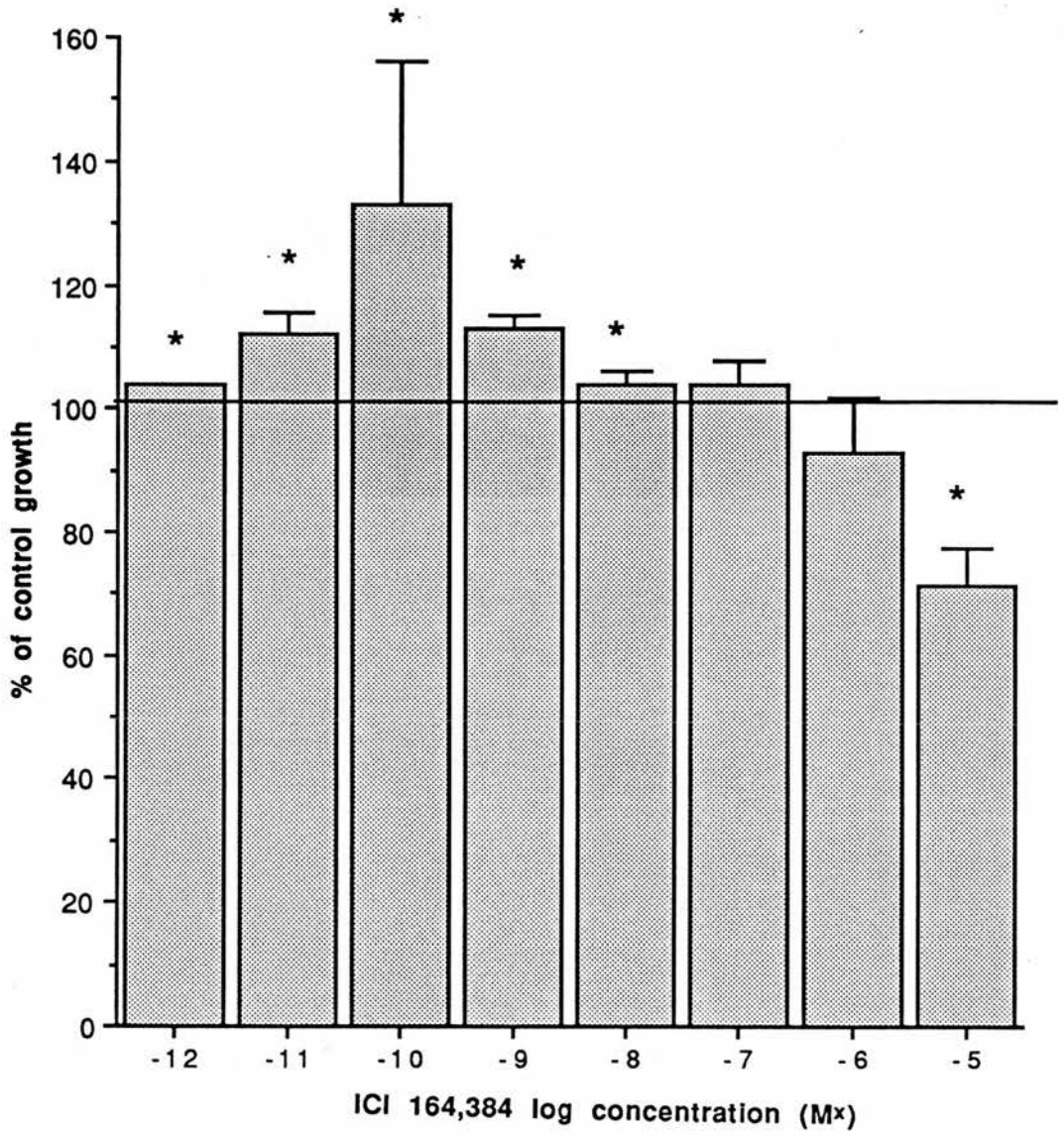

Figure 3.17. Effects of the exogenous addition of ICI 164,384 on the growth of PEO4 cell line. Cells were incubated in phenol red-free RPMI 1640 containing 5% dcs-FCS in the absence and presence of various concentrations (10^{-12}-10^{-5} M) of ICI 164,384 for 6 days. Cells were harvested by trypsinisation and counted using a Coulter Counter. In each experiment triplicate counts for each sample were taken, and each experimental condition was performed in quadruplicate. Results are expressed relative to growth of untreated cells. The figure shows the mean ± S.E. of 2 separate experiments. * $P < 0.05$, according to Students' t-test.

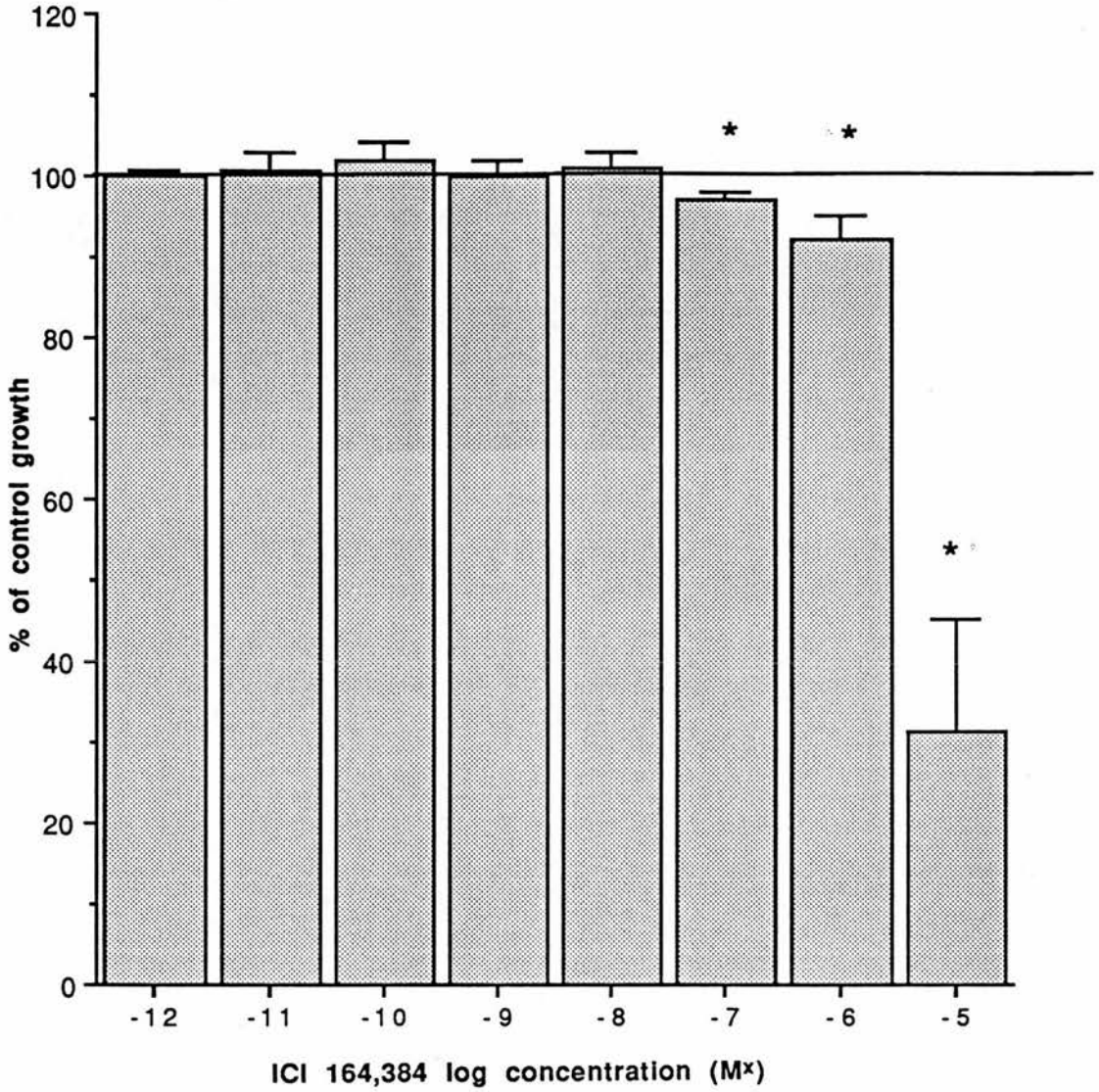

Figure 3.18. Effects of the exogenous addition of ICI 164,384 on the growth of the PEO14 cell line. Cells were incubated in phenol red-free RPMI 1640 containing 5% dcs-FCS in the absence and presence of various concentrations (10^{-12}-10^{-5} M) of ICI 164,384 for 6 days. Cells were harvested by trypsinisation and counted using a Coulter Counter. In each experiment triplicate counts for each sample were taken, and each experimental condition was performed in quadruplicate. Results are expressed relative to growth of untreated cells. The figure shows the mean ± S.E. of 2 separate experiments. * $P < 0.05$, according to Students' t-test.

3.3 Effect of EGF / TGF-α on Growth

A proportion of ovarian tumours express EGF receptors and some also secrete EGF or TGF-α (see Introduction). The growth of some ovarian malignancies may therefore be sensitive to EGF and TGF-α. It was therefore of interest to determine the effects of EGF and TGF-α on the growth of the three ovarian cell lines, PEO1, PEO4 and PEO14. Initially, the effects of these growth factors were investigated by performing cell counts after 7 days in culture with various concentrations of EGF and TGF-α. Then, in order to determine whether variation in incubation time altered the growth factor effects, experiments were performed in which cells were counted after 0, 2, 5 and 7 days in culture.

3.31 Effect of concentration of growth factors

PEO1 cell line

The effects of incubation for 7 days with EGF or TGF-α at concentrations varying between 0.001 and 10 nM are shown in figure 3.19. The data are shown as the percentage of cell numbers above control cells. Stimulatory effects were produced by EGF and TGF-α. Progressive increases in cell numbers were produced by concentrations of growth factor between 0.001 and 0.1 nM. Maximal stimulation of approximately 60% above control counts was produced by 0.01 and 0.1 nM. Concentrations of 1 and 10 nM were also associated with growth stimulation, but effects produced tended to be less marked.

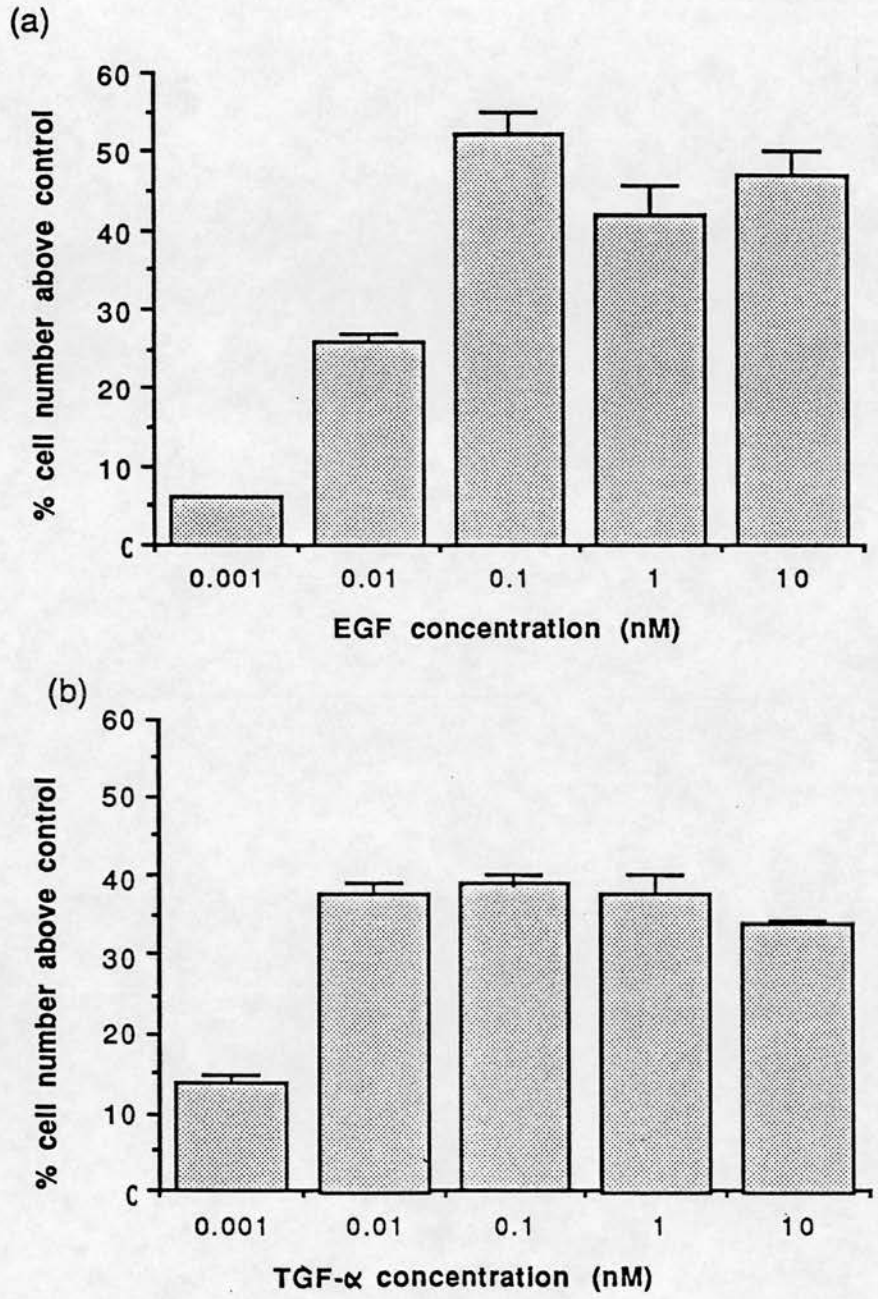

Figure 3.19. Effects of exogenous addition of (a) EGF and (b) TGF-α on the growth of PEO1 cells. Cells were incubated in phenol red free RPMI 1640 containing 5% dcs-FCS with the absence or presence of various doses of EGF or TGF-α (10^{-12}-10^{-8} M) for 7 days. Cells were harvested by trypsinisation and counted. Figure shows the results of a representative experiment with mean ± S.E of 4 counts for each condition. Results are expressed relative to growth of untreated cells. The experiment was performed on at least 3 separate occasions with similar results.

PEO4 cell line

The effects of EGF and TGF-α on the PEO4 cell line are shown in figure 3.20, and are essentially similar to those in the PEO1 cell line. Growth stimulation was produced at all concentrations of EGF and TGF-α tested. Increasing stimulation was produced by increasing concentrations of growth factors between 0.001 and 0.1 nM. Maximal effects of 60-70% above control growth were observed for the 0.1 nM concentrations. Higher concentrations ie 1 and 10 nM were also stimulatory, but effects were less than that produced by 0.1 nM.

PEO14 cell line

These results are shown in figure 3.21. Negligible responses to both growth factors were produced at 0.001 nM concentrations. Progressive increases in cell numbers were noted between 0.01 and 10 nM EGF and 0.1 and 10 nM TGF-α. The highest concentration tested (10 nM) produced maximal stimulation of approximately 20-30% above control cells for EGF and 30-40% for TGF-α.

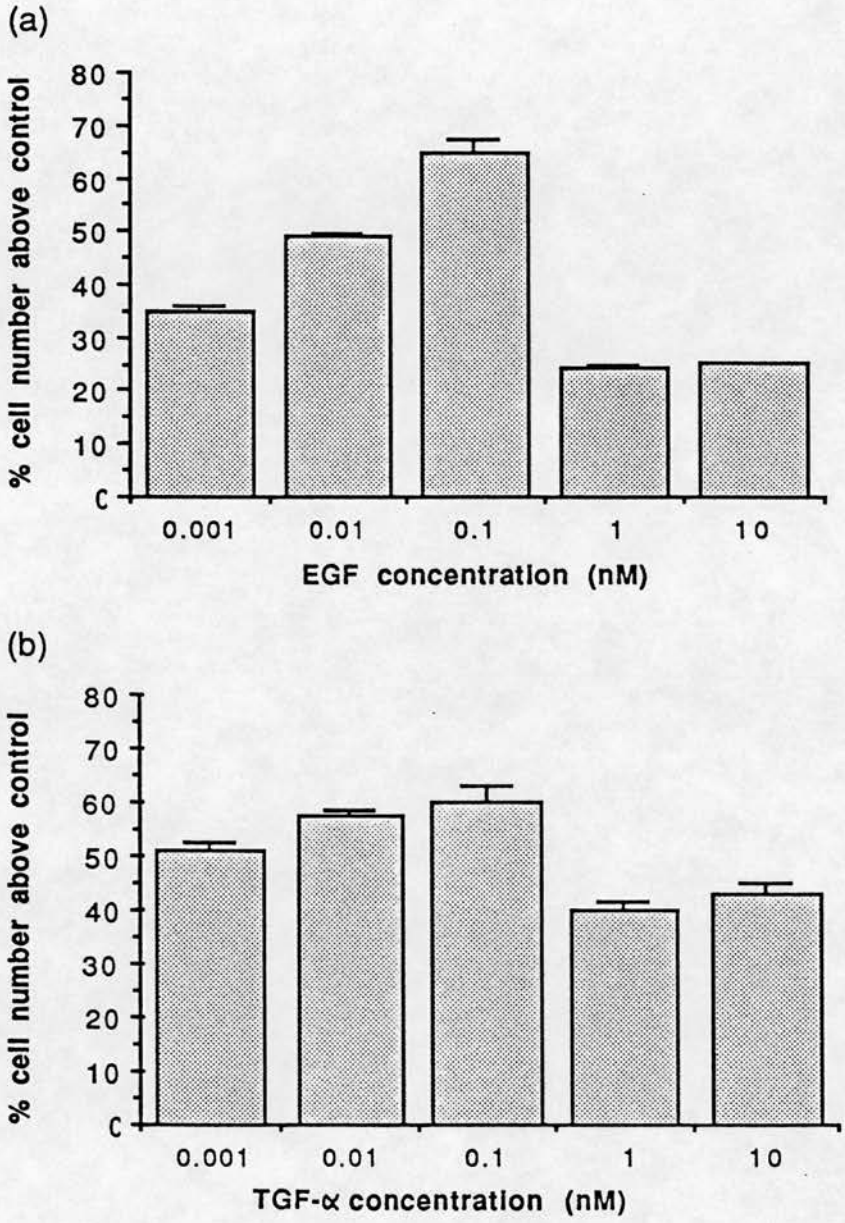

Figure 3.20. Effects of exogenous addition of (a) EGF and (b) TGF-α on the growth of PEO4 cells. Cells were incubated in phenol red free RPMI 1640 containing 5% dcs-FCS with the absence or presence of various doses of EGF or TGF-α (10^{-12}-10^{-8} M) for 7 days. Cells were harvested by trypsinisation and counted. Figure shows the results of a representative experiment with mean ± S.E of 4 counts for each condition. Results are expressed relative to growth of untreated cells. The experiment was performed on at least 3 separate occasions.

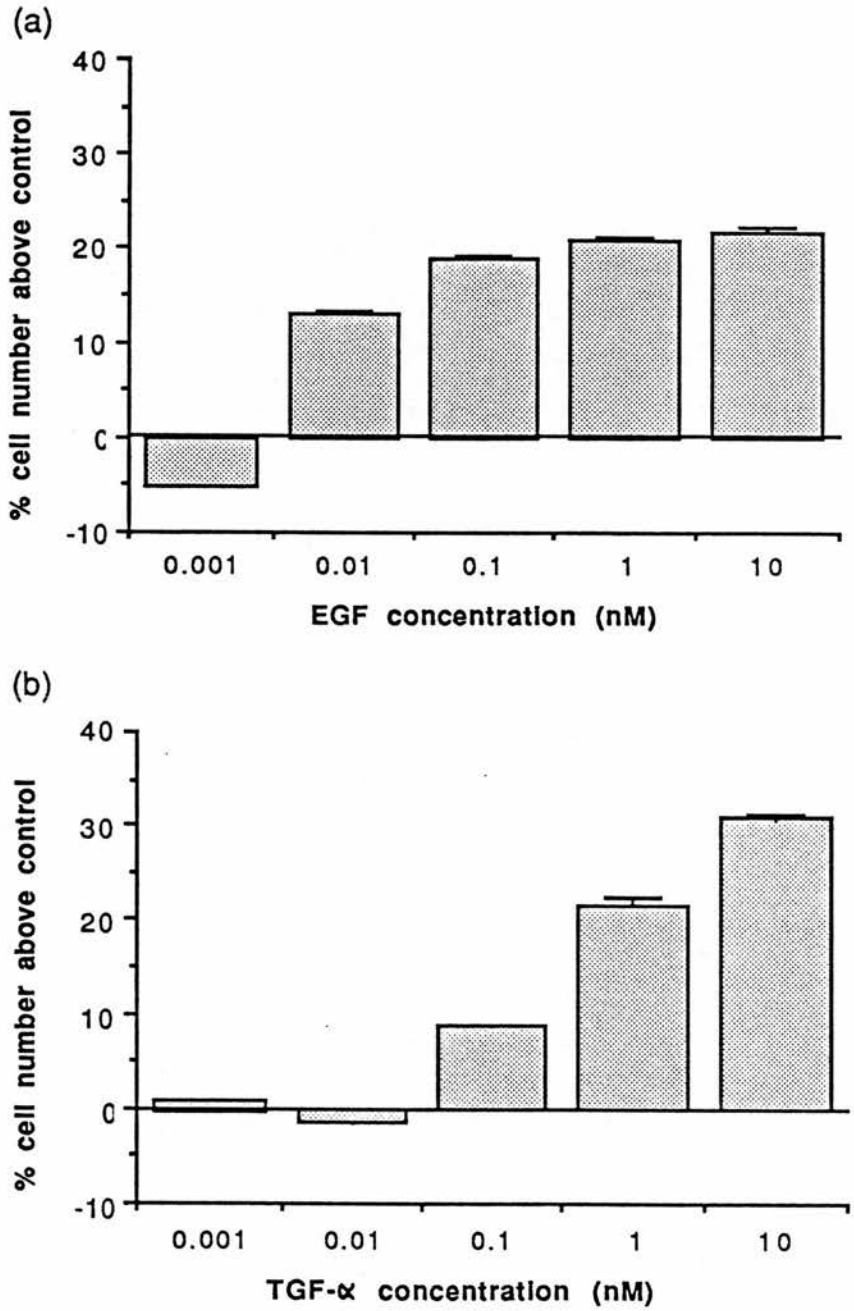

Figure 3.21. Effects of exogenous addition of (a) EGF and (b) TGF-α on the growth of PEO14 cells. Cells were incubated in phenol red free RPMI 1640 containing 5% dcs-FCS with the absence or presence of various doses of EGF or TGF-α (10^{-12}-10^{-8} M) for 7 days. Cells were harvested by trypsinisation and counted. Figure shows the results of a representative experiment with mean ± S.E of 4 counts for each condition. Results are expressed relative to growth of untreated cells. The experiment was performed on at least 3 separate occasions.

3.32 Effect of incubation time on EGF and TGF-α-stimulated growth

Representative growth curves for PEO4 and PEO14 cell lines are presented.

PEO4 cell line

The effects of EGF on PEO4 cell numbers over 7 days in culture are shown in figure 3.22. After two days in culture, EGF at all concentrations tested between 0.001 and 10 nM produced negligible effects on cell numbers compared with untreated cells. However, following 5 days in culture, clear stimulation of growth was produced by all but the lowest concentration (0.001 nM) of EGF. The 0.01 nM concentration produced lesser effects than those observed with concentrations between 0.1 and 10 nM. Similar effects on cell growth were produced after 7 days exposure to EGF, although at this time point the lowest concentration of 0.001 nM also produced a small growth stimulation.

PEO14 cell line

The effects of TGF-α on the growth of PEO14 cells over 7 days in culture are shown in figure 3.23. Similar effects were produced to those for EGF in PEO4 cells. No growth stimulation was produced at any concentration between 0.001 and 10 nMTGF-α following 2 days in culture. In addition, no stimulatory effects were produced by either 0.001 or 0.01 nM after 5 or 7 days in cuture. At all other concentrations between 0.1 and 10 nM, stimulation of growth was produced, following 5 days in culture.

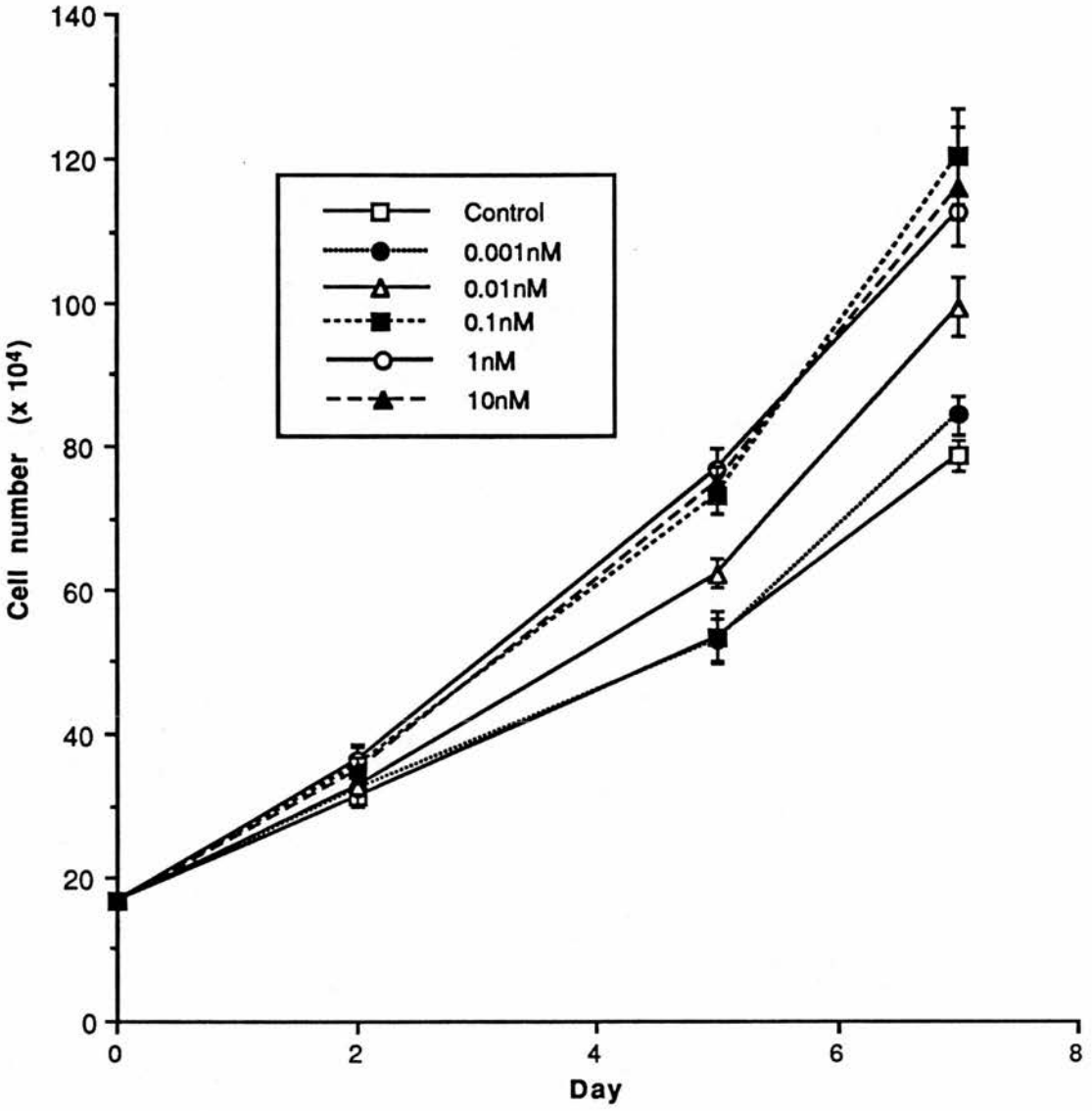

Figure 3.22. Typical growth curve demonstrating the effects of various concentrations of EGF on the growth of PEO4 cells in phenol red free RPMI 1640 containing 5% dcs-FCS. Cells were harvested and counted on days 0, 2, 5 and 7. Points shown are the means ± S.E of 4 values.

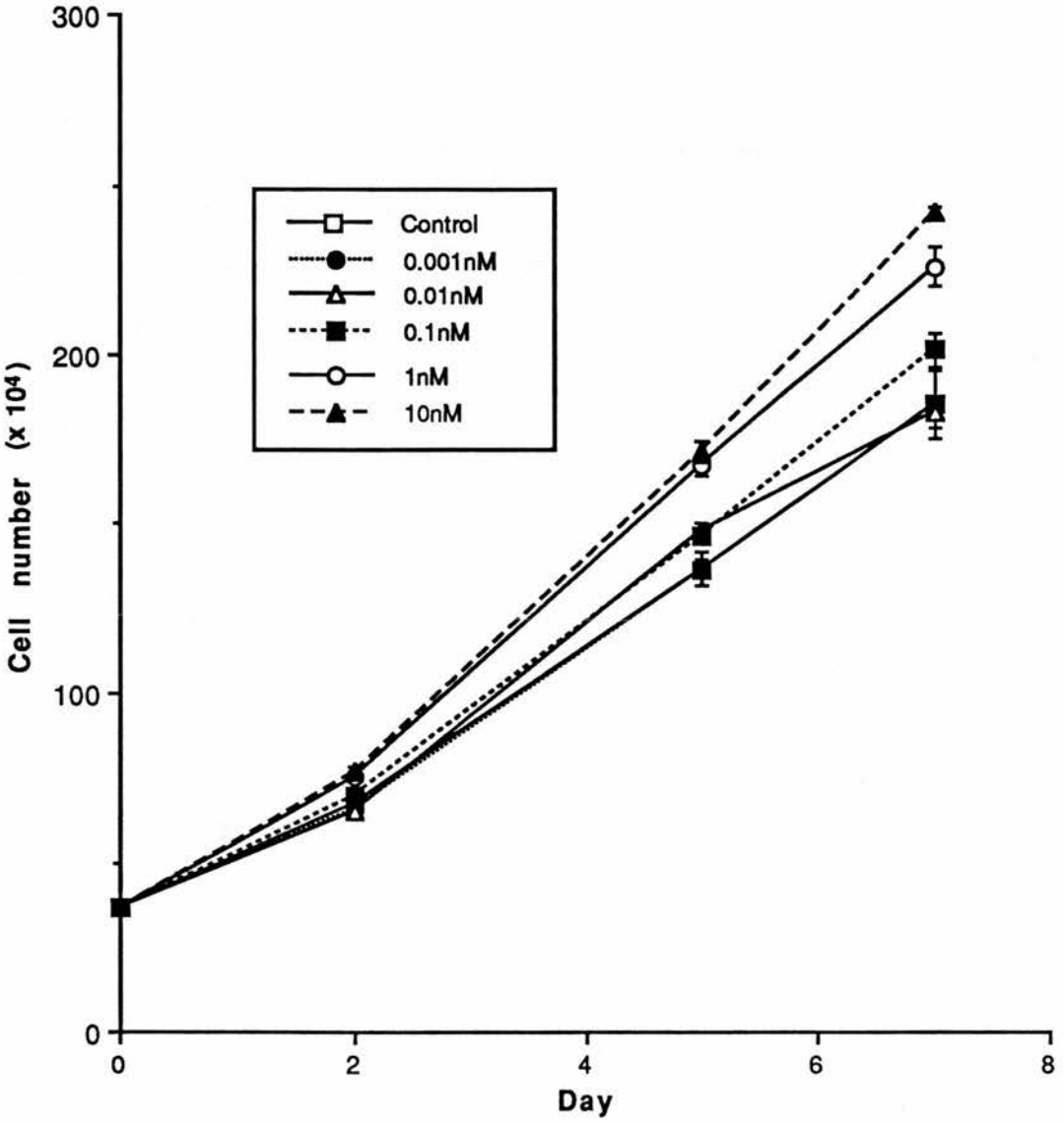

Figure 3.23. Typical growth curve demonstrating the effects of various concentrations of TGF-α on the growth of PEO14 cells in phenol red free RPMI 1640 containing 5% dcs-FCS. Cells were harvested and counted on days 0, 2, 5 and 7. Points shown are the means ± S.E of 4 values.

These effects were also evident after 7 days. The highest concentration of 10 nM produced maximal effects. The dose responses of the cells to the various concentrations are therefore comparable to those shown previously (figure 3.21).

3.33 Effects of EGF and TGF-α in the presence and absence of reduced serum concentration

In order to determine whether the presence of serum was essential for EGF and TGF-α growth stimulation, cells were cultured in RPMI 1640 containing 0.5% dcs-FCS or with hydrocortisone, insulin, transferrin and sodium selenite (HITS) in the presence and absence of growth factors. Effects on cell numbers following 7 days in culture are shown in tables 3.4 and 3.5.

Table 3.4. Effect of EGF and TGF-α on the growth rate of PEO1, PEO4 and PEO14 cells grown in phenol red-free RPMI 1640 containing 0.5% dcs-FCS. Cells were cultured in the presence and absence of growth factors for 7 days. Results are expressed as the % of growth above controls. Mean ± S.E of quadruplicate counts from at least one experiment are shown for each condition.

Cell line	EGF		TGF-α	
	1 nM	10 nM	1 nM	10 nM
PEO1	20 ±9	20 ±10	18 ±7	8 ±12
PEO4	7 ±4	5 ±4	60 ±10	13 ±15
PEO14	94 ±16	89 ±16	29 ±4	24 ±6

Table 3.5. Effect of EGF and TGF-α on the growth rate of PEO1, PEO4 and PEO14 cells grown in phenol red-free RPMI 1640 containing HITS. Cells were cultured in the presence and absence of growth factors for 7 days. Results are expressed as the % of growth above controls. Mean ± S.E of quadruplicate counts from at least one experiment are shown for each condition.

Cell line	EGF		TGF-α	
	1 nM	10 nM	1 nM	10 nM
PEO1	39 ±8	19 ±4	12 ±7	ND
PEO4	34 ±9	39 ±8	74 ±6	31 ±10
PEO14	41 ±13	17 ±6	11 ±8	27 ±13

ND=not done

The results show that all three ovarian carcinoma cell lines may be stimulated by EGF and TGF-α.

However, the results which were obtained in the presence of 0.5 % and in the absence of serum suggest that this response is not consistent, and that some differences in the potency of EGF and TGF-α may exist in the ovarian cancer cell lines.

3.34 Cell Cycle Analysis

As the growth of the three ovarian cell lines was stimulated by exogenous addition of EGF and TGF-α to the culture media, it was of interest to determine whether this was associated with effects on the cell cycle distributions. The cell lines were therefore cultured in phenol red free RPMI 1640 plus 5% dcs-FCS, in the presence and absence of 0.1 and 10 nM EGF or TGF-α. The percentages of cells in each phase of the cell cycle were calculated from cell cycle distribution plots, an example of which is shown in figure 3.24. The x-axis is an arbitrary scale representing DNA quantity and the y-axis shows the number of cells. The largest peak in the graph shows the G0/G1 phase, and the G2/M phase is represented by the smaller peak, which contains double the quantity of DNA. The S phase of the cell cycle is shown by the plateau between the two peaks, representing intermediate amounts of DNA. The percentages of cells in each stage of the cell cycle was calculated from the size of the peaks. The analysis on the flow cytometer was performed by Mr E Miller, Department of Clinical Oncology, Western General Hospital.

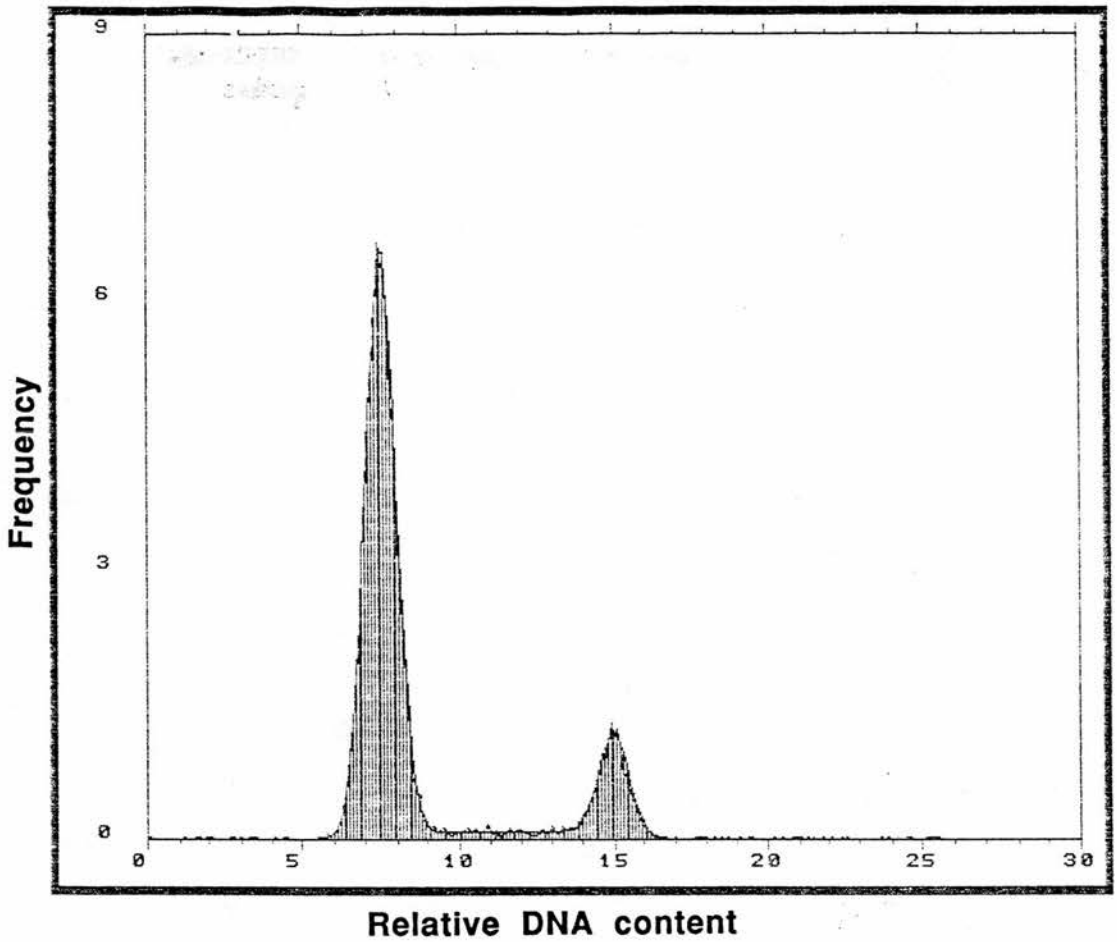

Figure 3.24. An example of cell cycle analysis of PEO1 cells cultured in phenol red free RPMI 1640 containing 5% dcs-FCS. Data is based on the analysis of 4 samples of 5000 cells. The x-axis shows the fluorescence of propidium iodide which represents the DNA within the cells, and the y-axis shows an arbitrary scale representing the number of cells containing each DNA quantity.

(i) For period of 72 hours following growth factor addition

Similar plots were examined at various times up to 72 hours following growth factor addition. Results are presented as percentage of cells in each phase of the cycle at various times and are shown in figs 3.25 to 3.33.

Before reviewing the specific effects in each cell line, it is useful to summarise the general trends observed. In all three cell lines, EGF and TGF-α produced similar changes in each of the phases of the cell cycle. Both concentrations of the growth factors induced the same response, although the change was of greater magnitude for the 10 nM concentration. Generally, EGF and TGF-α caused a decrease in the percentage of cells in the G0/G1 phase associated with an increase in the percentage of cells in S phase. This was followed by an increase in the percentage of cells in the G2/M stage of the cell cycle. The details of the extent, timing and duration of these effects in each cell line will now be discussed individually.

PEO1 cell line

The effects of time in culture in the presence and absence of EGF and TGF-α on the PEO1 cell cycle distributions are shown in figures 3.25 to 3.27. In the absence of growth factor addition the percentage of cells in the G0/G1 and S phases remained relatively constant over the 72 hour study period, whereas the percentage of cells in G2/M phase decreased during the initial 24 hours of the experiment. Thereafter, the proportion in G2/M phase remained constant. EGF and TGF-α produced decreases in the percentage of cells in G0/G1 and increases in the S phase, which were both clearly

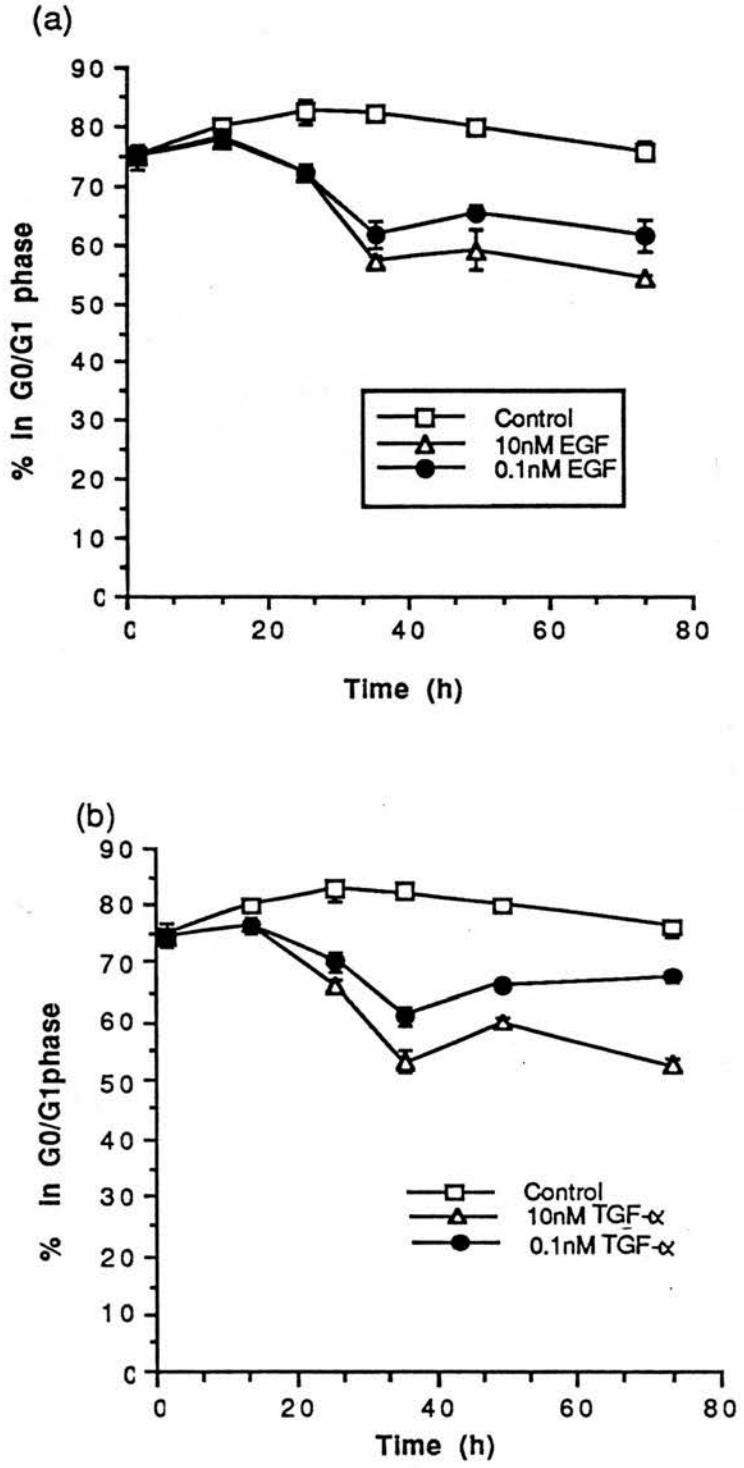

Figure 3.25 a and b Effects of (a) EGF and (b)TGF-α on the distribution of PEO1 cells in G0/G1 phase of the cell cycle. Cells were cultured for 72 hours in phenol red free RPMI 1640 containing 5% dcs-FCS in the presence or absence of 0.1 or 10 nM EGF or TGF-α Mean ± S.E for 4 values are shown from a representative experiment. The experiment was performed on at least 3 separate occasions with similar results.

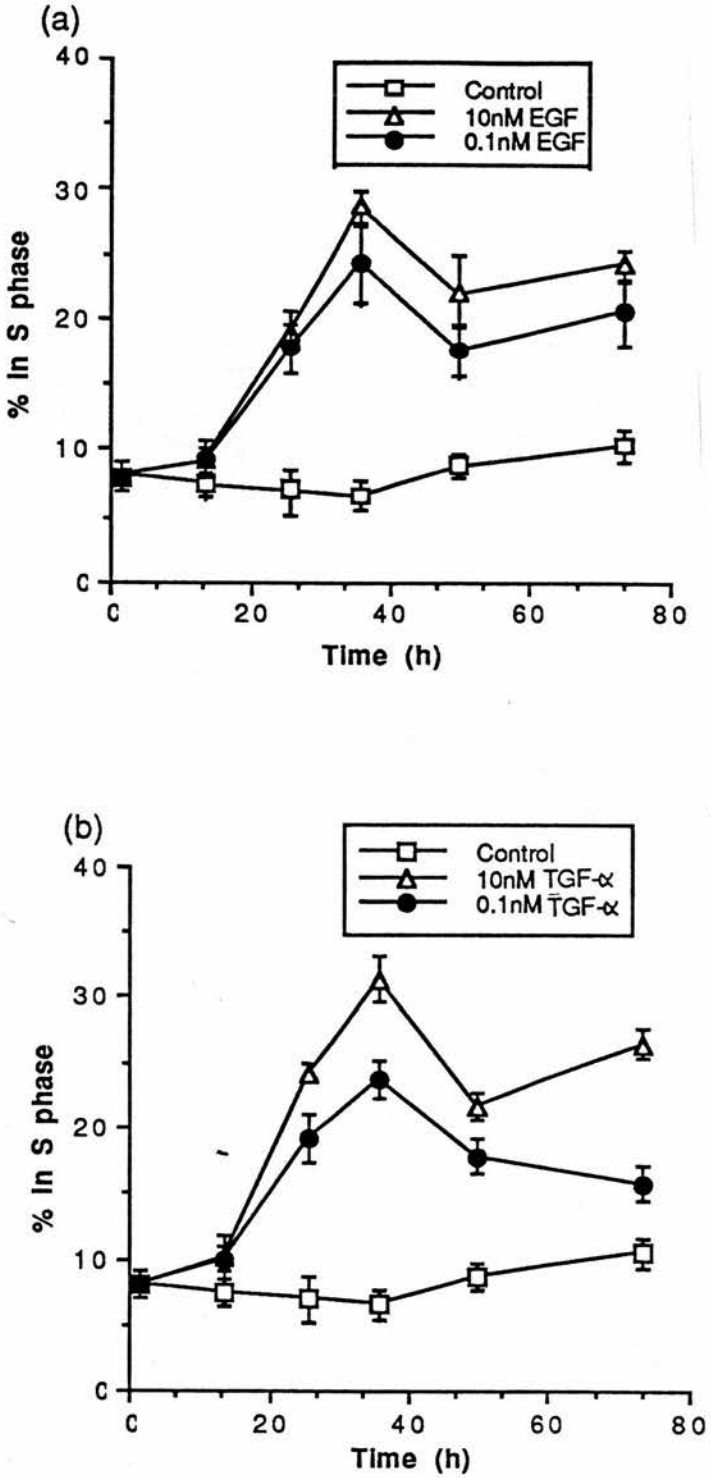

Figure 3.26 a and b Effects of (a) EGF and (b)TGF-α on the distribution of PEO1 cells in S phase of the cell cycle. Cells were cultured for 72 hours in phenol red free RPMI 1640 containing 5% dcs-FCS in the presence or absence of 0.1 or 10 nM EGF or TGF-α Mean ± S.E for 4 values are shown from a representative experiment. The experiment was performed on at least 3 separate occasions with similar results.

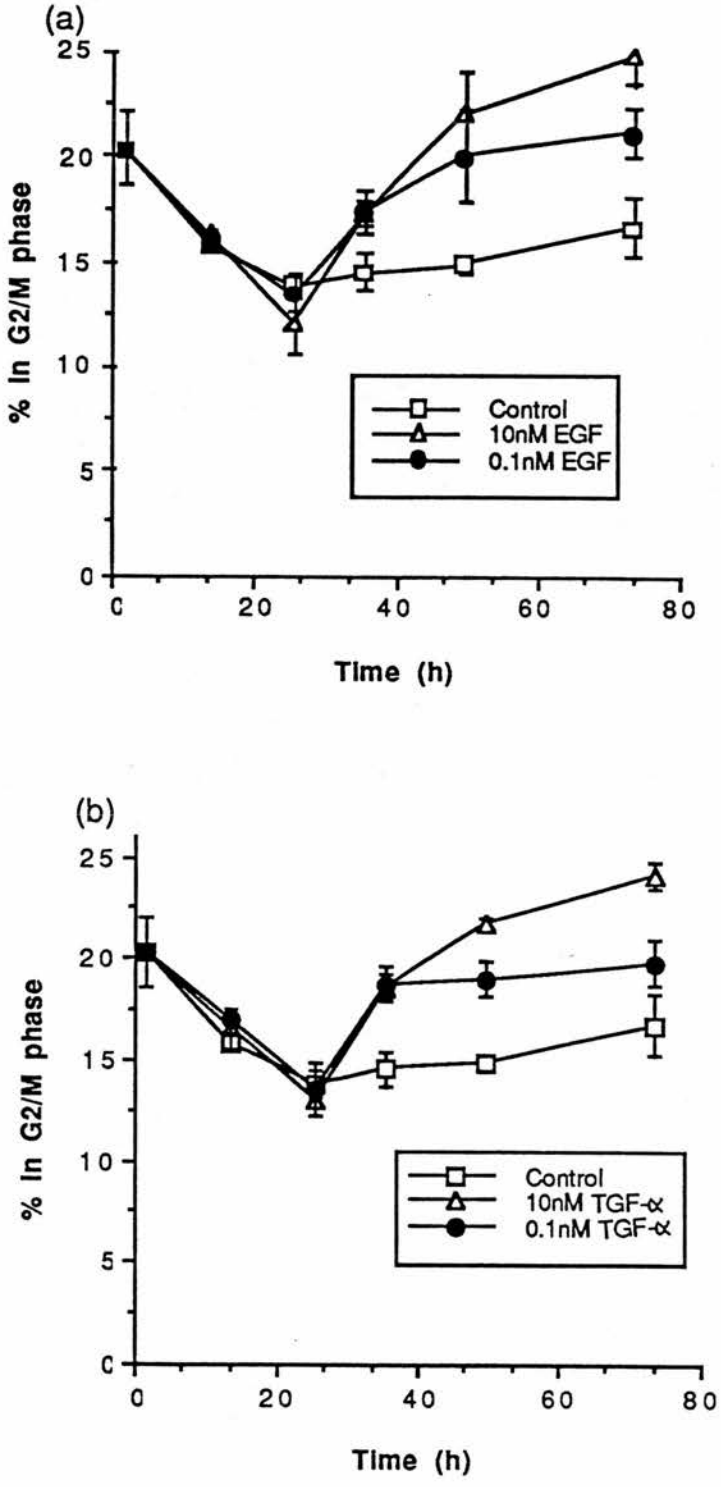

Figure 3.27 a and b Effect of (a) EGF and (b) TGF-α on the distribution of PEO1 cells in G2/M phase of the cell cycle. Cells were cultured for 72 hours in phenol red free RPMI 1640 containing 5% dcs-FCS in the presence or absence of 0.1 or 10 nM EGF or TGF-α Mean ± S.E for 4 values are shown from a representative experiment. The experiment was performed on at least 3 separate occasions with similar results.

seen 24 hours after addition of the growth factors. Both effects were maximal at 37 hours after the addition with the 10 nM concentrations decreasing the percentage of cells in G0/G1 and increasing the percentage in S phase by approximately 20% and 25%, respectively, compared with the control cell population. Beyond 37 hours the response generally diminished. Effects of EGF and TGF-α on the fraction of cells in G2/M phase were not observed until 37 hours, at which time an increase over control cells was present. This was maximal at the end of the study period when differences of around 7% were present between treated and control cells.

(ii) PEO4 cell line

The results for the PEO4 cell line are shown in figures 3.28-3.30. In the absence of EGF or TGF-α, the percentage of cells in G0/G1 and S phases remained constant for 36 hours. Thereafter, the percentage of cells in G0/G1 decreased progressively, and the percentage in S phase increased until 72 hours. As in the PEO1 cell line, the proportion of cells in the G2/M phase decreased initially, and then became relatively constant. although there was a tendency for an increase at 72 hours. Thus, cells treated with 10 nM EGF or TGF-α had approximately 10% less cells in G0/G1 phase 37 hours after their addition. Cell populations stimulated with growth factors showed a maximum increase of around 15% in S phase, 24 hours after addition, which decreased throughout the remaining length of the experiment and a 10% increase in the percentage of cells in G2/M was noted 37 hours after EGF/TGF-α addition, which had disappeared completely by 72 hours.

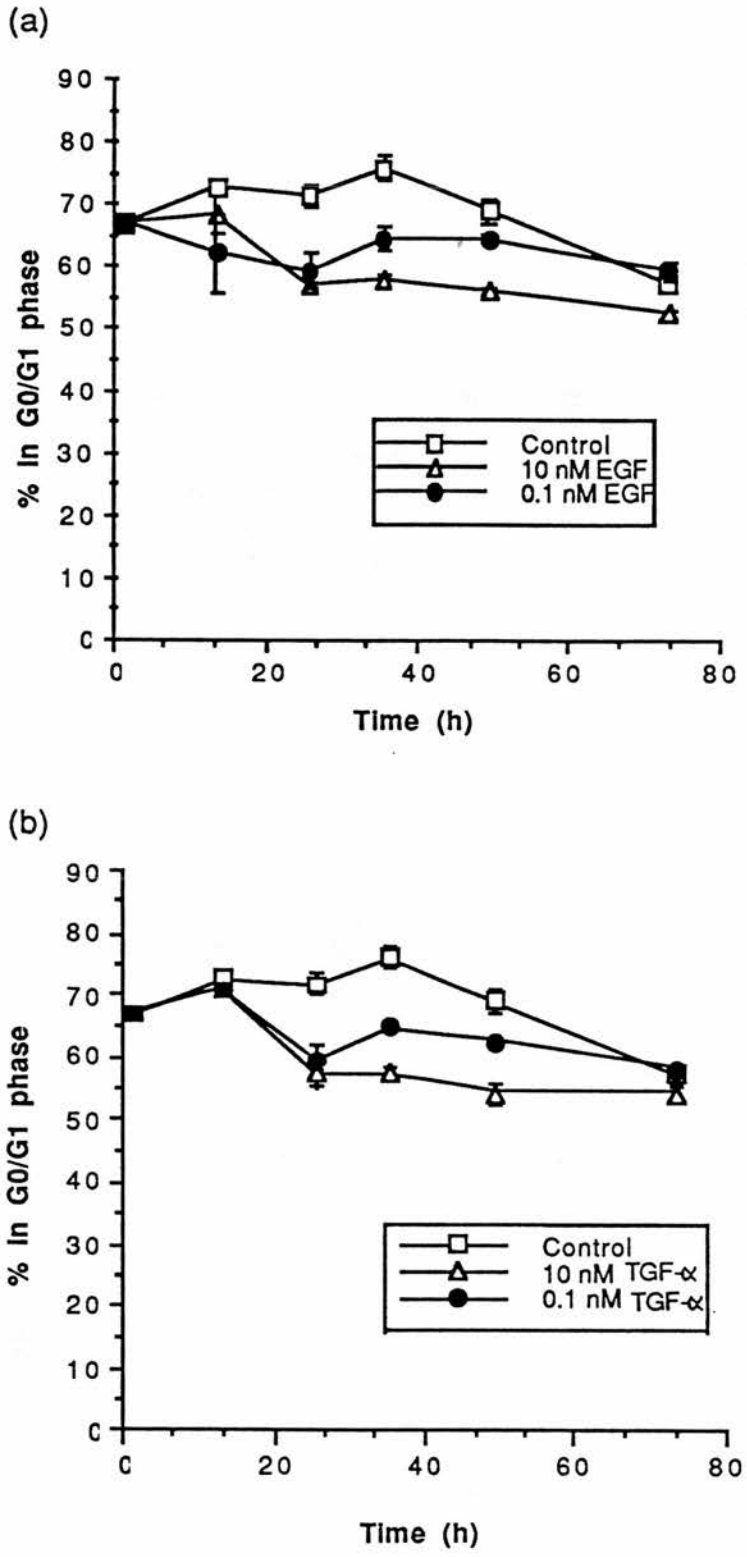

Figure 3.28 a and b Effects of (a) EGF and (b) TGF-α on the distribution of PEO4 cells in G0/G1 phase of the cell cycle. Cells were cultured for 72 hours in phenol red free RPMI 1640 containing 5% dcs-FCS in the presence or absence of 0.1 or 10 nM EGF or TGF-α. Mean ± S.E for 4 values are shown from a representative experiment. The experiment was performed on at least 3 separate occasions with similar results.

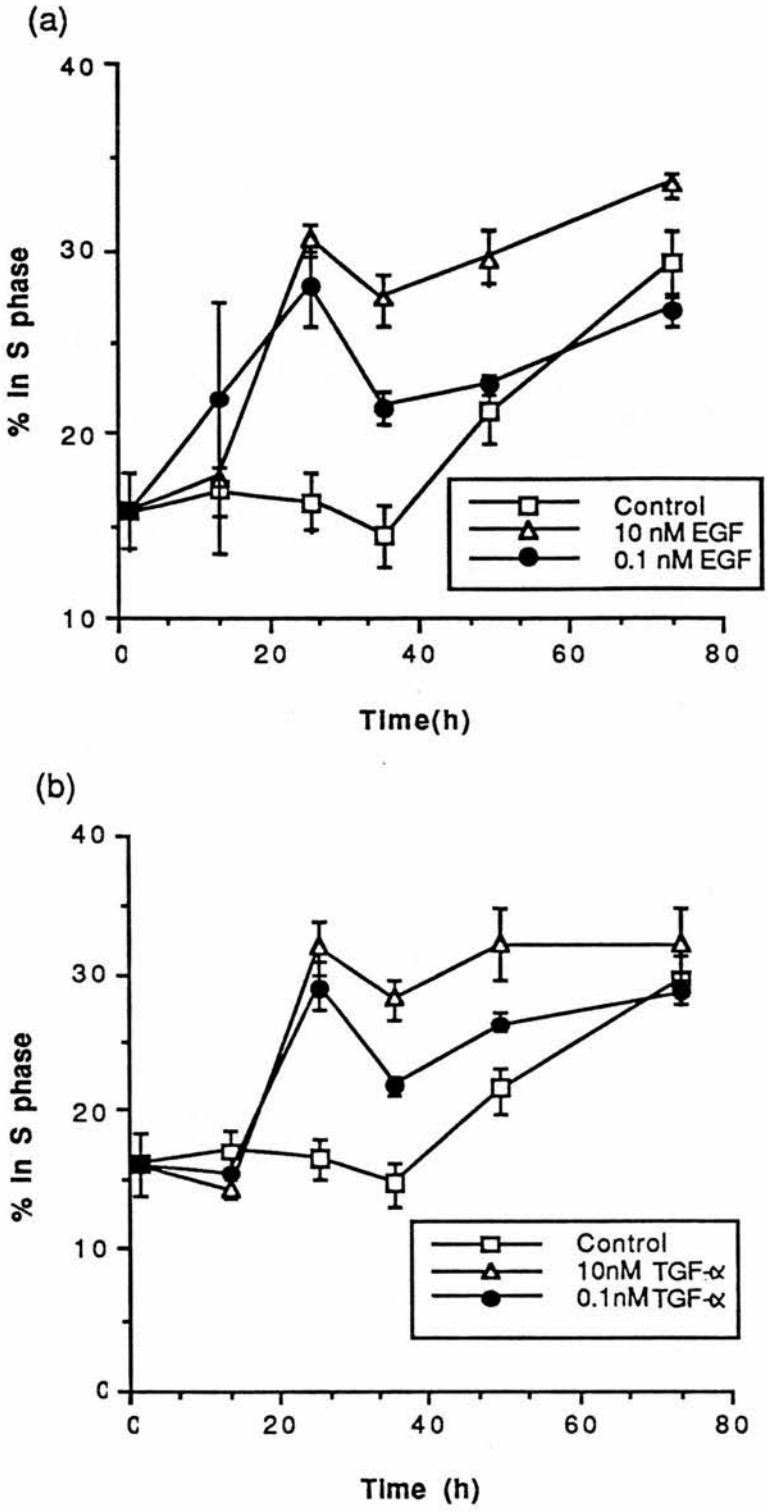

Figure 3.29 a and b Effects of (a) EGF and (b) TGF-α on the distribution of PEO4 cells in S phase of the cell cycle. Cells were cultured for 72 hours in phenol red free RPMI 1640 containing 5% dcs-FCS in the presence or absence of 0.1 or 10 nM EGF or TGF-α Mean ± S.E for 4 values are shown from a representative experiment. The experiment was performed on at least 3 separate occasions with similar results.

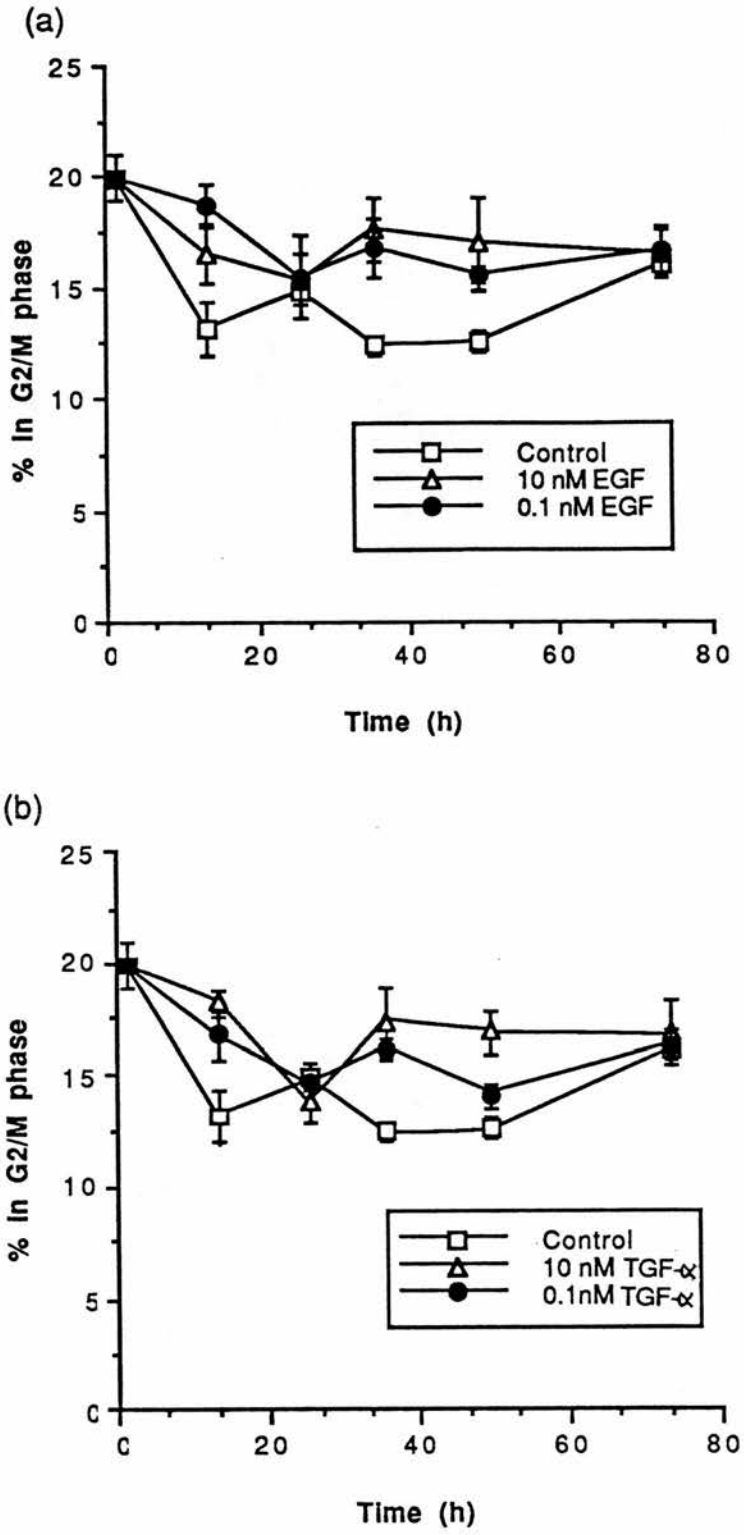

Figure 3.30 a and b Effects of (a) EGF and (b) TGF-α on the distribution of PEO4 cells in G2/M phase of the cell cycle. Cells were cultured for 72 hours in phenol red free RPMI 1640 containing 5% dcs-FCS in the presence or absence of 0.1 or 10 nM EGF or TGF-α. Mean ± S.E for 4 values are shown from a representative experiment. The experiment was performed on at least 3 separate occasions with similar results.

Overall, the changes in the percentage of cells in the G0/G1 phase of the cell cycle produced by EGF and TGF-α followed a similar sequence as in PEO1 cells, but decreases were smaller. Also the increases in the fraction of cells in G2/M phases were not as defined in PEO4 as in PEO1.

PEO14 cell line

These results are shown in figures 3.31-3.33. Some variation was observed in the percentages of control cells in the phases of the cell cycle during the study period. A progressive decrease in the percentage of cells in the G0/G1 phase of the cycle occurred between 12 and 36 hours, after which the proportion remained constant. The percentage of cells in the S phase showed an initial decrease, and then increased until around 36 hours, and remained constant for the remaining study period. The proportion of cells in the G2/M phase showed the same overall changes as the S phase, although the increase occured 12 hours later. Incubation with EGF and TGF-α produced decreases in the percentage of cells in the G0/G1 phase, with a maximal effect of 15-20% which occurred after 24-36 hours. After this time the response diminished. Similarly, maximal effects on the S phase were noted at 24 hours, although a 15-20% increase above control was produced and then decreased during the study period. Changes in the G2/M fraction were also evident by 24 hours, although maximal effects of 15% increases were not observed until 36 hours. The response had virtually disappeared at 72 hours.

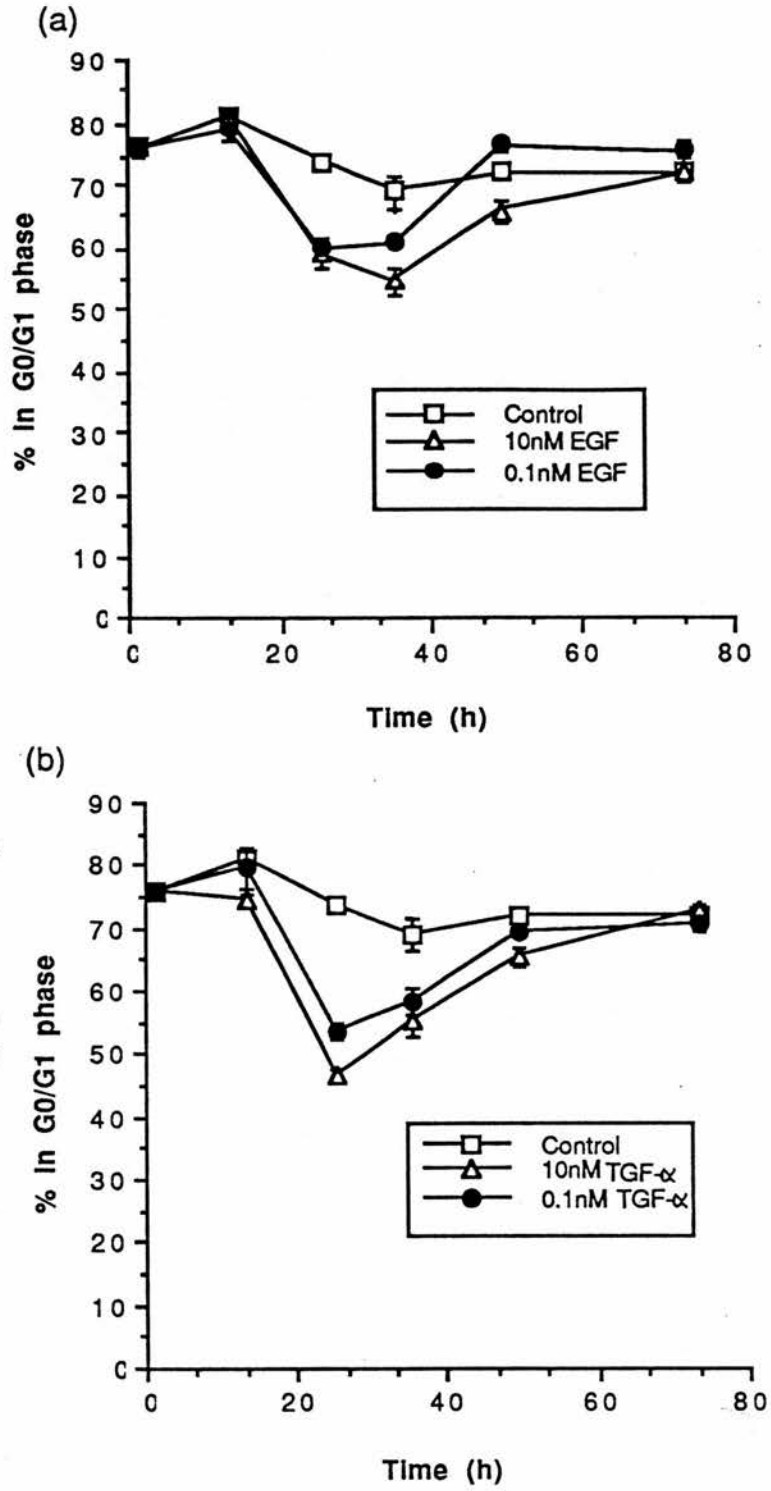

Figure 3.31 a and b Effects of (a) EGF and (b) TGF-α on the distribution of PEO14 cells in G0/G1 phase of the cell cycle. Cells were cultured for 72 hours in phenol red free RPMI 1640 containing 5% dcs-FCS in the presence or absence of 0.1 or 10 nM EGF or TGF-α. Mean ± S.E for 4 values are shown from a representative experiment. The experiment was performed on 3 separate occasions with similar results.

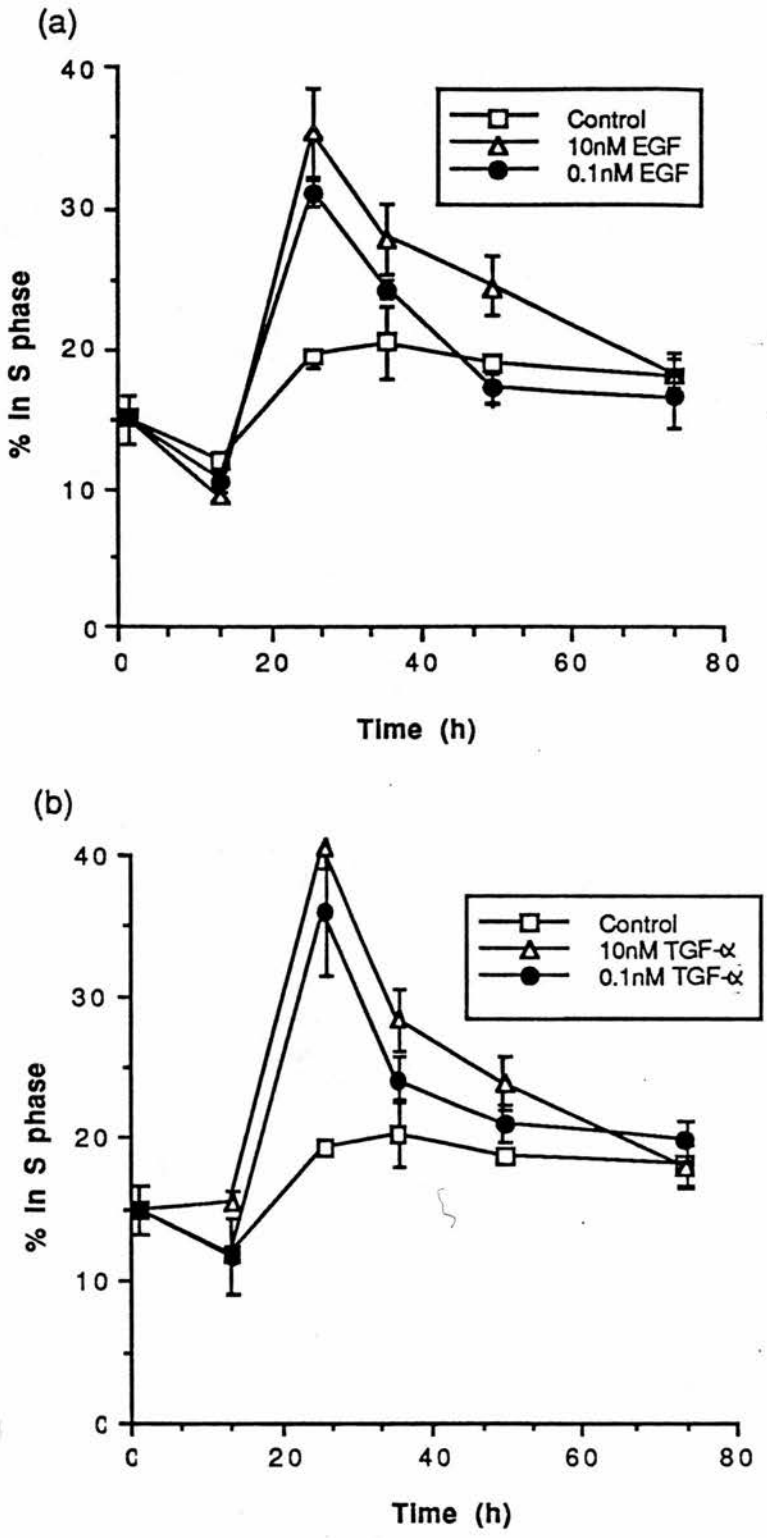

Figure 3.32 a and b Effects of (a) EGF and (b) TGF-α on the distribution of PEO14 cells in S phase of the cell cycle. Cells were cultured for 72 hours in phenol red free RPMI 1640 containing 5% dcs-FCS in the presence or absence of 0.1 or 10 nM EGF or TGF-α. Mean ± S.E for 4 values are shown from a representative experiment. The experiment was performed on 3 separate occasions with similar results.

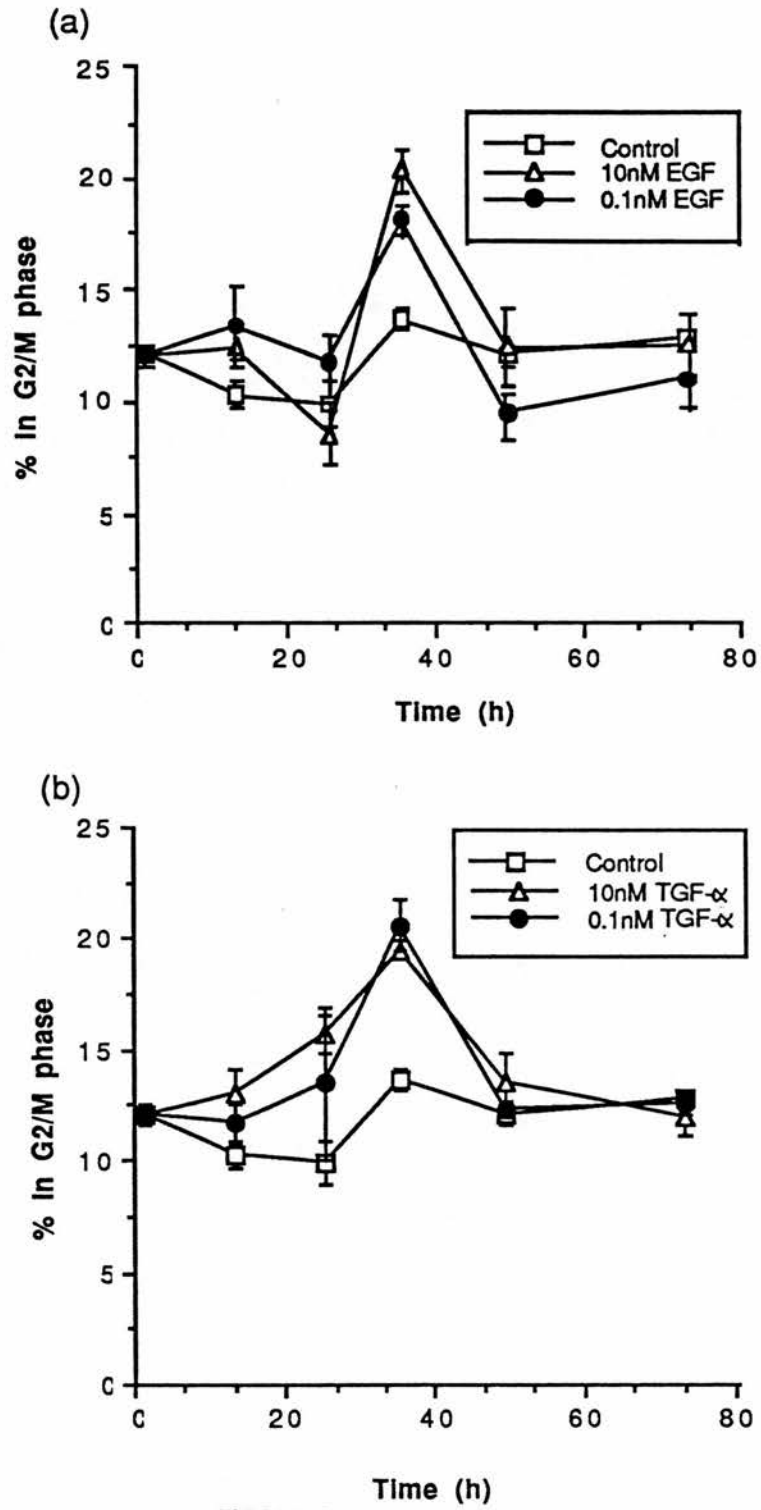

Figure 3.33 a and b Effects of (a) EGF and (b) TGF-α on the distribution of PEO14 cells in G2/M phase of the cell cycle. Cells were cultured for 72 hours in phenol red free RPMI 1640 containing 5% dcs-FCS in the presence or absence of 0.1 or 10 nM EGF or TGF-α Mean ± S.E for 4 values are shown from a representative experiment. The experiment was performed on 3 separate occasions with similar results.

(ii) Effect of replenishment of media on cell cycle analysis over 7 days

The results from a single experiment, to follow the changes in the cell cycle distribution of PEO1 cells produced by 10 nM EGF over a 7 day culture period, are shown in figure 3.34. This was performed to attempt to correlate cell cycle changes over the same time period as in the growth experiments. Media was replenished on days 2 and 5, as in growth experiments, to test whether feeding with fresh media and growth factor would further alter distributions. In the absence of EGF, the percentages of cells in G0/G1 and S phases fluctuated during the 7 day period, whereas the proportion in G2/M phase remained constant over the study period. Incubation with EGF produced decreases in the percentage of cells in G0/G1 phase, which diminished after 5 days in culture, to a value 5% lower than control cells, a decrease which was maintained to 7 days. Culture with EGF caused an increase in the S phase after 2 days, which had virtually diminished by days 5 and 7. In the presence of EGF the percentage of cells in G2/M phase remained relatively constant until 5 days, at which time a 7% increase occurred. Similar changes in the cell cycle distributions could be detected using the conditions employed to measure growth. Therefore replenishment of media did not alter these effects.

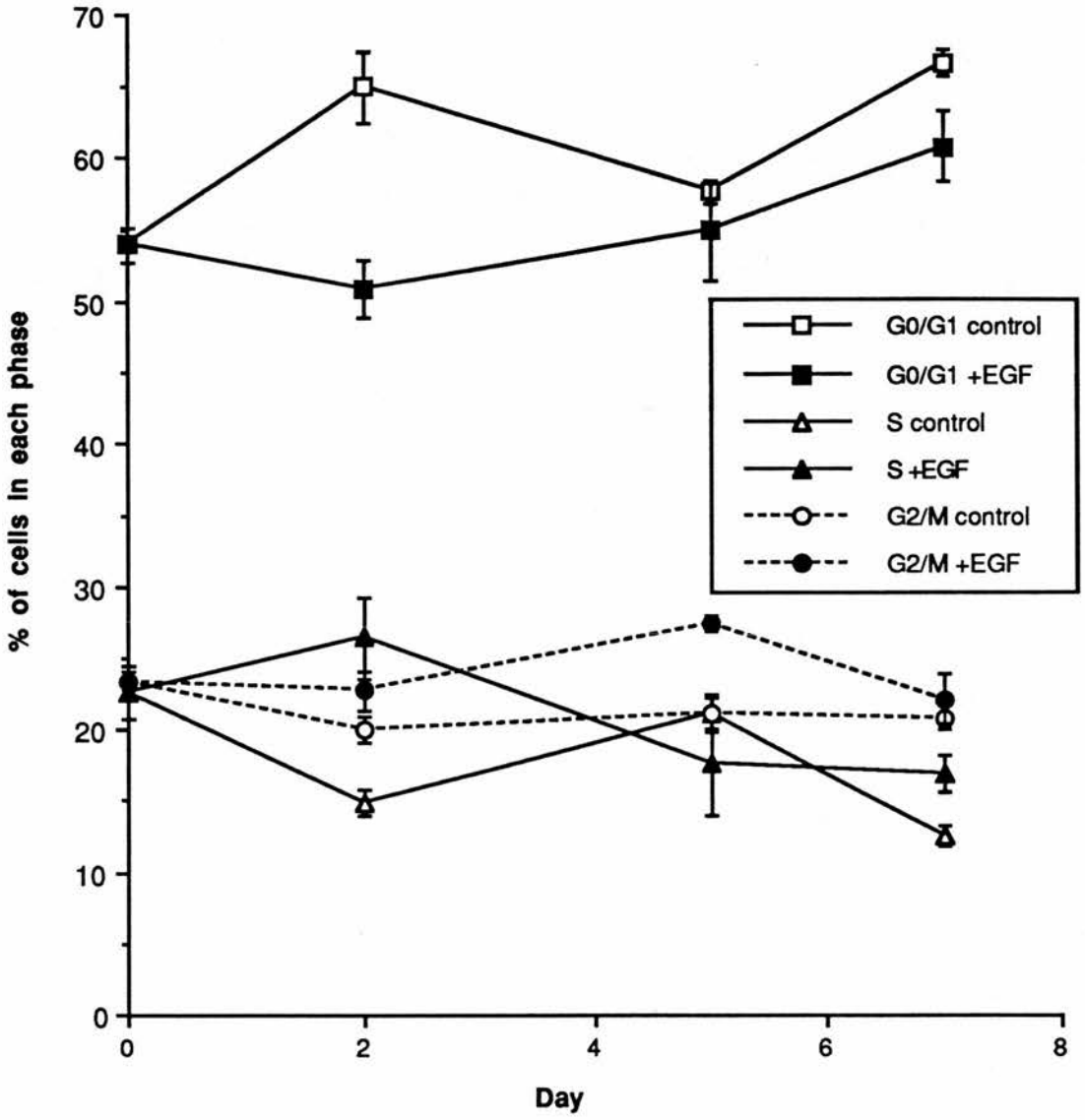

Figure 3.34. Effects of 10 nM EGF on the distribution of PEO1 cells in G0/G1, S and G2/M phase of the cycle. Cells were cultured for 7 days in phenol red free RPMI 1640 containing 5% dcs-FCS in the presence or absence of the growth factor. Media was replenished on days 2 and 5. Mean ± S.E of 4 values are shown.

3.4 Measurement of EGF receptors

As the growth of all three human ovarian cell lines was stimulated by the exogenous addition of EGF or TGF-α to the culture media, the expression of EGF receptors by these cell lines was investigated, to determine whether growth sensitivity correlated with the presence of EGF receptors on the cell surface. Assays were developed for detection by ligand binding and immuncytochemistry.

3.41 Ligand binding

(i) Validation of method

Before the ligand binding assay was used routinely to measure EGF receptors on the ovarian cell lines, a number of steps in the method were investigated to test whether these were optimal.

(ia) Influence of incubation time and temperature on specific binding

Previous experiments performed by Dr R A Hawkins in the Department of Surgery (Royal Infirmary, Edinburgh) suggested that a 90 min incubation at 26°C was optimal for the measurement of EGF receptors on primary breast tumours. In order to determine whether this was also optimal for the ovarian cell lines, the time course of binding of ^{125}I EGF was examined by incubating "membranes" from the PEO1 cell line at three different temperatures, 4°C, 26°C and 37°C, with a fixed concentration of ligand (approximately 0.22 nM), chosen to saturate high affinity sites (Dr R A Hawkins, personal communication). These results are shown in figure 3.35. At 4°C there

appeared to be two phases of binding, an initial phase in which approximately 0.002 nM was bound in 5-10 min, and a second phase in which the binding steadily increased up to 0.035 nM at 300 min. As for the 4°C incubation temperature, a large proportion of binding of ^{125}I EGF was present after 5-10 min incubation at 37°C. The specific binding increased further to approximately 0.006 nM between 90-120 min, after which a decreased level of binding was noted consistently. At 26°C, binding was again rapid during the initial 5-10 min of incubation and reached a maximum at around 60 min. Maximum binding was greater than that observed for the 4°C and 37°C incubations. After 60 min a period of reduced binding of 0.008 nM was maintained up to 300 min.

In the light of these results, it was decided to use a 90 min incubation at 26°C for all subsequent experiments.

(ib) Specificity of binding

The specificity of binding of ^{125}I EGF was tested on a "membrane" preparation of PEO1 cells incubated in the presence of varying concentrations of non-radioactive peptides, and is shown in figure 3.36. Human EGF, murine EGF and human TGF-α produced displacement of ^{125}I EGF which was similar. All concentrations of the growth factors examined (6.25-300 nM) displaced approximately 50% of the binding. No consistent displacement of ^{125}I EGF binding occurred with of 17 β-oestradiol, insulin and NGF at any concentration tested.

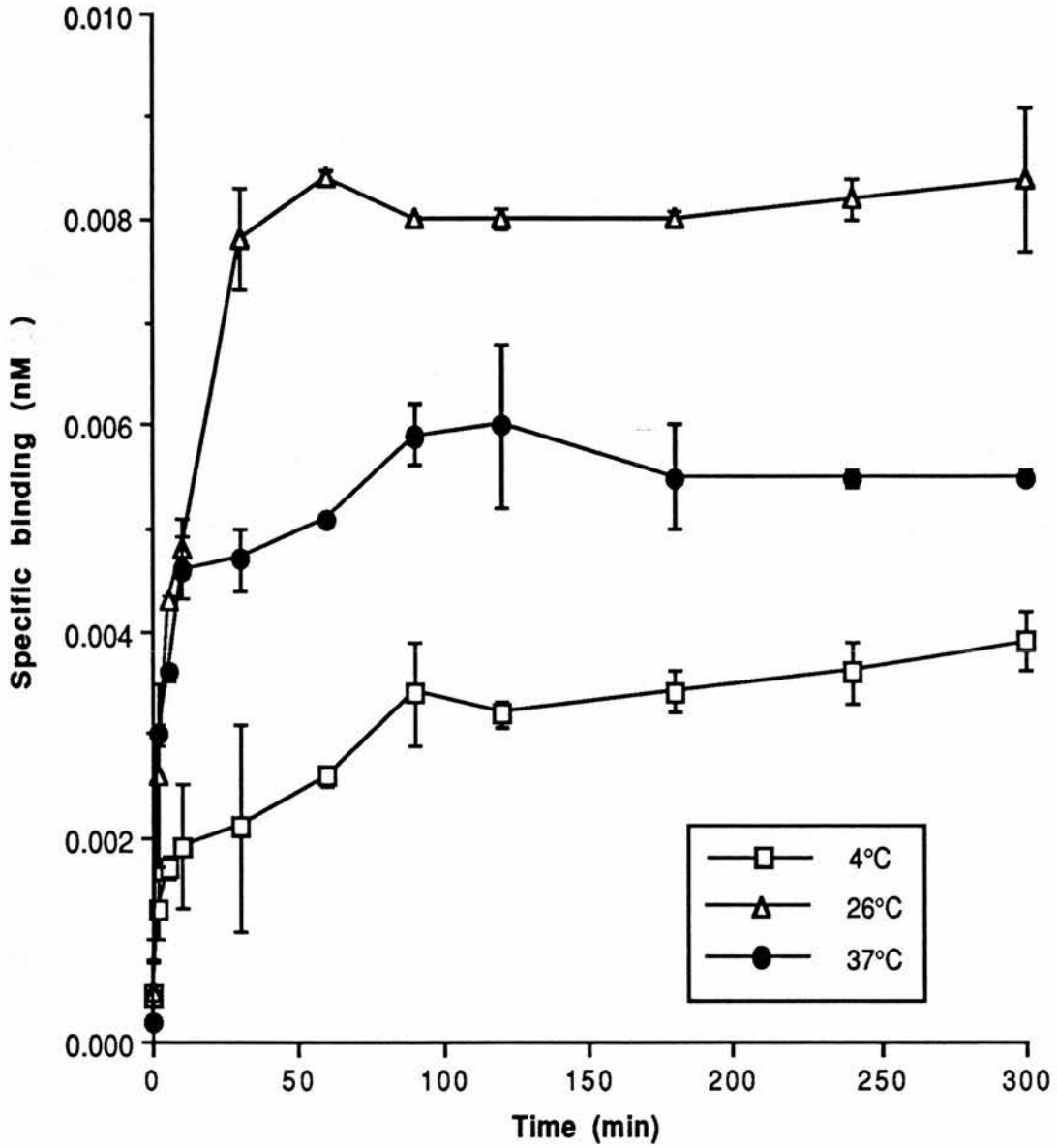

Figure 3.35. Effect of varying time and temperature of incubation period on the binding of ^{125}I EGF to PEO1 cell "membranes". "Membranes" were incubated with 125,000 cpm (approximately 0.22 nM) for varying times (0-300 min) at 4°C, 26°C or 37°C. Non-specific binding was estimated by incubation with 100-fold excess of unlabelled EGF. Points on graph represent mean ± S.E of triplicate values.

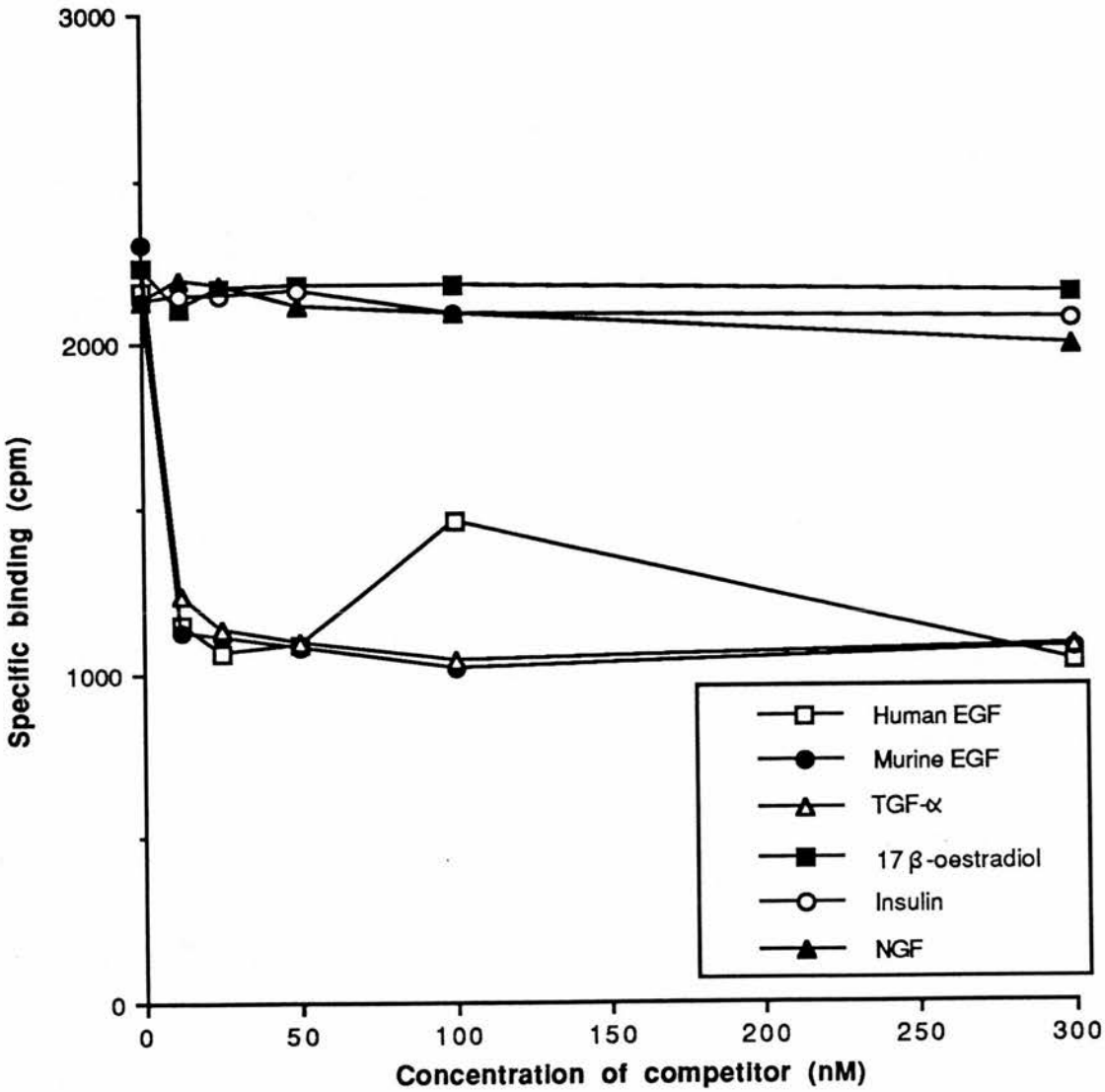

Figure 3.36. Specificity of binding of ^{125}I EGF to PEO1 cells. Membranes were incubated with 35,000 cpm ^{125}I EGF (approximately 0.04 nM) in the presence or absence of competing peptide (6.25-300 nM) for 90 min at 26°C.

(ii) Scatchard analysis

(iia) Calculation of binding site concentration

"Membranes" were incubated with a fixed concentation of ^{125}I EGF, 10,000 cpm (approximately 0.01 nM) and increasing concentrations of unlabelled EGF (0.024-300 nM), for 90 min at 26°C. Scatchard analysis of the binding data obtained from a "membrane" preparation of rat liver, used as a positive control, resulted in a curve, a typical example of which is shown in figure 3.37. Since this probably represents two binding components, binding data was analysed using the computer method of Hetherington (Sainsbury *et al.*, 1985, Nicholson *et al.*, 1988) to assign, fit and calculate the slopes and x-axis intercepts. The intercept with the x-axis of line A represents high affinity sites and the intercept of line B represents low affinity sites. The inverse of the slopes represent the Kd.

(iib) Relationship of tissue concentration to binding site concentration measured by Scatchard analysis

The effect of varying the tissue concentration (2-66 mg /ml wet weight of tissue) on the specific binding of ^{125}I EGF was investigated using a stock of pooled rat liver "membrane" preparations. The effects of assaying varying amounts of tissue on specific binding to high and low affinity sites are shown in figures 3.38 and 3.39. There was a linear relationship between specific binding for low affinity sites with a high correlation coefficient of 0.968 from regression analysis. The relationship between binding to high affinity sites was less clear and therefore had a lower correlation coefficient.

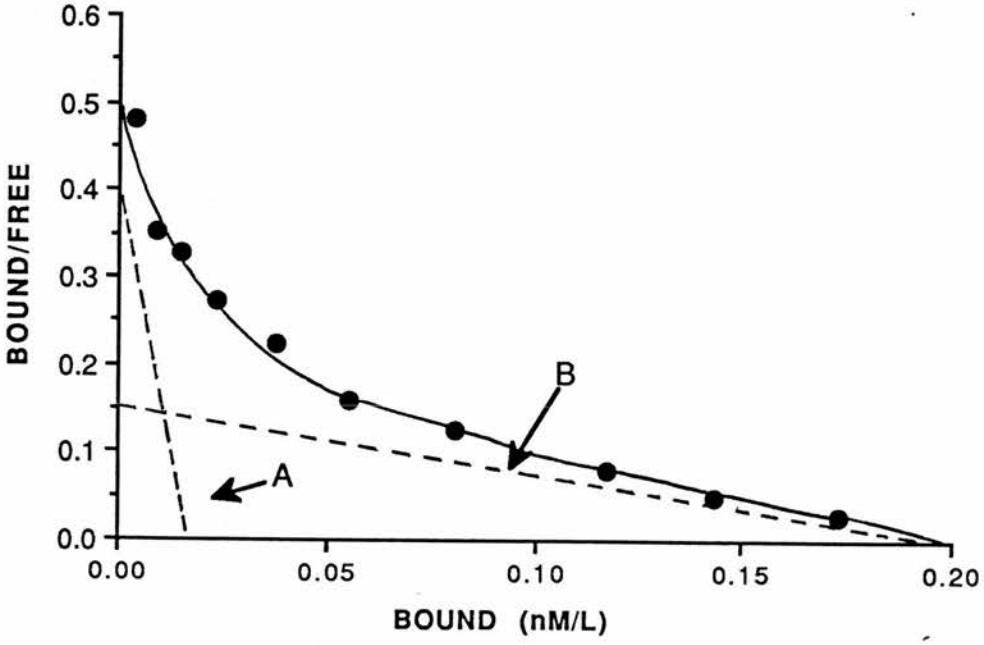

Figure 3.37. An example of Scatchard analysis of ^{125}I EGF binding to the "membrane" fraction of rat livers. Membranes were incubated with a fixed concentration of ^{125}I EGF (0.01 nM equivalent to approximately 10,000 cpm) and varying concentrations of unlabelled EGF (0.024-300 nM) for 90 min at 26°C. A non-linear binding plot was obtained representing two components of binding. The inverse of the gradient of line A is equivalent to the dissociation constant of the high affinity binding component, whereas the inverse of the gradient of line B is the constant for the lower affinity binding sites. The intercepts of the lines with the x-axis represent the number of high and low binding sites. Thus, the Kds obtained from the plots are 0.04 and 1.2 nM for the high and low affinity components respectively and represent 0.02 and 0.19 nM/L specific binding of EGF.

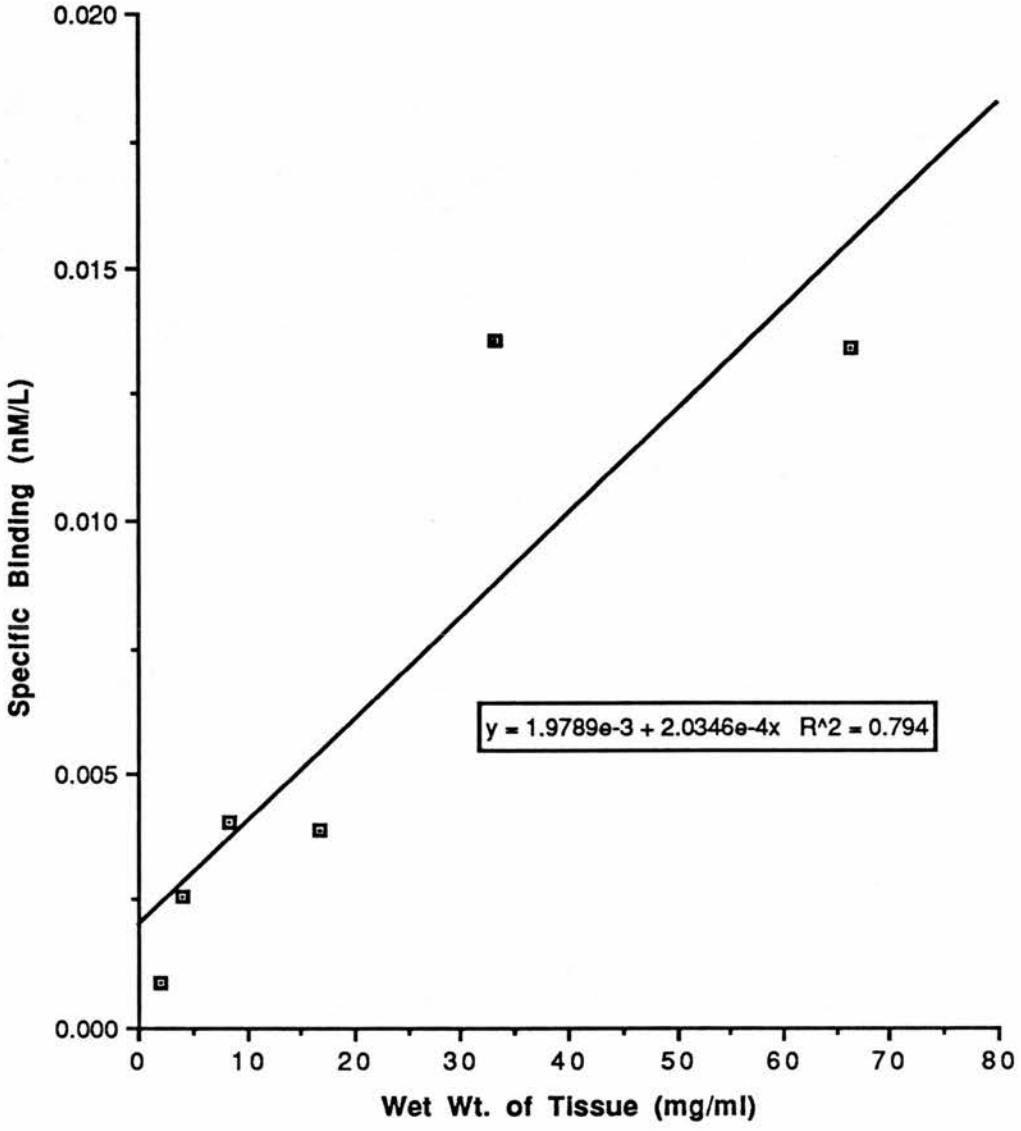

Figure 3.38. Relationship of specific binding to high affinity sites, calculated by Scatchard analysis, to amount of tissue. Various concentrations of rat liver membranes (2-66 mg / ml wet weight tissue) were incubated with 10,000 cpm (approximately 0.01 nM) ^{125}I EGF for 90 min at 26°C. Binding data was analysed using the computer method of Hetherington. Points on the graph represent the mean of 2 values. The experiment was performed on 2 separate occasions.

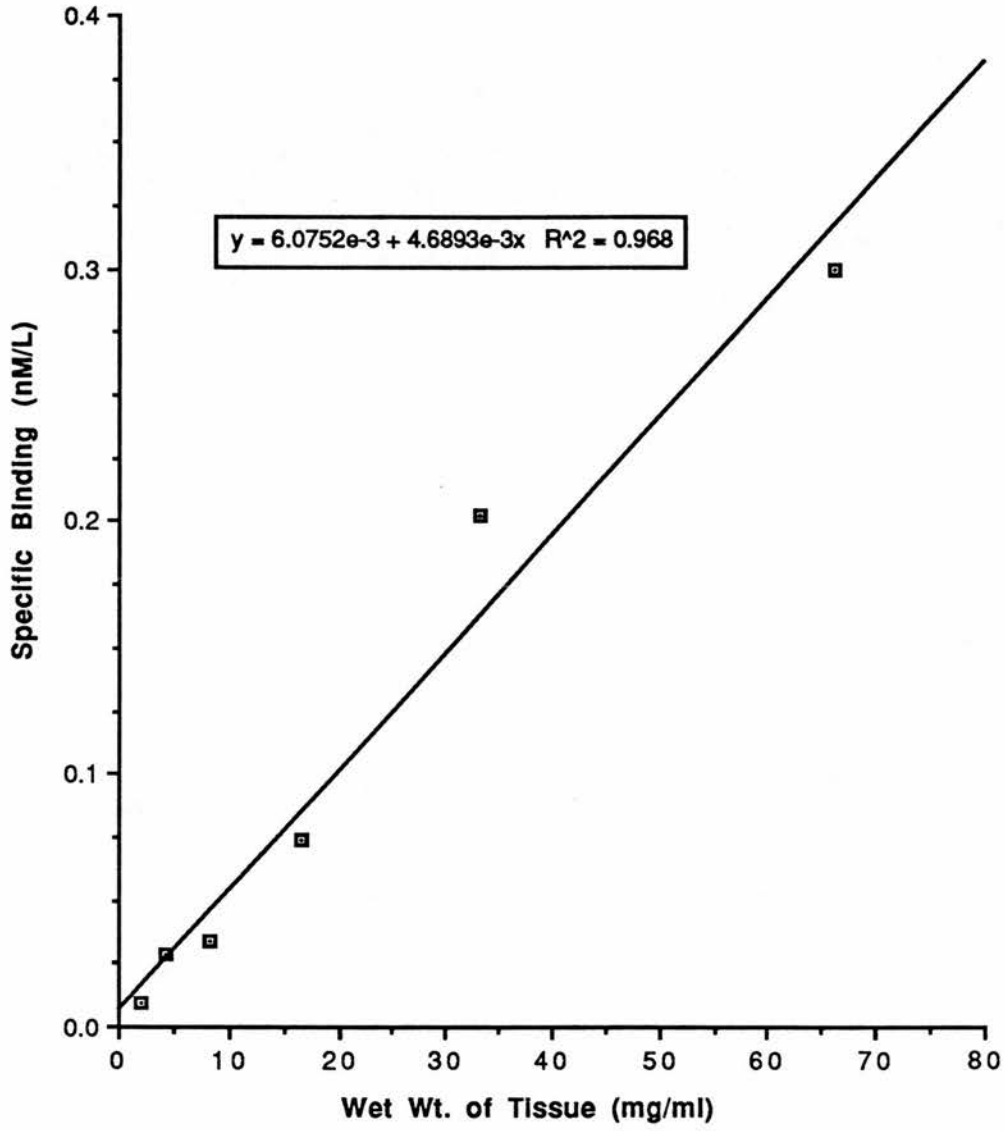

Figure 3.39. Relationship of specific binding of ^{125}I EGF to low affinity sites, calculated by Scatchard analysis, to amount of tissue. Various concentrations of rat liver membranes (2-66 mg / ml wet weight tissue) were incubated with 10,000 cpm (approximately 0.01 nM) ^{125}I EGF for 90 min at 26°C. Binding data was analysed using the computer method of Hetherington. Points on the graph represent the mean of 2 values. The experiment was performed on 2 separate occasions.

(iii) EGF receptor expression on ovarian cell lines

(iiia) Cells cultured in media containing 5% dcs-FCS

The binding of ^{125}I EGF was determined on the "membrane" preparations of PEO1, PEO4 and PEO14 ovarian adenocarcinoma cell lines cultured in phenol red free RPMI 1640 containing 5% dcs-FCS, and also in the A431 and NIH-H69 cell lines, as positive and negative controls. The range of values obtained for the five cell lines are shown in table 3.6, and examples of Scatchard plots of the ovarian carcinoma cell lines, PEO1, PEO4 and PEO14 are shown in figures 3.40-3.42. In each case, inspection of the curves revealed non-linear curves suggestive of two components of binding. For the three ovarian cell lines the dissociation constants were similar, between 0.02-0.18 nM for the high affinity, and 0.12-4.8 nM for the lower affinity binding. The number of binding sites tended to be higher in the PEO1 cell line than in PEO4 and PEO14 cells. Approximately 70-80 fold greater levels of total binding were observed in the A431 cells compared with PEO1, PEO4 and PEO14. The proportion of high affinity sites was very small in comparison with the total number of binding sites in the A431 cell line compared with the ovarian cell lines, although the dissociation constants of binding were similar.

Table 3.6. Levels of expression of high and low affinity EGF receptors (fmol/mg protein), and dissociation constants on the ovarian cell lines cultured in phenol red-free RPMI 1640 containing 5% dcs-FCS. Cell "membranes" were incubated with a fixed concentration, 10,000 cpm (approximately 0.02 nM), of ^{125}I EGF and increasing concentrations of unlabelled EGF (0.024-100 nM) for 90 min at 26°C. Non-specific binding was estimated by incubation with 300 nM of unlabelled EGF. Binding data was analysed according to Scatchard (1949). The table shows the mean values ± S.E. for the number of experiments which are indicated (n). Ranges of values obtained are shown in brackets.

Cell line	n	High affinity site(fmol/mg protein)	Kd (nM)	Low affinity sites(fmol/mg protein)	Kd (nM)
PEO1	5	24±19 (3-60)	0.12 ± 0.04 (0.08-0.18)	135±59 (64-229)	1.8 ± 1.0 (0.12-2.8)
PEO4	3	3.6 ±4.0 (1.4-9.3)	0.09 ±0.03 (0.05-0.12)	51±20 (36-80)	2.8 ±1.9 (0.12-4.76)
PEO14	3	1±0.4 (0.7-1.5)	0.04 ±0.01 (0.02-0.05)	93.5 ±32.7 (48-121)	1.9 ±0.1 (1.7-2.0)
A431	2	277.4 ±244 (32.9-522)	0.18 ±0.01 (0.18-0.19)	7717 ±1509 (6208-9227)	5.4 ±0.6 (4.8-5.9)
NIH-H69	3	0±0 (0-0)	-	0±0 (0-0)	-

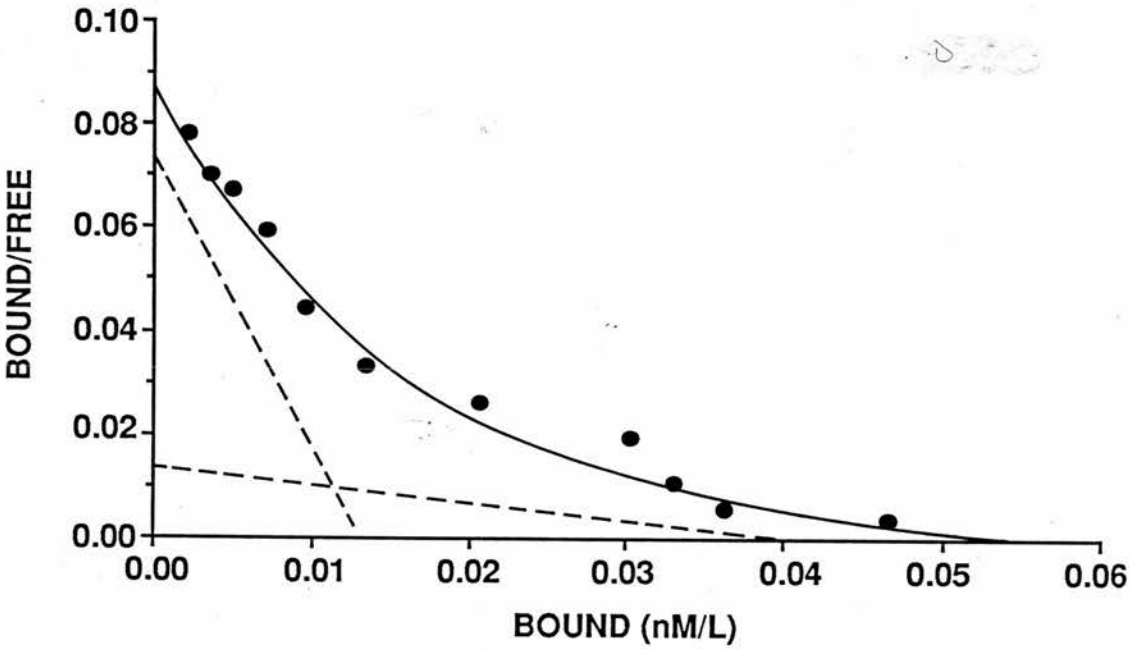

Figure 3.40. Scatchard analysis of ^{125}I EGF binding to "membrane" fraction of PEO1 cells. Cells were incubated with a fixed concentration of ^{125}I EGF (0.01 nM eqivalent to approximately 10,000 cpm) and varying concentrations of unlabelled EGF (0.024-300 nM) for 90 min at 26°C. A non-linear binding plot was obtained representing two components of binding. The Kds obtained from the plots are 0.18 nM and 2.8 nM for the high and low affinity components respectively and represent 22 and 70 fmol/mg protein of specific binding of EGF.

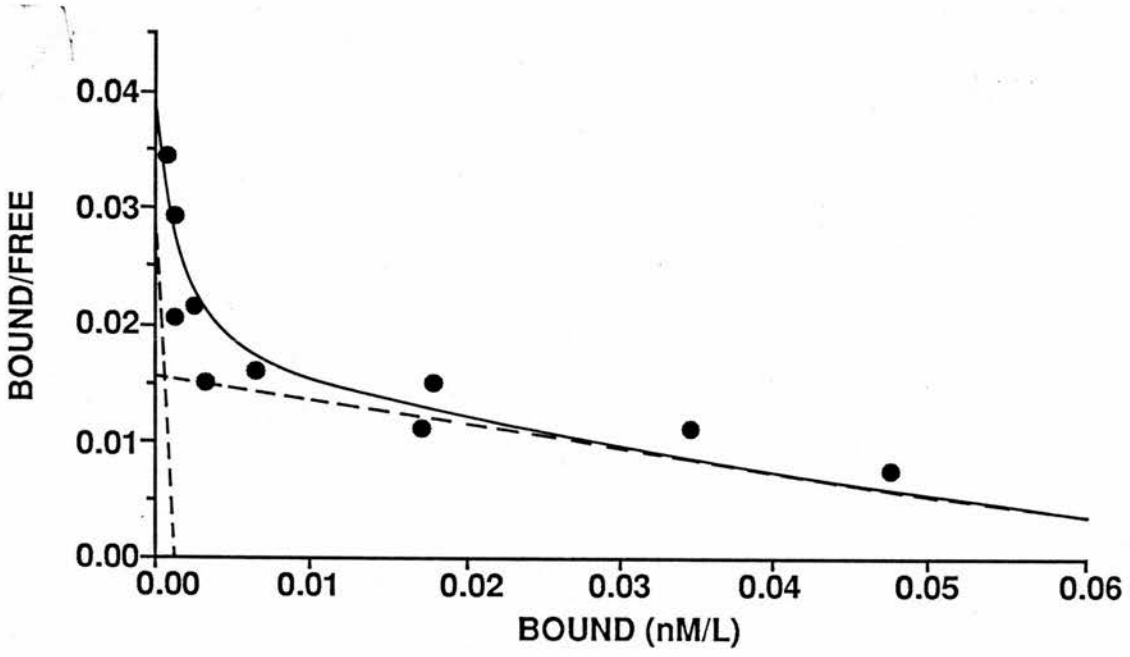

Figure 3.41. Scatchard analysis of ^{125}I EGF binding to "membrane" fraction of PEO4 cells. Cells were incubated with a fixed concentration of ^{125}I EGF (0.01 nM eqivalent to approximately 10,000 cpm) and varying concentrations of unlabelled EGF (0.024-300 nM) for 90 min at 26°C. A non-linear binding plot was obtained representing two components of binding. The Kds obtained from the plots are 0.05 nM and 4.8 nM for the high and low affinity components respectively and represent 1.4 and 80 fmol/mg protein of specific binding of EGF.

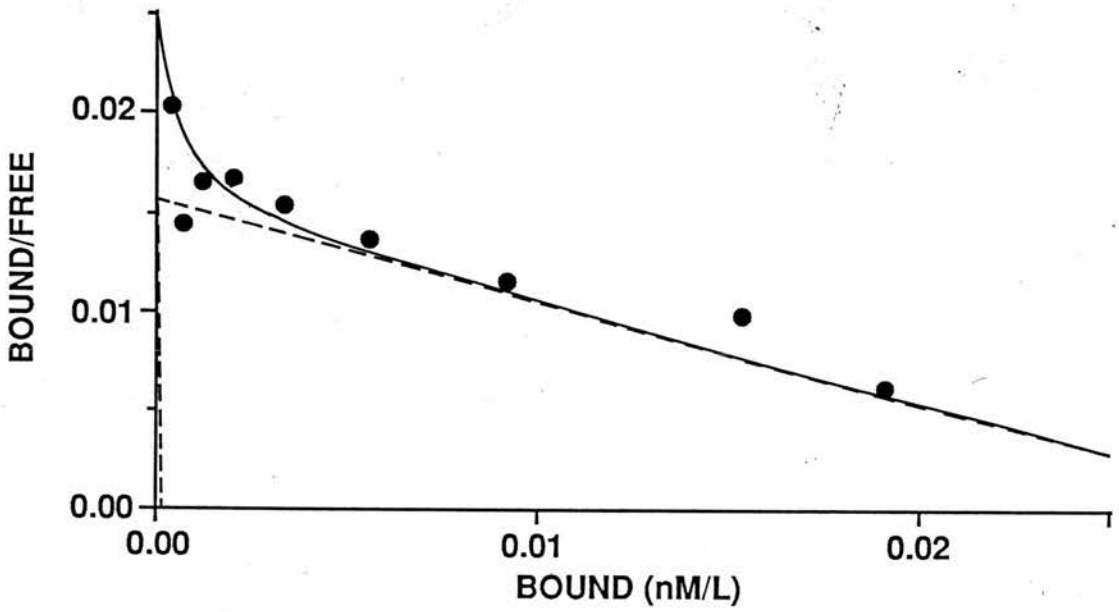

Figure 3.42. Scatchard analysis of ^{125}I EGF binding to "membrane" fraction of PEO14 cells. Cells were incubated with a fixed concentration of ^{125}I EGF (0.01 nM eqivalent to approximately 10,000 cpm) and varying concentrations of unlabelled EGF (0.024-300 nM) for 90 min at 26°C. A non-linear binding plot was obtained representing two components of binding. The Kds obtained from the plots are 0.05 nM and 2.0 nM for the high and low affinity components respectively and represent 1.5 and 50 fmol/mg protein of specific binding of EGF.

(iiib) Cells cultured in serum free media

To investigate whether the presence of serum had any effect on the expression of EGF receptors, the binding of ^{125}I EGF to "membrane " preparations of PEO1, PEO4 and PEO14 cells which had been cultured in the absence of serum in phenol red free RPMI 1640 containing HITS, was also analysed. Results are shown in table 3.7. Non-linear Scatchard plots were detected as previously obtained for cells grown in media containing 5% dcs-FCS (data not shown). The values for high and low affinity binding sites and the dissociation constants were within the same range as those observed in the presence of serum

except for PEO14 cells which have an increased number of binding sites which show greater dissociation constants.

Table 3.7. Levels of expression of high and low affinity EGF receptors (fmol/mg protein) and binding affinties (nM) on the ovarian cell lines, cultured in phenol red-free RPMI 1640 containing HITS. Binding was performed as described in table 3.6 and analysed according to Scatchard (1949). Results from one experiment shown.

Cell line	High affinity sites (fmol/mg protein)	Kd (nM)	Low affinity sites (fmol/mg protein)	Kd (nM)
PEO1	14	0.22	75.8	3.4
PEO4	5.4	0.26	20.7	4.2
PEO14	10.3	0.16	375	8.8

(iiic) Effect of culturing cells in the presence of EGF

EGF exerts its biological action by binding to specific receptors on the cell membrane. Down-regulation of the EGF receptors is a consequence of this (see Introduction). Therefore, the effect of EGF on the level of EGF receptor expression was investigated by Scatchard analysis of the binding data of PEO1 cells, to determine whether changes in receptor number could be detected. Cells were cultured in phenol red free RPMI 1640 containing 5% dcs-FCS in the presence and absence of 1 nM EGF for 5 days. The results from the Scatchard analysis are shown in table 3.8. The presence of EGF in the culture media produced a decrease in the number of high and low affinity binding sites, but had no effect on the dissociation of binding constants.

Table 3.8. Effect of incubation with EGF on the high and low affinity EGF receptor levels and dissociation constants on PEO1 cells. Cells were cultured in phenol red-free RPMI 1640 containing 5% dcs-FCS in the presence or absence of 1 nM EGF for 5 days. Cell "membranes " were incubated with a fixed concentration, 10,000 cpm (approximately 0.02 nM) of ^{125}I EGF and increasing concentrations of unlabelled EGF (0.024-100 nM) for 90 min at 26°C. Non-specific binding was estimated by incubation with 300 nM of unlabelled EGF. Binding data was analysed according to Scatchard (1949). EGF receptor levels from one experiment are shown.

Media	High affinity sites (fmol/mg protein)	Kd (nM)	Low affinity sites (fmol/mg protein)	Kd (nM)
Control	16.3	0.13	148.9	2.9
+ EGF	7.2	0.10	72.8	2.2

(iiid) Effect of culturing cells in the presence of oestrogen

It is believed that oestrogen may mediate some of its growth stimulatory effects by altering the secretion of growth factors from target cells. Therefore, it is possible that oestrogen may interact with the EGF growth regulatory pathway and alter the level of expression of EGF receptors. The effect of culturing PEO1, PEO4 and PEO14 cells with 17 β-oestradiol on the level of EGF receptor expression, was investigated. The cell lines were cultured in phenol red free RPMI 1640 containing 5% dcs-FCS in the presence or absence of 10^{-8} M 17 β-oestradiol for 5 days. Results of the Scatchard analysis of the binding data are shown in table 3.9. The level of high and low affinity EGF receptors detected in the two ER-positive cell lines, PEO1 and PEO4, fell in the presence of oestrogen in the culture media by approximately 50% compared with untreated cells. The dissociation of binding constants for the high affinity sites on both cell lines were unaffected, but the constant for the lower affinity sites in PEO1 cells was reduced from 3 to 1.5 nM. Culture with 17 β-oestradiol had no effect on the EGF receptor levels or affinity of the ER-negative cell line PEO14.

Table 3.9. Effect of incubation with oestrogen on the high and low affinity EGF receptor levels, and binding affinities on PEO1, PEO4 and PEO14 cell lines. Cells were cultured in phenol red-free RPMI 1640 containing 5% dcs-FCS in the presence or absence of 10^{-8} M 17 β-oestradiol. Binding was performed as described in table 3.8 and analysed according to Scatchard (1949). Results from one experiment are shown.

Cell line/ Media	High affinity sites (fmol/mg protein)	Kd (nM)	Low affinity sites (fmol/mg protein)	Kd (nM)
PEO1/control	16.3	0.13	148.9	2.9
PEO1/+ E_2	6.9	0.10	83.5	1.5
PEO4/control	9.3	0.12	38.6	3.4
PEO4/+ E_2	2.7	0.10	13.0	2.8
PEO14/control	0.7	0.02	121.0	1.9
PEO14/+E_2	0.7	0.04	112.0	1.7

3.42 Immunocytochemistry

Ligand binding is a quantitative assay for EGF receptors. However, no information is obtained regarding the heterogeneity of expression. Immunocytochemical techniques are only semi-quantitative, but they are able to measure the proportion of cells which possess EGF receptors on their surface. Therefore, the distribution of the EGF receptors on the ovarian cell lines was investigated using two methods of immunocytochemical analysis, indirect horse-radish peroxidase immunocytochemistry and flow cytometry employing a fluorescent label.

(i) Static cytometry

(ia) Titration of antibodies

The assays for EGF receptors were based on an indirect immunoperoxidase technique employing two different monoclonal antibodies, EGFR1 (directed against the external domain of the receptor), and F4 (directed against a small portion of the cytoplasmic domain). The optimal dilutions of antibodies were determined by titrating against the vulval carcinoma cell line A431, which is known to overexpress EGF receptors (Merlino *et al*, 1984) and the small cell lung cancer cell line NIH-H69, which does not express EGF receptors (Gamou *et al*, 1987; Haeder *et al*, 1988). The dilution of antibody at which the strongest intensity of staining of A431 cells was noted, without staining present in the NIH-H69 cells was chosen to be optimal. Collective results from 3 separate experiments are shown in tables 3.10 and 3.11. The EGFR1 antibody showed positive staining in NIH-H69 cells at concentrations greater than 1:100, and reduced reactivity against A431 cells at

concentrations less than 1:100. Therefore, this concentration was used in further studies on the ovarian carcinoma cell lines.

Table 3.10. Titration of the EGFR1 monoclonal antibody using a positive control cell line, A431 and a negative control cell line, NIH-H69. Percentage of cells scored as positive are shown.

	Cell Line	
Antibody Dilution	**A431**	**NIH-H69**
	% cells positve	
Neat	>90	30-40
1: 2	>90	30-40
1: 5	>90	20-30
1:10	>90	<20
1:100	>90	0
1:500	60-70	0
1:1000	<10	0

The monoclonal antibody F4 was tested against the same cell lines at similar concentrations to that used in EGFR1. At concentrations greater than 1:50 positive staining was observed in the majority of NIH-H69 cells. The percentage of cells stained in the positive control cell line was reduced at concentrations less than 1:50. Thus F4 was used routinely at this dilution.

Table 3.11. Titration of F4 monoclonal antibody against a positive control cell line, A431 and a negative control cell line, NIH-H69. Percentage of cells scored positive are shown.

	Cell Line	
Antibody Dilution	**A431**	**NIH-H69**
	% cells positive	
Neat	>90	>90
1:10	>90	>90
1:50	>90	0
1:100	80-90	0
1:500	80-90	0

(ib) EGF receptor expression on PEO1, PEO4 and PEO14 cells

The results of the immunocytochemical detection of EGF receptors using EGFR1 and F4 antibodies against the ovarian carcinoma and control cell lines are shown in table 3.12. Results are also included for the positive contol, CAM 5.2, an antibody directed against cytokeratins 8, 18 and 19, and for the negative control, tris buffer. The intensity of staining is expressed relative to that observed in the A431 cell line. Examples of the staining are shown in figures 3.43-3.48. Using the F4 antibody , EGF receptors could be detected in greater than 90% of the cells in the three ovarian lines. Staining was weak in comparison to A431 cells. PEO1 and PEO4 cell lines also showed positive staining with the EGFR1 monoclonal antibody. The staining in PEO1 cells was stronger than in PEO4 cells but less than that observed in the A431 cell line. No positive staining occurred in the PEO14 cell line with the EGFR1 antibody.

Table 3.12. Expression of EGF receptors detected by an indirect immunoperoxidase staining technique. The monoclonal antibodies were used at dilutions of 1:100 (EGFR1) and 1:50 (F4). A positive control antibody, CAM 5.2 and tris buffer as a negative control were included in each staining run. In addition, the A431 and NIH-H69 cell lines were included. Table shows percentage of cells stained positively and intensity of staining, represented by - absent, + weak, ++ moderate, +++, strong. Each cell line was tested on at least three separate occasions.

	% cells positive			
	Monoclonal Antibody			
Cell Line	**EGFR1**	**F4**	**CAM5.2**	**Tris**
PEO1	>90	>90	>90	-ve
	+	+	+++	
PEO4	>90	>90	>90	-ve
	+	+	+++	
PEO14	-ve	>90	>90	-ve
		+	+++	
A431	>90	>90	>90	-ve
	+++	+++	+++	
NIH-H69	-ve	-ve	>90	-ve
			+++	

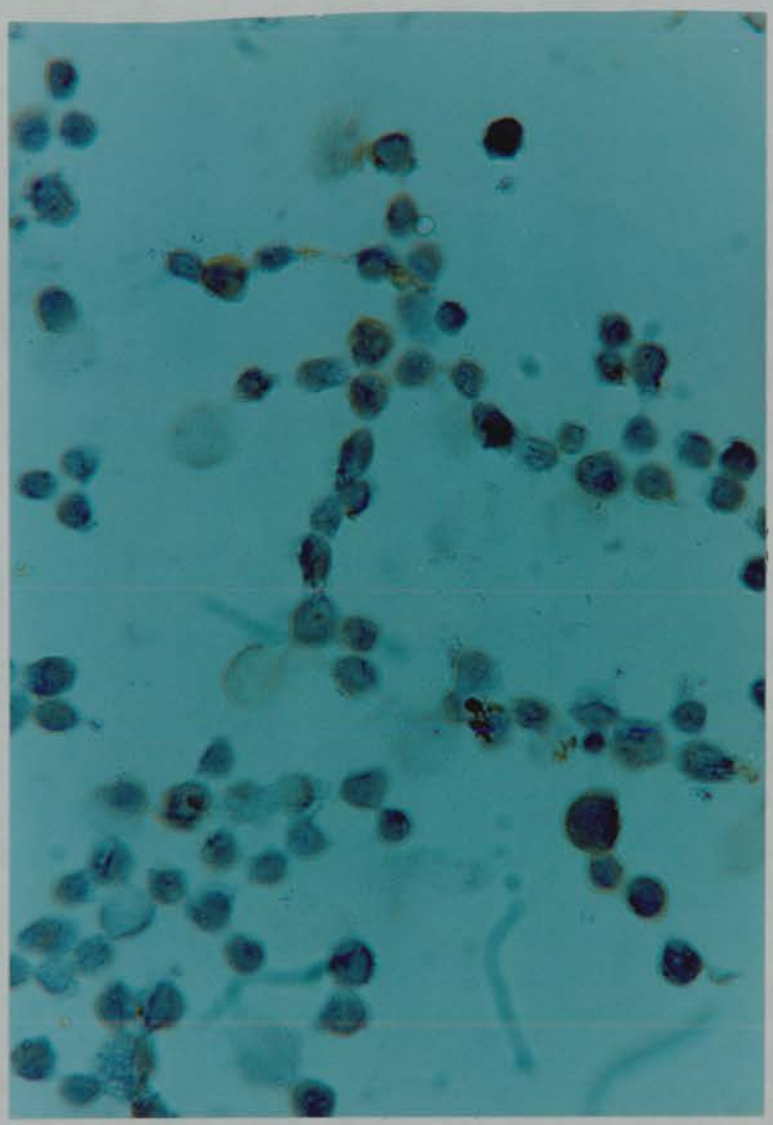

Figure 3.43. Illustration of weak positive staining with the F4 (1:50) antibody in greater than 90% of PEO1 cells. EGF receptors were detected using indirect immunoperoxidase staining with avidin-biotin complex and 3, 3-diaminobenzidine tetrahydrochloride (DAB) as substrate.

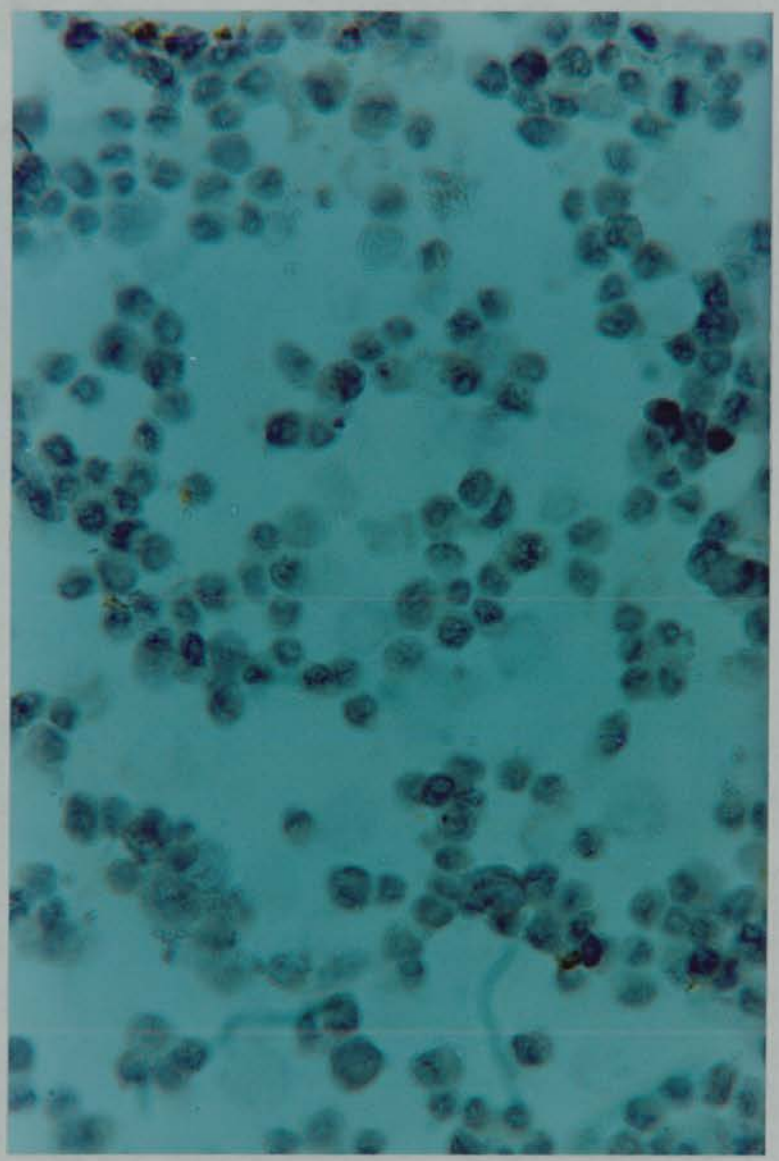

Figure 3.44. Illustration of weak positive staining with the F4 (1:50) antibody in greater than 90% of PEO4 cells. EGF receptors were detected using indirect immunoperoxidase staining with avidin-biotin complex and DAB as substrate.

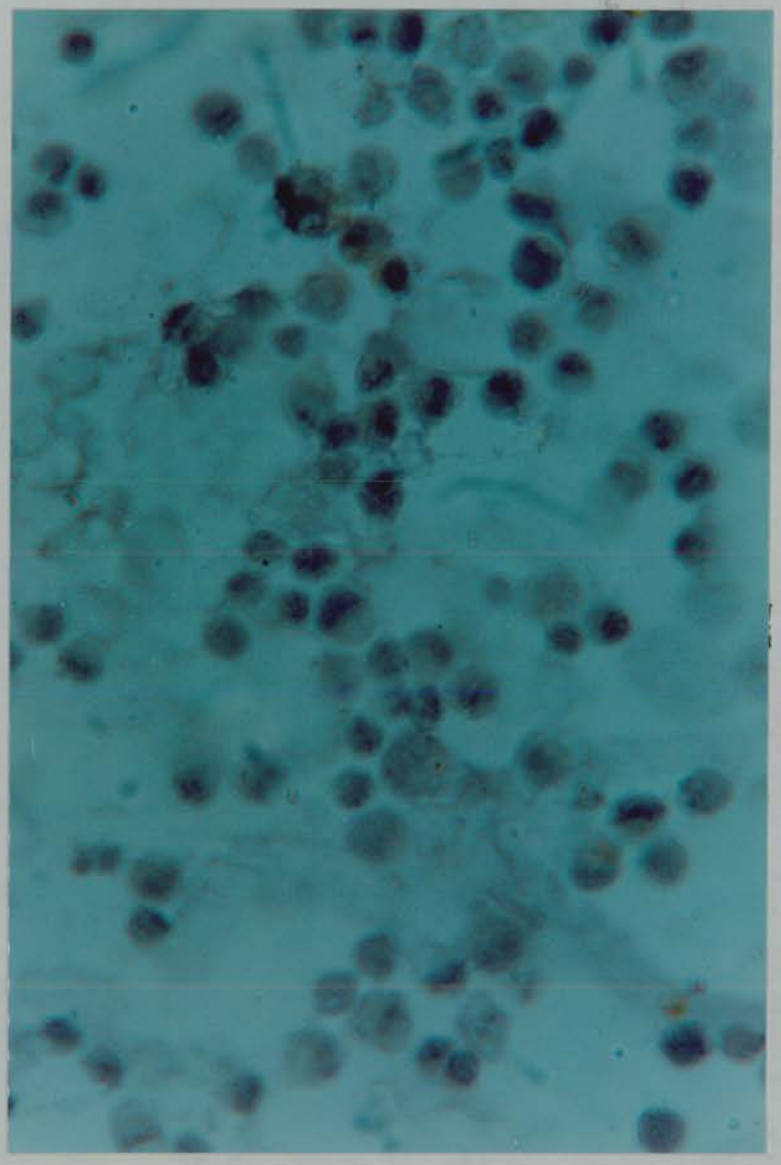

Figure 3.45. Illustration of positive staining with F4 (1:50) antibodies in PEO14 cells. The positive staining with F4 was weak and present in greater than 90% of cells. EGF receptors were detected using indirect immunoperoxidase staining with avidin-biotin complex and DAB as substrate.

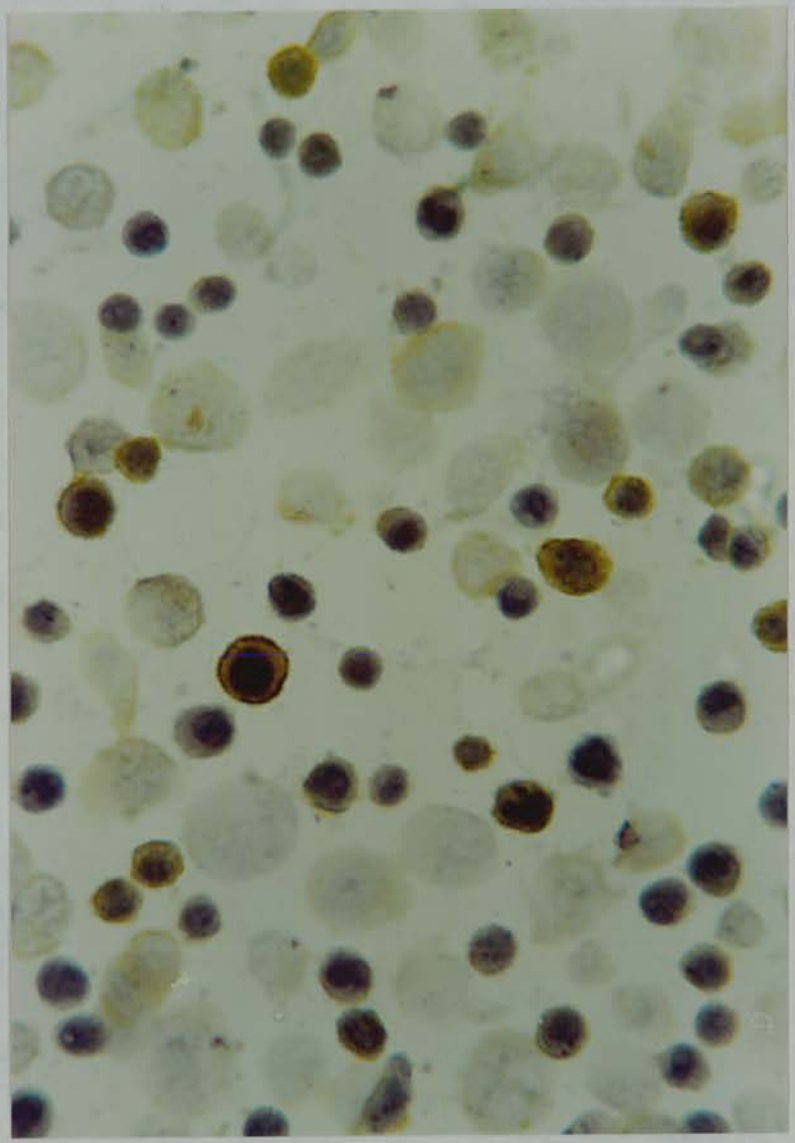

Figure 3.46. Illustration of strong positive staining with the F4 (1:50) antibody in the positive control cell line, A431. EGF receptors were detected using indirect immunoperoxidase staining with avidin-biotin complex and DAB as substrate.

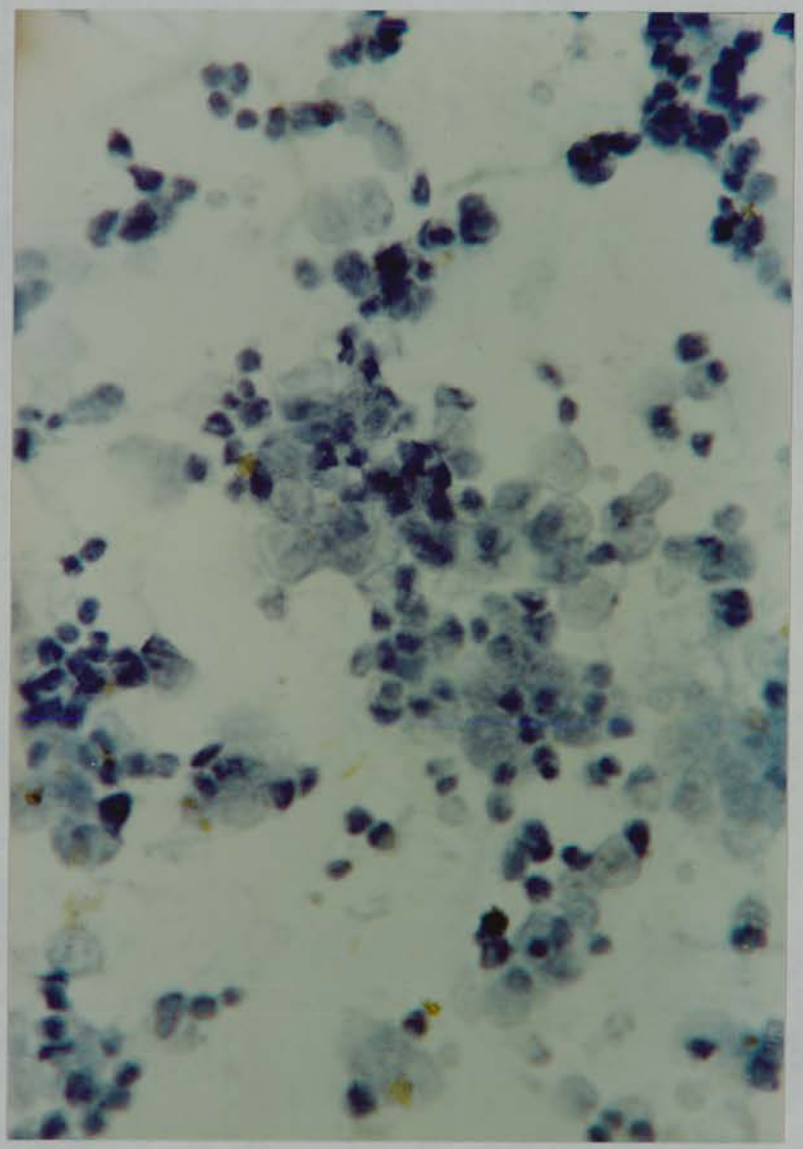

Figure 3.47. Illustration of negative staining with the F4 (1:50) antibody in the negative control cell line, NIH-H69. An indirect immunoperoxidase staining technique with avidin-biotin complex and DAB as substrate was employed.

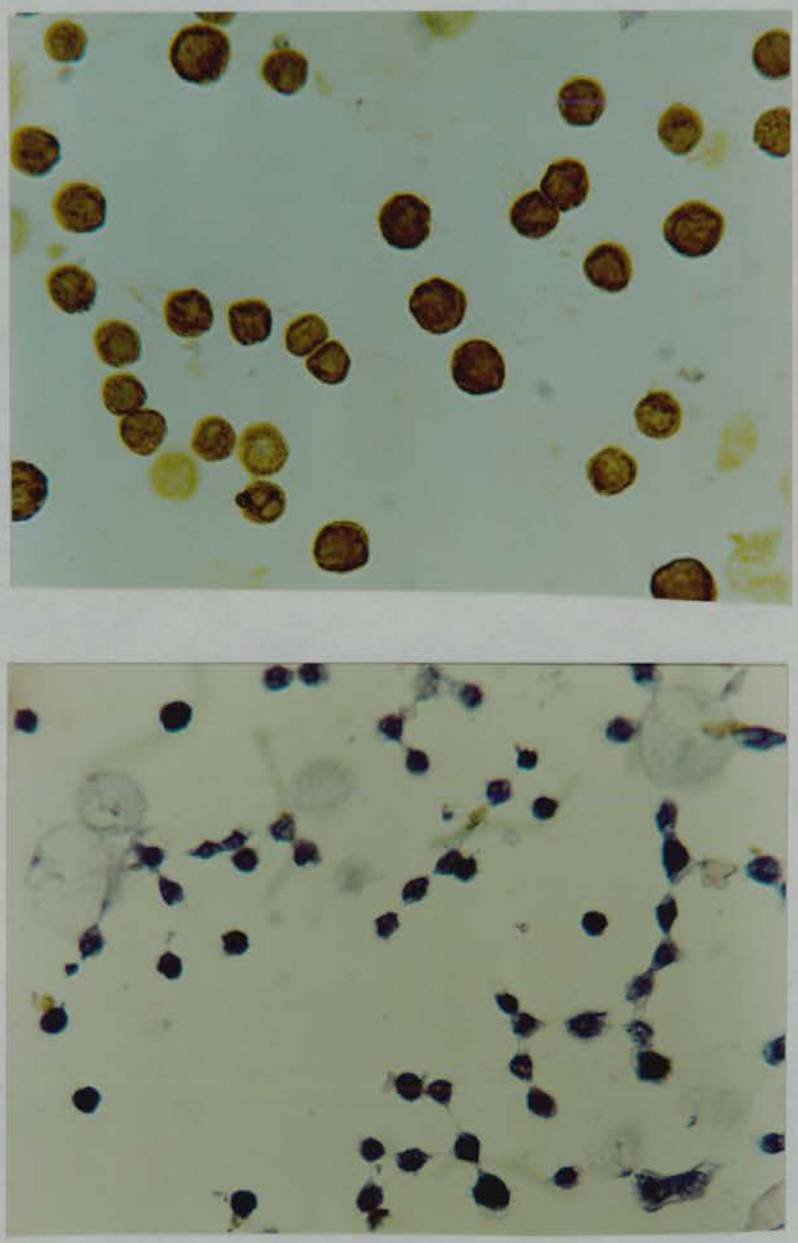

Figure 3.48. Illustration of (a) positive staining with the positive control antibody, CAM 5.2, and (b) negative staining with tris buffer, in PEO1 cells, using indirect immunoperoxidase staining with avidin-biotin complex and DAB as substrate.

(ii) Flow cytometry

(iia) Effect of varying EGFR1 concentration

An experiment was performed to determine the optimum dilution of EGFR1 monoclonal antibody to be used in experiments to study EGF receptor expression by flow cytometry. Concentration of antibody was varied, and the percentage of cells staining positively above background, and the intensity of staining were measured in PEO1, PEO4 and PEO14 cells, harvested by trypsinisation. The flow cytometric analysis was performed by Mr E Miller, Department of Clinical Oncology. Background staining was determined by estimation of the percentage of cells positively stained and the intensity of that staining in the absence of the EGFR1 monoclonal antibody. Results are shown in tables 3.13 a and b. In all three cell lines, the 1:2 antibody dilution produced the largest percentage of positively stained cells and the greatest staining intensity. Both parameters decreased with dilution of the antibody. For PEO1 cells, the decrease in percentage of positive cells with antibody dilution was small compared with that in intensity of staining. Ideally, in the light of these results, the 1:2 dilution of antibody would have been used routinely to determine EGF receptors of the ovarian cell lines. However, due to the limited quantity of antibody, it was necessary to utilise the 1:5 dilution for routine analysis. At the 1:5 dilution no positive staining was detected on the negative control cell line NIH-H69.

Table 3.13 a. Effect of varying the dilution of EGFR1 monoclonal antibody on the percentage of cells staining positive for EGF receptors, above background.

	Antibody Dilution			
Cell Line	**1:2**	**1:5**	**1:10**	**1: 20**
PEO1	98.3	98.2	98.2	96. 8
PEO4	89.6	82.0	72.1	61. 5
PEO14	60.1	29.4	24.9	18.9

Table 3.13 b. Effect of varying the dilution of EGFR1 monoclonal antibody on the intensity of fluorescence above background.

	Antibody Dilution			
Cell Line	**1:2**	**1:5**	**1:10**	**1:20**
PEO1	3.62	3.37	3.26	2.98
PEO4	2.18	1.74	1.67	1.59
PEO14	3.06	2.73	2.44	2.25

(iib) Effect of different methods of cell harvesting

To determine if the method of cell harvesting affected the ability to detect EGF receptors, PEO4 cells were grown in culture for 5 days and harvested using trypsin/versene solution, EDTA alone or by scraping with a cell scraper. The percentage of cells stained positive for the EGF receptor and the intensity of staining following each treatment are shown in table 3.14. Detachment with trypsin/versene solution yielded a cell population which had a higher proportion of cell staining, with a higher staining intensity, than populations of cells prepared by harvesting by either scraping or EDTA treatment. Incubation with trypsin/versene was therefore chosen as the routine method for harvesting cells for flow cytometric analysis.

Table 3.14. Effect of different methods of cell harvesting on percentage of PEO4 cells staining positive for EGF receptors with EGFR1 antibody used at 1:5 dilution, and the intensity of fluorescence obtained, relative to background staining.

Treatment	% staining	Intensity
Trypsin	60.3	3. 93
Scraping	19.0	2. 37
EDTA	6.6	1.97

(iic) EGF receptor expression by ovarian cell lines

The PEO1, PEO4 and PEO14 cell lines were cultured in phenol red-free RPMI 1640 containing 5% dcs-FCS for 5 days, and the intensity of staining and percentage of cells stained with EGFR1 (1:5) was measured. A positive control cell line, A431, and negative control line, NIH-H69, were also included. The expression of EGF receptors of cells were measured on 7 separate occasions. Representative plots showing intensity of staining in the three ovarian cell lines are shown in figure 3.49. A summary of the results obtained are shown in Table 3.15.

Table 3.15. EGF receptor levels on PEO1, PEO4 and PEO14 cells measured by flow cytometry. Intensity of green fluorescence and % of cells stained above background with EGFR1 (1:5) were determined. Background staining was obtained in the absence of the primary antibody. Mean ± S.E. are shown for 7 separate measurements.

Cell line	% of cells stained	Intensity of staining
PEO1	97.1	3.3
	±2.3	±0.2
PEO4	66.2	2.2
	±13	±0.3
PEO14	64.8	2.8
	±18	±0.2

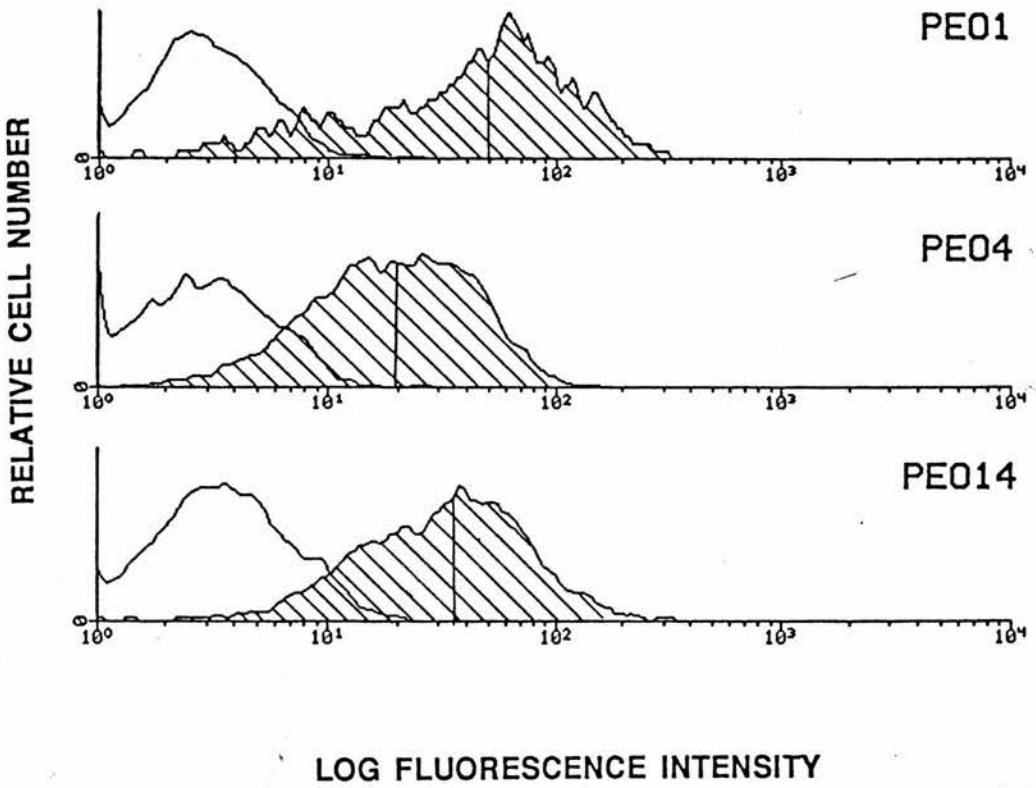

Figure 3.49. Relative fluorescence intensities in the cell lines following incubation with EGFR1 (1:5), followed by incubation with sheep anti-mouse FITC. Background fluorescence (where no primary antibody was included) is indicated by the open curve. The median value for the EGF receptor staining is indicated by the vertical line in the shaded curve. Of the ovarian cell lines, PEO1 cells showed the greatest intensity of staining, and the highest percentage of cells expressing EGF receptors. The intensity of staining obtained was approximately 15 times less than that of the positive control cell line A431. The percentages of cells in the PEO4 and PEO14 cell lines stained positively were similar, although PEO14 cells had a greater intensity of staining. No positive staining was detected on the negative control cell line NIH-H69.

Of the ovarian cell lines, PEO1 cells showed the greatest intensity of staining, and the highest percentage of cells expressing EGF receptors. The intensity of staining obtained was approximately 15 times less than that of the positive control cell line A431. The percentage of cells in the PEO4 and PEO14 cell lines stained positively were similar, although PEO14 cells had a greater intensity of staining. No positive staining was detected on the negative control cell line NIH-H69.

The variations in the percentages of cells stained and the intensity of staining between the 7 experiments are illustrated in figure 3.50. Large variations in the percentages of cells expressing EGF receptors were found between experiments for the PEO4 and PEO14 cell lines. The intensity of staining was more consistent between experiments.

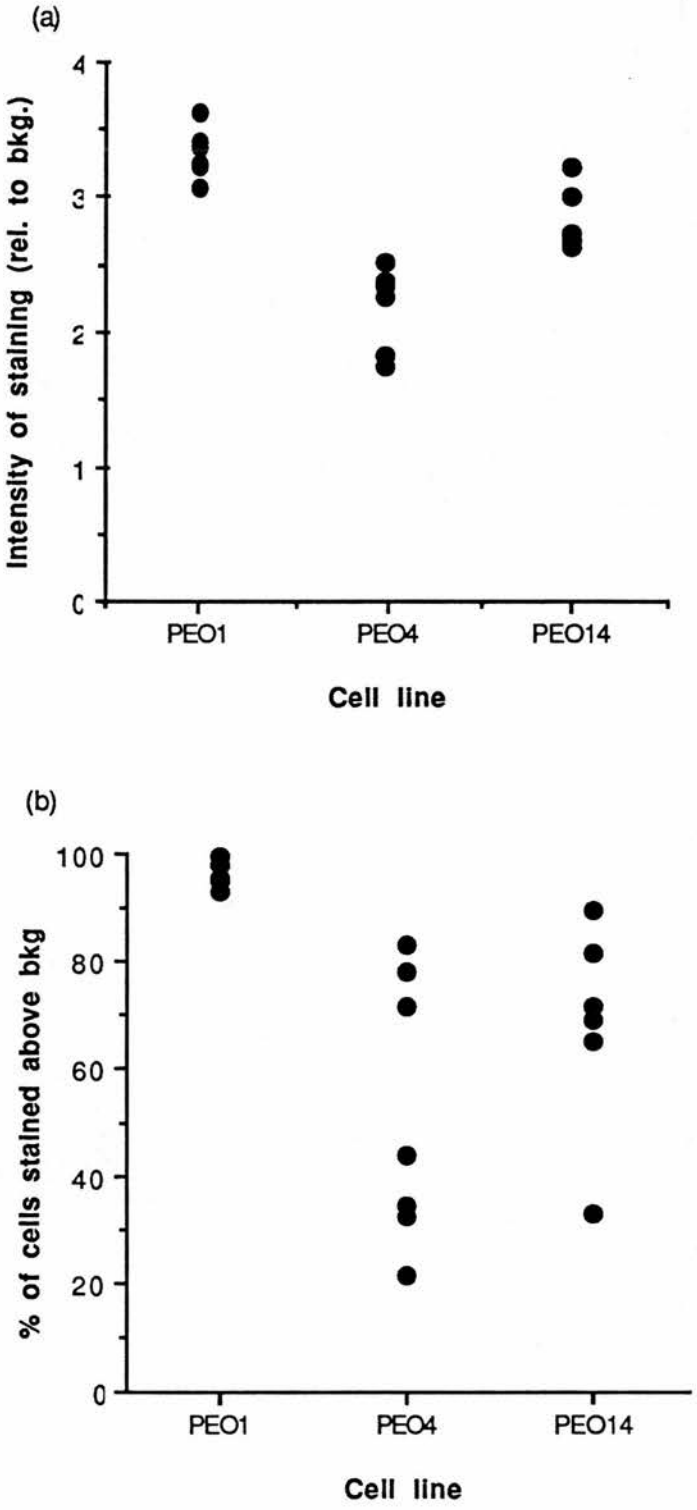

Figure 3.50. Variation in (a) intensity of staining and (b) % of cells stained between experiments in PEO1, PEO4 and PEO14 cells. Figure shows the results from 7 separate measurements.

(iid) Effects of culturing with EGF/TGF-α

The effects of the exogenous addition of 10 nM EGF and TGF-α on EGF receptor expression by PEO1, PEO4 and PEO14 cell lines were investigated. Initially, cells were cultured in phenol red free RPMI 1640 containing 5% dcs-FCS with or without addition of growth factors, and the intensity of staining and percentage of cells stained with EGFR1 (1:5) was measured after 5 days. The changes in the percentages and intensity of staining were then examined in more detail over the 5 day culture period. Measurements were taken daily.

The intensity of staining and the percentage of cells stained following 5 days in culture are shown in tables 3.16 and 3.17. In general, EGF and TGF-α produced decreases in the percentages of cells stained and the intensity of fluorescence in all three cell lines. EGF and TGF-α at 10 nM induced a similar magnitude of response.

Table 3.16. Intensity of staining with EGFR1 (1:5) above background after 5 days in culture with various additions to phenol red free RPMI 1640 containing 5% dcs-FCS. EGF, TGF-α and 17 β-oestradiol were tested at the concentration of 10^{-8} M. Table shows means and standard errors. The effects of oestrogen and EGF were tested on three occasions and the effect of TGF-α on two occasions. Separate controls for each group of experiments are shown.

	Addition to Media					
Cell Line	**-**	**EGF**	**-**	**TGF-α**	**-**	**E_2**
PEO1	3.29	1.91	3.3	2.04	3.30	3. 16
	±0.1	±0.2	±0.1	±0.0	±0.2	±0. 1
PEO4	2.41	1.60	2.45	1.29	2.19	1.74
	±0.1	±0.3	±0.1	±0.1	±0.2	±0. 2
PEO14	2.96	1.30	2.84	1.75	2.70	2. 75
	±0.2	±0.3	±0.2	±0.2	±0.1	±0. 1

Table 3.17. Percentage of cells stained with EGFR1 (1:5) above background after 5 days in culture with various additions to phenol red-free RPMI 1640 containing 5% dcs-FCS. EGF, TGF-α and 17 β-oestradiol were tested at 10^{-8} M concentration. Table shows the mean and standard errors. The effects of EGF and oestrogen were tested on 3 separate occasions and the effects of TGF-α on two occasions. Separate controls for each group of experiments are shown.

	Addition to media					
Cell line	**-**	**EGF**	**-**	**TGF-α**	**-**	**E_2**
PEO1	97.3	32.8	96.3	16.1	96.9	94.4
	±1.9	±35	±1.5	±0.3	±2.9	±4
PEO4	47.6	17.4	49.7	3.1	45.5	25.9
	±23	±16.6	±28.2	±0.8	±15.7	±17
PEO14	80.1	8.9	79.4	10.2	67.2	69.6
	±8.4	±8.8	±10.2	±3.7	±3	±3

In all three cell lines, both the intensity and percentage of cells staining decreased within 24 hours if cultured with EGF compared with controls. The details of the magnitude and timing of responses will now be dicussed in more detail for the individual cell lines.

PEO1 cell line

The changes in intensity and percentage of staining with EGFR1 in PEO1 cells in the presence and absence of 10 nM EGF are shown in figure 3.51. The intensity of staining with EGFR1 antibody remained constant during the first 3 days of the study period, and then decreased on day 4. The percentage of cells which were stained positive was constant during the 5 days. Incubation with EGF produced a dramatic decrease in relative intensity of staining above background, from 4 to almost zero after 24 hours in culture. The intensity of staining remained at this level for a further 24 hours and then increased to 50% of the control value on day 3. For the remaining study period, the intensity of staining with EGFR1 was maintained at this value. The initial decrease in intensity was accompanied by a decrease in the percentage of PEO1 cells expressing EGF receptors to 40%. The percentage of cells staining with EGFR1 then increased with time in culture with EGF. Progressive increases in the proportion of cells positively stained were produced after 48 hours in culture reaching approximately 60% compared with control cells on day 5.

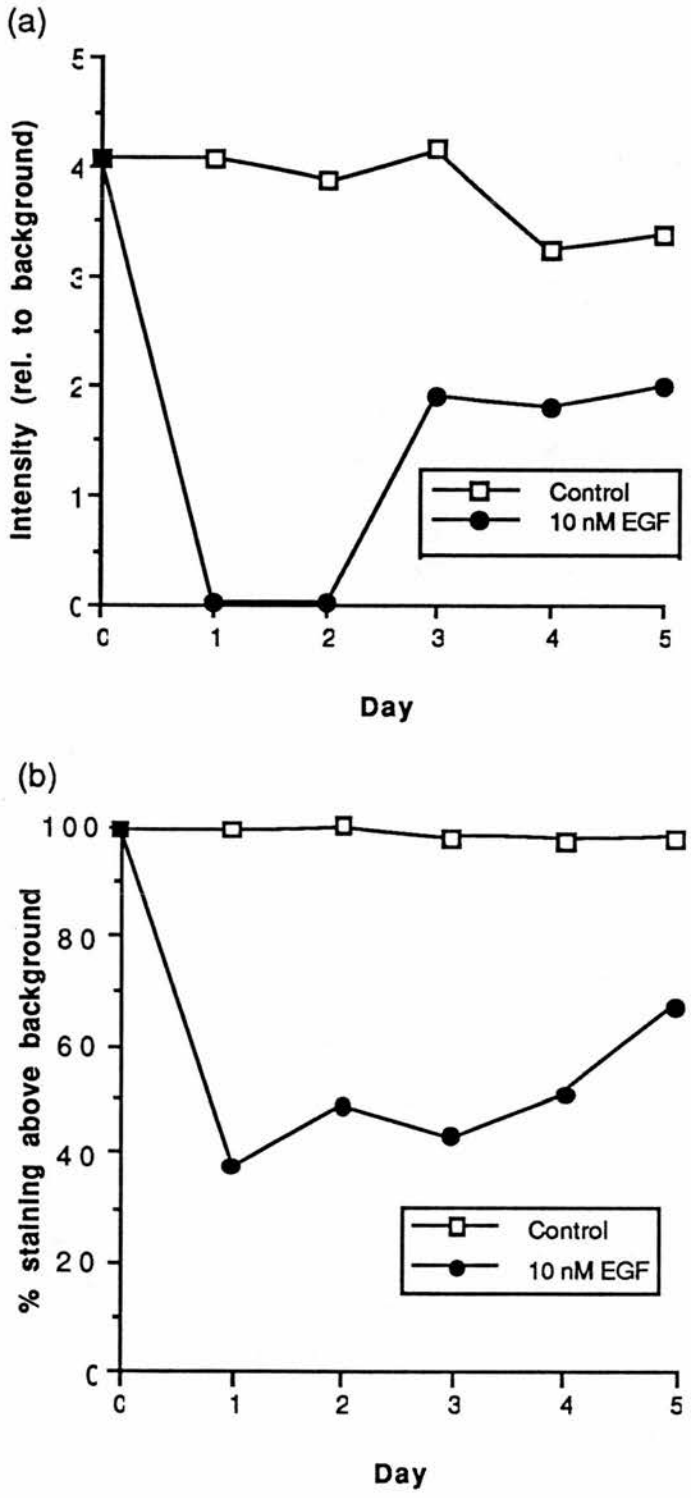

Figure 3.51 The effect of incubation with EGF on EGF receptors on PEO1 cells measured by flow cytometry. (a) Intensity of green fluorescence and (b) % of cells stained with EGFR1 monoclonal antibody (1:5) following incubation in phenol red free RPMI 1640 plus 5% dcs-FCS, with or without the addition of 10 nM EGF for 5 days are shown. Points on the graph represent the mean of 2 measurements. The experiment was repeated on 3 separate occasions with essentially the same results.

PEO4 cell line

Results for the PEO4 cell line are shown in figure 3.52. In the absence of EGF, the intensity of staining increased after 48 hours in culture and then remained essentially constant, whereas the percentage of cells stained increased steadily with time in culture. As in the PEO1 cell line, incubation with EGF produced a decrease in the intensity of staining and in the percentage of cells which were stained positive with EGFR1. The observed decrease in the intensity of staining in the PEO4 cell line was less marked than for PEO1 cells. After 24 hours culture with EGF, the intensity decreased from around 2 to 1.5 compared to control cells, and remained at that value for the remaining period of study. The percentage of cells expressing EGF receptors dropped by around 40% to 20% within the first 24 hours of study, fluctuating around that value for the remaining 4 days in culture.

PEO14 cell line

These results are shown in figure 3.53. The intensity of staining and the percentage of cells stained with EGFR1 fluctuated with time in culture. As in the other two cell lines, EGF produced decreases in both of these parameters in PEO14 cells. The decrease in staining intensity was not as rapid as in PEO1 cells, reaching lowest values of almost zero after 48 hours in culture. However, the intensity of staining increased after 3 days in culture, to approximately 50% of controls, as observed in PEO1 cells. The decrease in the percentage of PEO14 cells stained followed a similar time course to that in PEO4 cells, showing an initial large decrease to 10%

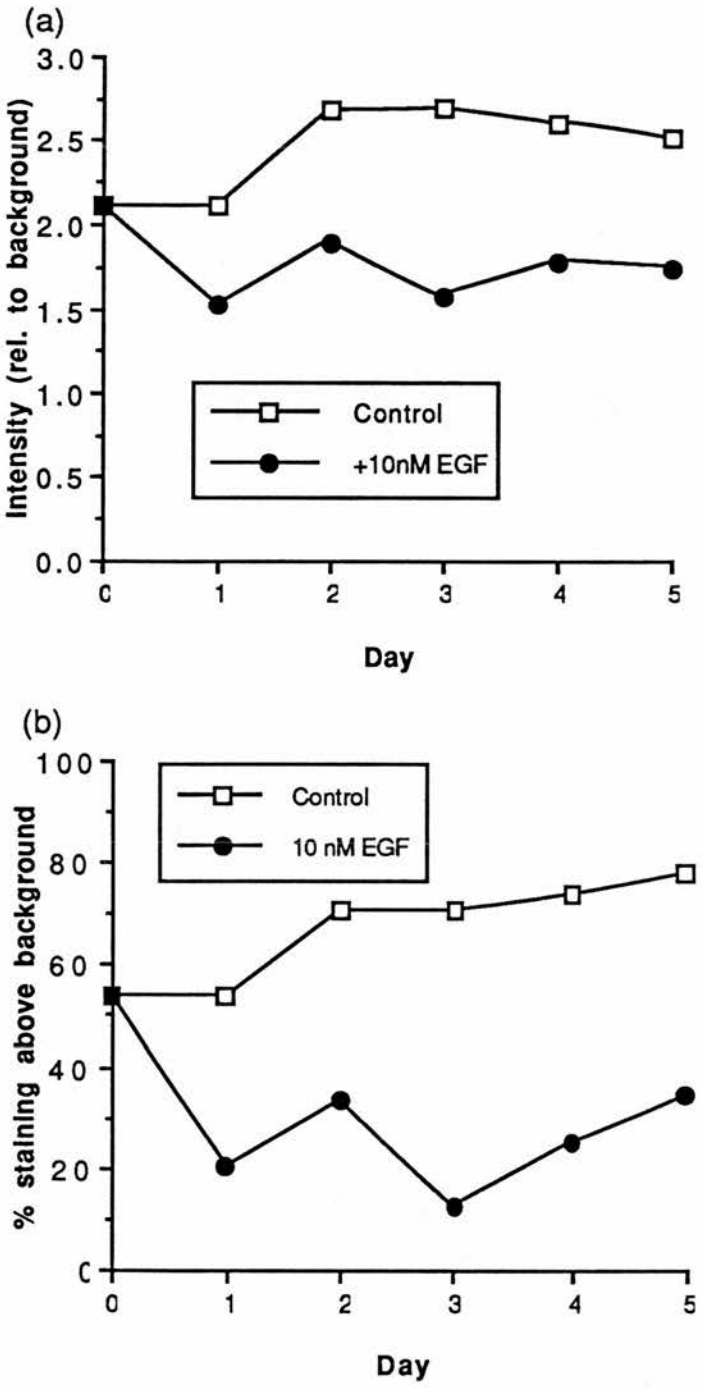

Figure 3.52. The effect of incubation with EGF on EGF receptors on PEO4 cells measured by flow cytometry. (a) Intensity of green fluorescence and (b) % of cells stained with EGFR1 monoclonal antibody (1:5) following incubation in phenol red free RPMI 1640 plus 5% dcs-FCS, with or without the addition of 10 nM EGF for 5 days are shown. Points on the graph represent the mean of 2 measurements. The experiment was repeated on 3 separate occasions with essentially the same results.

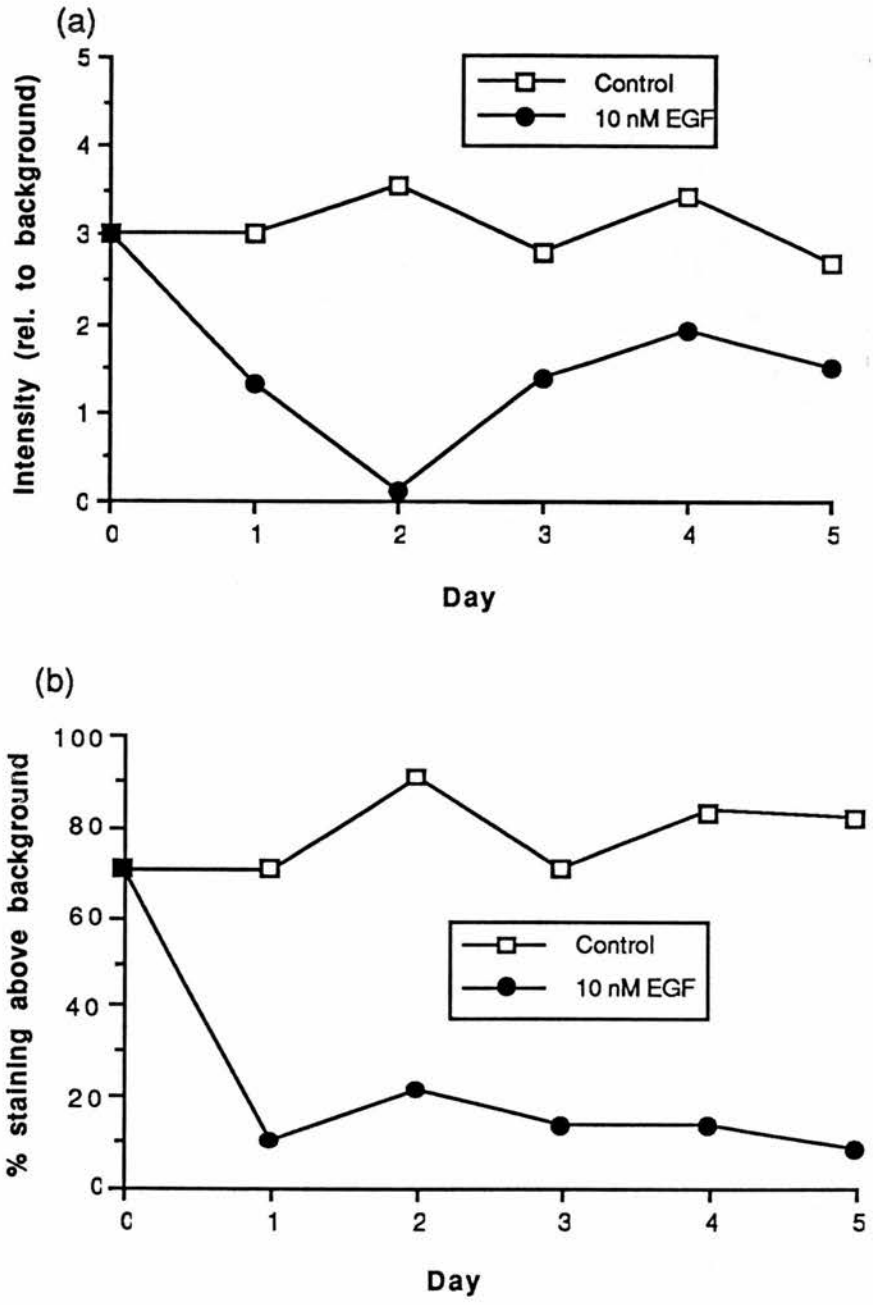

Figure 3.53. The effect of incubation with EGF on EGF receptors on PEO14 cells measured by flow cytometry. (a) Intensity of green fluorescence and (b) % of cells stained with EGFR1 monoclonal antibody (1:5) following incubation in phenol red free RPMI 1640 plus 5% dcs-FCS, with or without the addition of 10 nM EGF for 5 days are shown. Points on the graph represent the mean of 2 measurements. The experiment was repeated on 3 separate occasions with essentially the same results.

compared with control cells, which was maintained for 5 days.

(iie) Effects of culturing with oestrogen

As for the effects of incubation with EGF, experiments to investigate the effects of 10^{-8} M 17 β-oestradiol on EGF receptors were initially performed by determining the level of staining with EGFR1 antibody following 5 days in culture. The effects of oestrogen on the intensity of staining and the percentage of cells stained in the two ER-positive cell lines, PEO1 and PEO4 and the ER-negative cell line, PEO14 are shown in tables 3.16 and 3.17. In the PEO1 cell line, the intensity of staining and the percentage of cells which were stained with EGFR1 were essentially not altered by incubation with 17 β-oestradiol. In contrast to the PEO1 cell line, 17 β-oestradiol produced decreases in both the intensity of staining and the percentage of cells stained in the PEO4 cell line. The percentage decreased by approximately 50% and the intensity by 20%, compared with approximate decreases of 80% and 33% induced by EGF and TGF-α. No change in the pattern of expression of EGF receptors occurred in ER-negative PEO14 cells.

The changes in intensity of staining and percentage of cells stained with the EGFR1 antibody in the PEO4 cell line, were examined in more detail. Results from culture in the absence and presence of 10^{-8} M 17 β-oestradiol over 5 days are shown in figure 3.54. Measurements were performed daily.

In the absence of oestrogen, the intensity of staining increased after 48 hours in culture. This remained constant for the remaining 3 days, whereas the percentage of cells stained increased steadily with time in culture (as described in figure 3.52). Incubation with 10^{-8} M 17 β-oestradiol produced a decrease in both the intensity and percentage of cells stained. Both decreases did not occur until cells had been incubated in the presence of 17 β-oestradiol for 3 days, in comparison with the effects of EGF which were visible after 24 hours (figure 3.52). The decreases in the intensity of staining and in the percentage of cells stained were approximately 0.3 and 10% less than those observed by culture with EGF.

(iif) Effects of culturing with tamoxifen

As the EGF receptor expression of PEO4 cells was modulated by incubation with 10^{-8} M 17 β-oestradiol, it was of interest to investigate the effects of the anti-oestrogen, tamoxifen. Measurements were taken daily during a 5 day period of culture in the presence and absence of 10^{-6} M tamoxifen. These results are shown in figure 3.55. The percentage of cells stained and the intensity of staining of cells varied throughout during the 5 days in culture. Nevertheless, incubation with tamoxifen was associated with decreases in both the intensity of staining and the percentage of cells which were stained with EGFR1. These decreases were not apparent until after 3 days in culture, similar to effects observed with oestrogen (figure 3.54). However, the

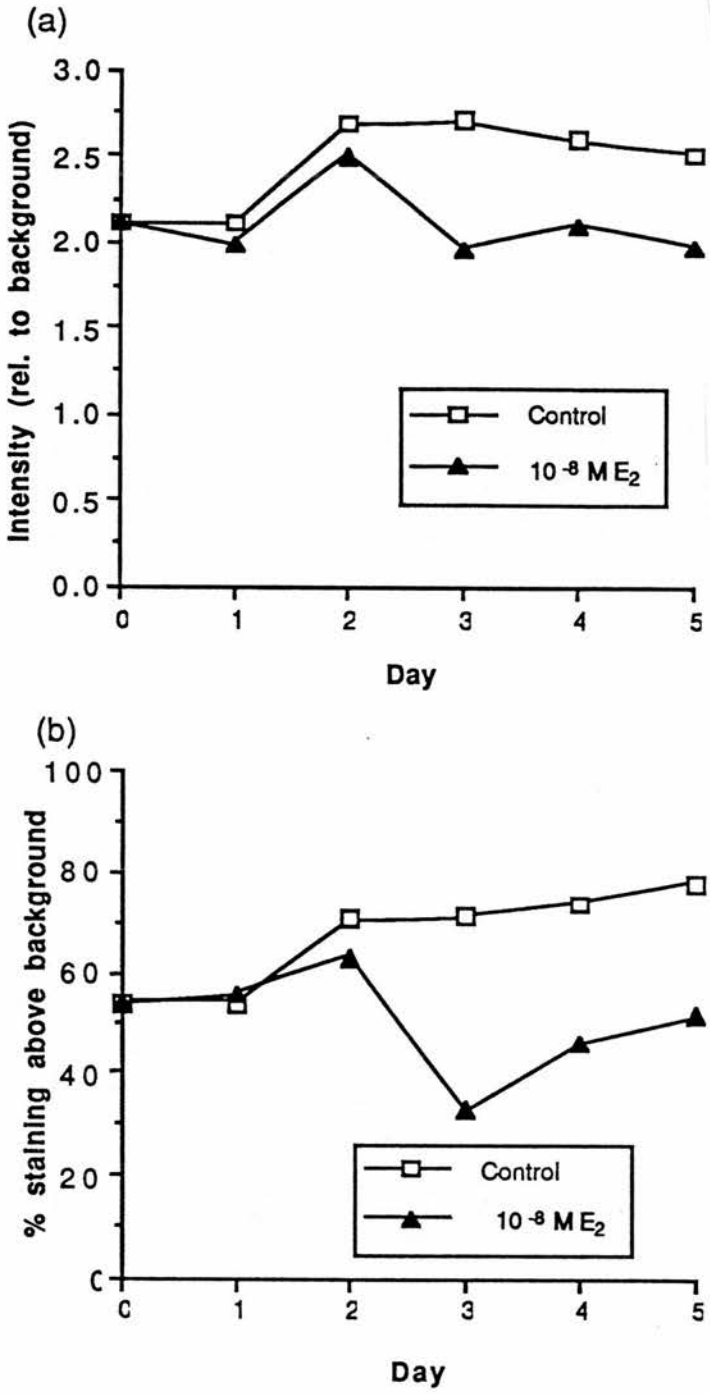

Figure 3.54. The effect of incubation with 17-β oestradiol on EGF receptors on PEO4 cells measured by flow cytometry. (a) Intensity of green fluorescence and (b) % of cells stained with EGFR1 monoclonal antibody (1:5) following incubation in phenol red free RPMI 1640 plus 5% dcs-FCS in the presence or absence of 10^{-8} M 17-β oestradiol are shown. Points on the graph represent the mean of 2 measurements. The experiment was performed on two separate occasions.

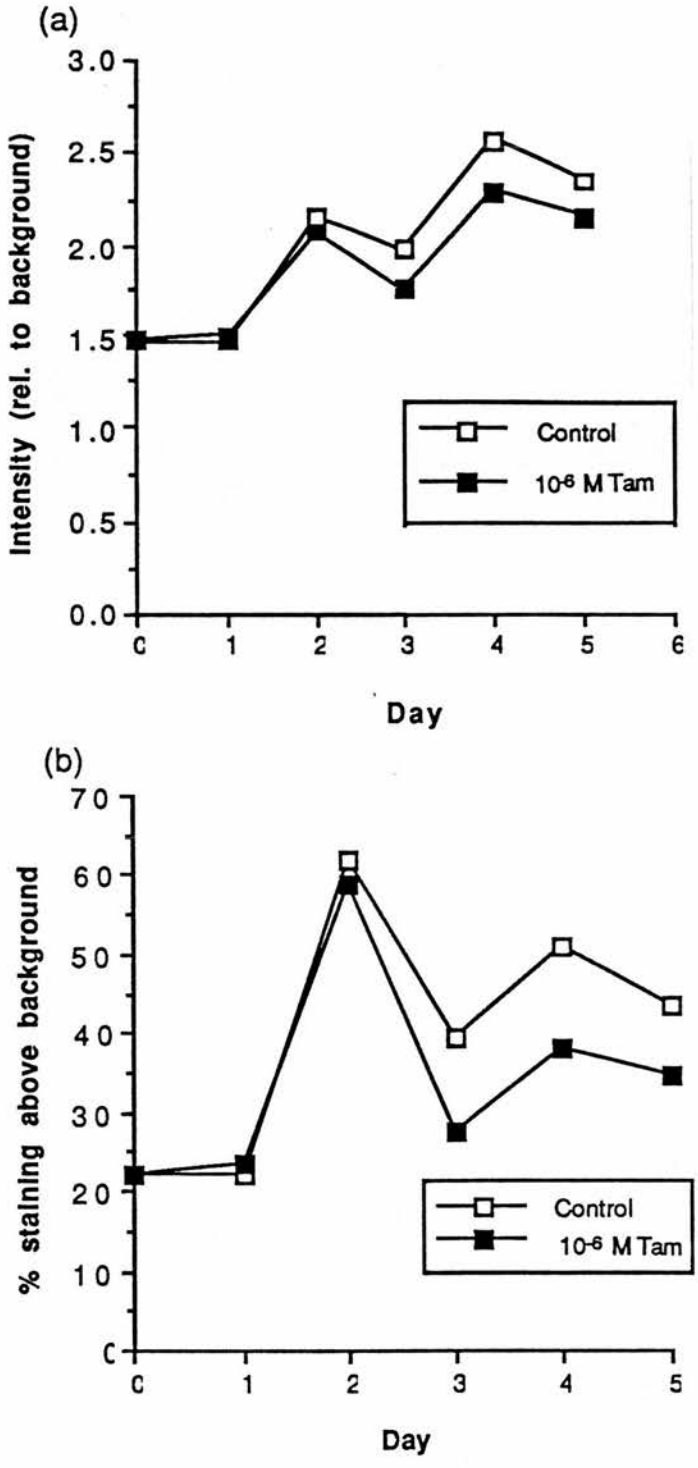

Figure 3.55. The effect of incubation with tamoxifen on EGF receptors on PEO4 cells measured by flow cytometry. (a) Intensity of green fluorescence and (b) % of cells stained EGFR1 monoclonal antibody (1:5) following incubation in phenol red free RPMI 1640 plus 5% dcs-FCS in the presence or absence of 10^{-6} M tamoxifen are shown. Points on the graph represent the mean of 2 measurements. The experiment was performed on two separate occasions.

decreases in intensity and percentage of cells staining were not as great as those in the presence of oestrogen.

(iig) Expression of EGF receptors at various stages in the cell cycle

In order to determine whether EGF receptor expression varied during the cell cycle, both parameters were measured on the same population of cells in a single flow cytometric analysis. This study was performed on at least 5 separate occasions. Representative two dimensional plots for the three ovarian carcinoma cell lines are shown in figure 3.56. The x-axis shows the DNA quantities reflected by the intensity of fluorescence of propidium iodide, and the y-axis shows green fluorescence representing amount of EGFR1 monoclonal antibody bound to cells. The lines represent areas of equivalent intensities of staining for both parameters. No differences in EGF receptor expression were apparent in the various stages of the cell cycle.

Previous results showed that 10 nM EGF modulated EGF receptor expression in all three cell lines and that incubation with oestrogen had a similar effect in PEO4 cells. Dual labelling experiments were performed to determine if these modulations represented changes in specific phases of the cell cycle. Dual labelling plots for PEO4 cells cultured in the presence of EGF and oestrogen for 5 days are shown in figure 3.57. Overall vertical shifts downward in the plots were observed, representing a decrease in the

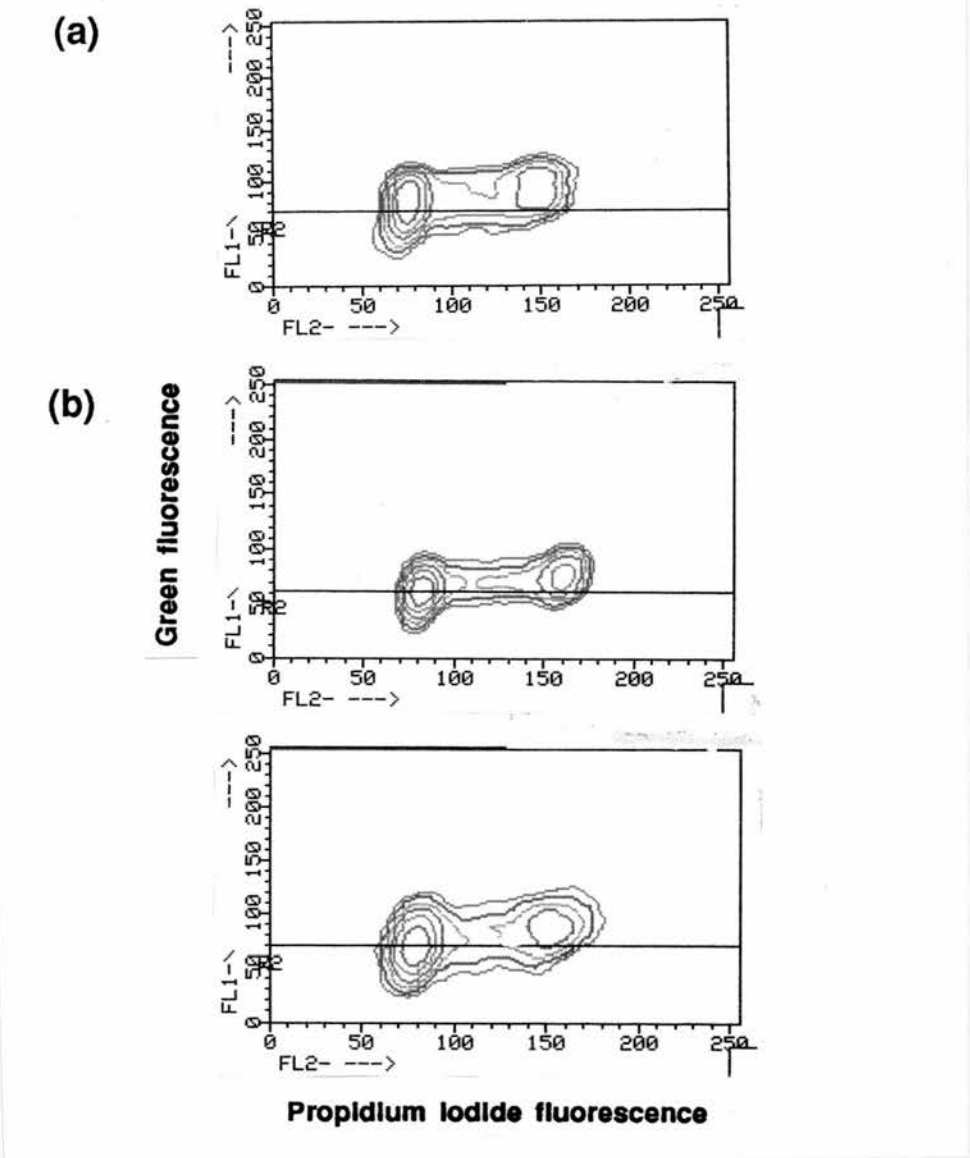

Figure 3.56. Expression of EGF receptors during the G0/G1, S and G2/M phases of the cell cycle after 5 days in culture in RPMI 1640 containing 5% dcs-FCS. Two dimensional plots of green fluorescence, which is equivalent to binding of EGFR1, against red fluorescence of propidium iodide which stains DNA, are shown for (a) PEO1, (b) PEO4 and (c) PEO14 cell lines. Contour lines show areas of equal staining intensity.

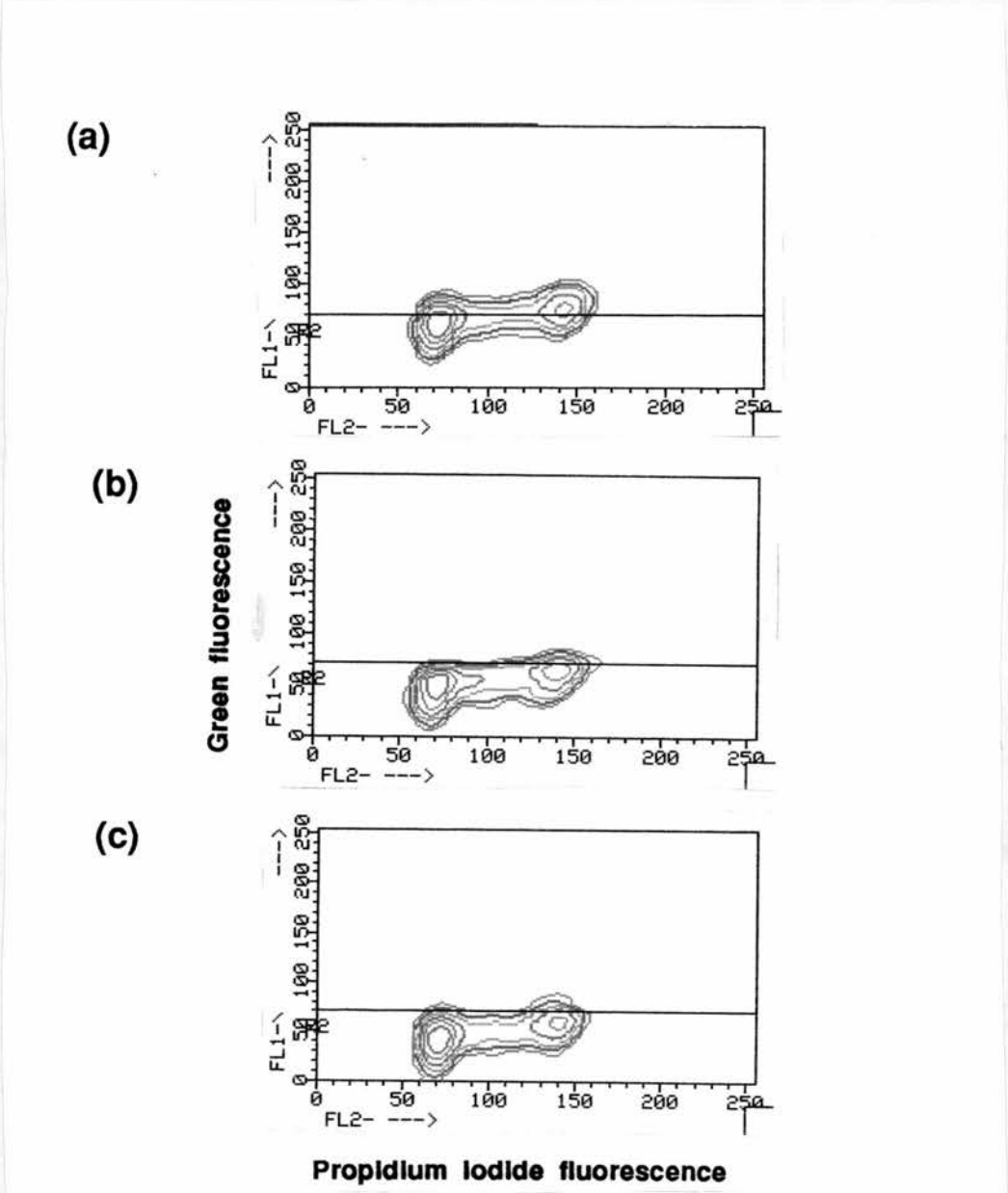

Figure 3.57. Effects of the exogenous addition of 10 nM EGF and 10^{-8} M 17 β-oestradiol on the expression of EGF receptors during the G0/G1, S, and G2/M phases of the cell cycle in PEO4 cells after 5 days in culture. Two dimensional plots of green fluorescence, which is equivalent to binding of EGFR1, against red fluorescence of propidium iodide which stains DNA, are shown of (a) control cells, (b) EGF and (c) 17 β-oestradiol treated cells.

intensity of staining, consistent with previous results (figures 3.52 and 3.54), but no change was seen in the pattern of fluorescence of propidium iodide compared with control cells. Therefore, incubation with EGF and oestrogen produced negligible effects on the temporal expression of its receptors.

3.5 Measurement of EGF activity produced by PEO1, PEO4 and PEO14 ovarian carcinoma cell lines

The three ovarian cell lines express EGF receptors and are stimulated by the exogenous addition of EGF and TGF-α. If the cells secrete EGF and/or TGF-α, an autocrine or paracrine growth regulation pathway may exist. The presence of EGF-like activity in the conditioned media of the cells was investigated using a radioimmunoassay (RIA) specific for human EGF and a radioreceptor assay (RRA) which would potentially detect all ligands including EGF and TGF-α which bind to the EGF receptor.

3.51 RIA

Conditioned media collections and the RIA were performed by Mr W N Scott and Dr J M S Bartlett, Department of Clinical Oncology, Western General Hospital, Edinburgh. Media was conditioned for 48 hours, lyophilised, reconstituted in a small volume and dialysed against PBS. Material was present in the conditioned media which competed for the antibody with radioactive EGF. Dilutions of the conditioned media which had been concentrated 100 fold were made to verify that the EGF activity diluted in parallel with the known concentrations of EGF. Figure 3.58 illustrates the parallelism between the various displacement curves, verifying that it was human EGF-like material that was being detected. Results of the RIA are shown in table 3.18.

The concentrations of EGF-like material in the conditioned media were determined by comparison with the standard curve. Values were calculated from the mean of points on the linear part of the curve.

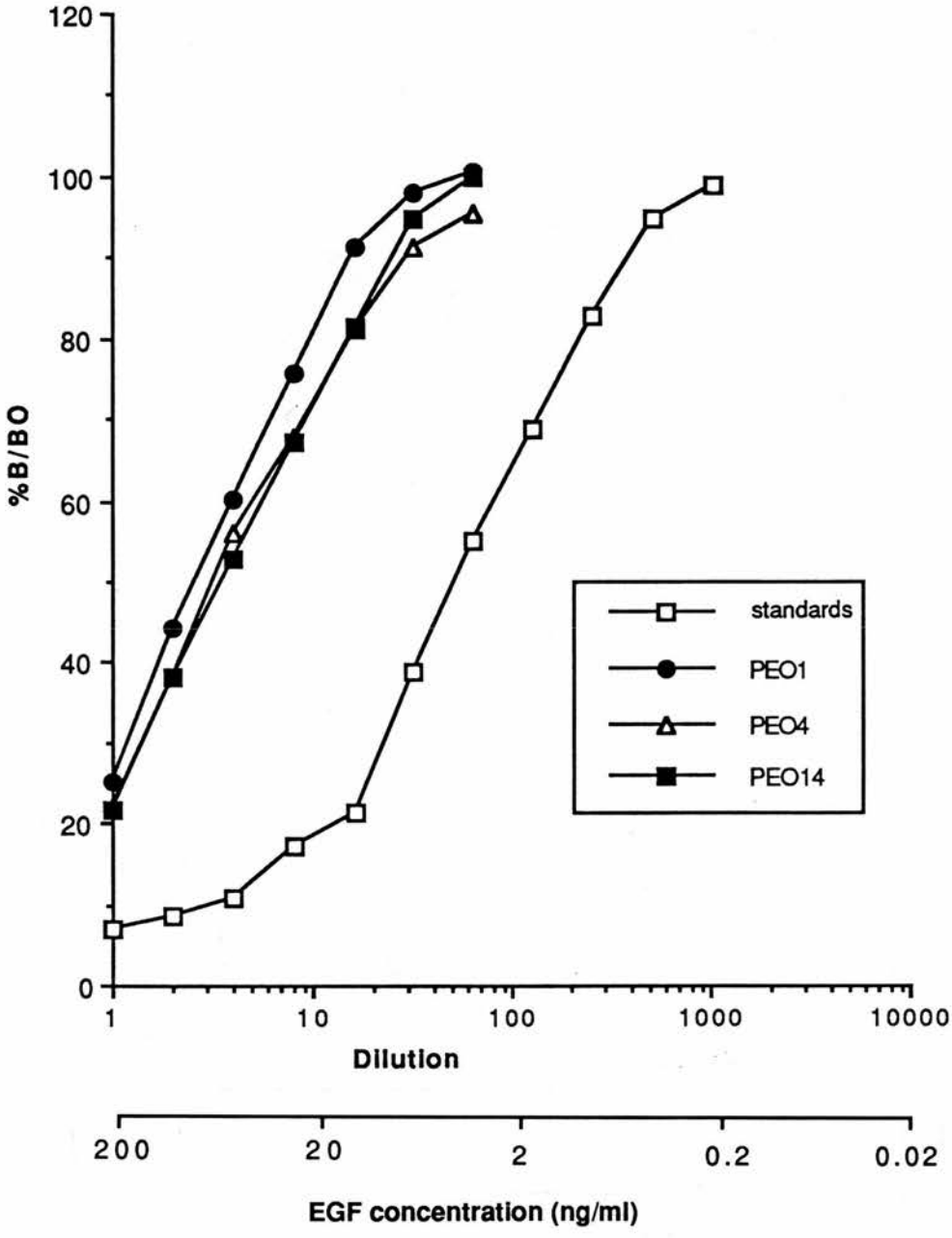

Figure 3.58. Detection of human EGF by RIA in conditioned media of PEO1, PEO4 and PEO14 cells. Each point represents the mean of duplicate measurements.

Dilutions (1:2, 4, 8, 16, 32 and 64) of conditioned media which had been concentrated 100 fold, were made to determine whether activity diluted in parallel with the EGF standards.

Table 3.18. Secretion of EGF-like material by PEO1, PEO4 and PEO14 ovarian carcinoma cell lines. The presence of EGF-like material in phenol red-free RPMI 1640 containing 5% dcs-FCS which had been conditioned for 48 h was detected using a RIA specific for human EGF. Results are expressed as ng of EGF/ml of media. Values in the table represent the mean ± S.E. of 4 separate measurements.

Cell line	EGF concentration (ng/ml)
PEO1	0.133 ±0.02
PEO4	0.242 ±0.05
PEO14	0.158 ±0.07

Bovine FCS contained no material which cross-reacted with the anti-human EGF antibody used in these studies.

3.52 Radio Receptor Assay (RRA)

The RIA for EGF does not cross-react with murine EGF or human TGF-α therefore potentially will specifically measure EGF amounts in the conditioned media Using rat liver as a source of EGF receptors, a RRA will detect quantities of material which will compete with ^{125}I EGF for binding to EGF receptors. Therefore the RRA will potentially detect EGF-like factors in addition to human EGF such as murine EGF, TGF-α and amphiregulin. If the conditioned

media samples contained more EGF-like activity than was detected by the RIA, then it would seem likely that the cells secreted other EGF-like factors in addition to EGF.

Stock rat liver "membranes " were incubated with a fixed concentation of ^{125}I EGF and increasing concentrations of unlabelled EGF. From this, a standard curve of displacement of binding was plotted. The quantity of EGF-like activity in the conditioned media of PEO1 and PEO14 cells was estimated by incubating samples of the media with ^{125}I EGF and the rat liver "membranes". In addition, concentrated, unconditioned RPMI 1640 containing 5% dcs-FCS was analysed for ^{125}I EGF displacement activity. In order to determine that any displacement was produced by EGF-like factors, and not by other competing substances in the media, dilutions of the samples were made to verify that the activity was diluted in parallel with the EGF standards.

The binding of ^{125}I EGF rat liver "membranes" following incubation with known concentrations of EGF and various dilutions of unconditioned and conditioned RPMI 1640 are shown in figure 3.59. Incubation with unconditioned RPMI 1640 containing 5% dcs-FCS produced displacement of ^{125}I EGF. However, the activity did not dilute in parallel with EGF. The media conditioned by PEO1 and PEO14 cells (concentrated 100 fold) also displaced the binding of the ligand, but again failed to dilute in parallel with the EGF standards. It was therefore not possible to quantitate the EGF-like

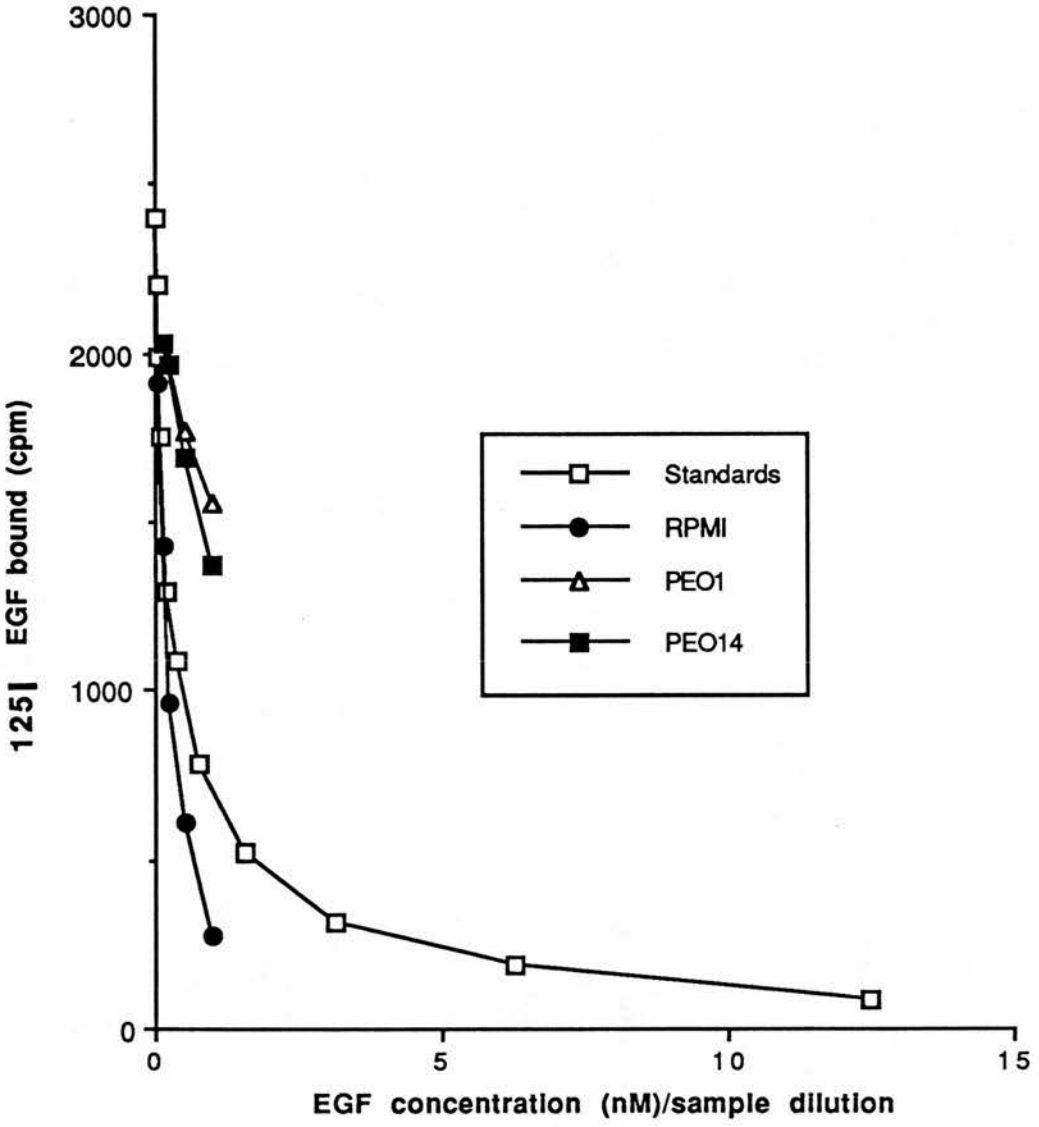

Figure 3.59. Displacement of ^{125}I EGF binding to rat liver "membranes" by EGF (0.024-625 nM) and various dilutions (neat, 1:2, 1:4, 1:8, 1:16) of phenol red free RPMI 1640 containing 5% dcs-FCS and media conditioned by PEO1 and PEO14 cells.

activity present in the samples.

3.53 Effects of incubation with EGF or TGF-α antisera

Previous experiments performed by ICI had shown that antisera raised against EGF or TGF-α could inhibit the EGF/TGF-α stimulated [^{3}H]-thymidine incorporation into NIH-3T3 cells. Using the same antisera, the ability to block the growth stimulatory effects of EGF and TGF-α in the ovarian cell lines was investigated. Initial experiments were performed in the presence of 5% dcs-FCS in phenol red free RPMI 1640.

(i) EGF antiserum on EGF-stimulated growth

The ability of the antisera against EGF to block EGF growth stimulation was tested at two concentrations (diluted 10^4 and 10^3), in the presence of two concentrations of EGF (0.01 and 0.1 nM) in the PEO4 and PEO14 cell lines. A limited supply of the antiserum prevented the experiment being performed in all three ovarian cell lines. The PEO4 and PEO14 cell lines were chosen as they differ in their sensitivy to EGF, and may therefore react differently in the presence of the antiserum.

PEO4 cell line

The effects of antiserum against EGF on the EGF-growth stimulation of PEO4 cells are shown in figure 3.60. The antiserum was tested at 10^4 and 10^3 dilutions. Incubation with 0.01 and 0.1 nM EGF produced stimulation of growth, consistent with previous experiments (figure 3.20). At the 10^3

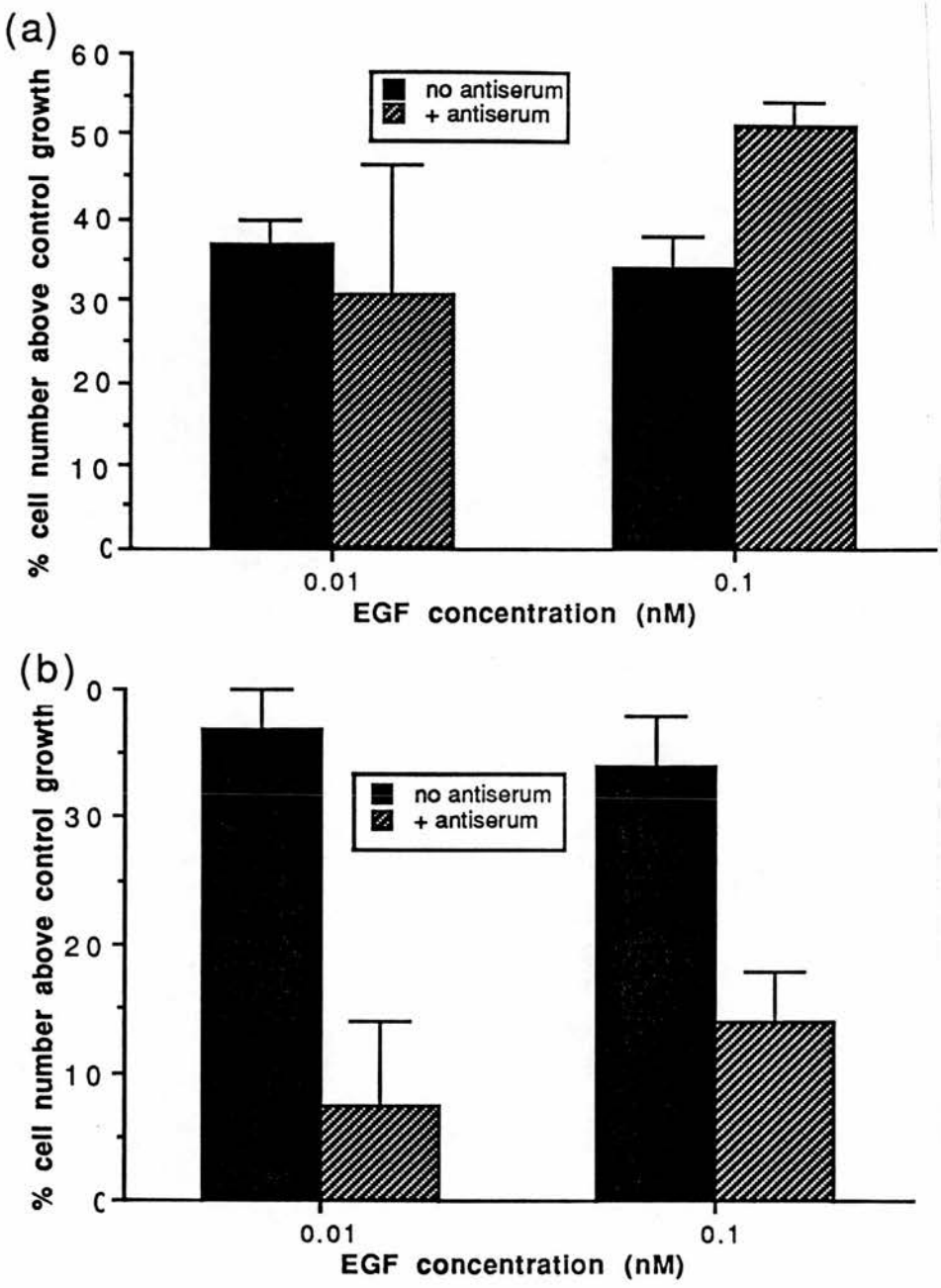

Figure 3.60. Effect of EGF antiserum on the EGF-stimulated growth in PEO4 cells. Cells were incubated in phenol red free RPMI 1640 containing 5% dcs-FCS plus EGF (0.01 or 0.1 nM), in the presence or absence of anti-EGF antiserum, diluted (a) 10^4 or (b) 10^3, for 4 days. Cells were harvested by trypsinisation and counted. The figures show mean ± S.E. for 2 separate experiments. Each value represents the mean of quadruplicate counts. Results are expressed as % of cell number above control growth, where controls represent untreated cells.

dilution, the presence of antiserum reduced the stimulation of growth produced by 0.01 and 0.1 nM EGF, whereas the 10^4 dilution did not reduce the EGF-growth stimulation. In the presence of 0.1 nM EGF, the antiserum produced a slight growth stimulation above that observed only in the presence of EGF alone.

PEO14 cell line

The results for the PEO14 cell line are shown in figure 3.61. As in the PEO4 cell line, incubation with 0.01 and 0.1 nM EGF produced stimulation of growth. The presence of antiserum at 10^3 dilution completely blocked the stimulatory effects of EGF and produced a further decrease in cell growth to values below the rate of growth of untreated cells (in the absence of EGF). Incubation with antiserum at 10^4 dilution reduced the EGF-induced growth stimulation produced by 0.01 and 0.1 nM EGF, but effects were not as marked as those observed with the 10^3 dilution.

(ii) TGF-α antiserum on TGF-α stimulated growth

Due to a limited supply of antiserum the ability of the antiserum to block the TGF-α growth stimulation was investigated in the PEO4 cell line only. The effects of incubation with 0.01, 0.1 and 1 nM TGF-α in the presence and absence of antiserum against TGF-α (diluted 1:2000) following 4 days in culture are shown in figure 3.62. Incubation with TGF-α produced stimulation of growth consistent with previous results (figure 3.20). The antiserum

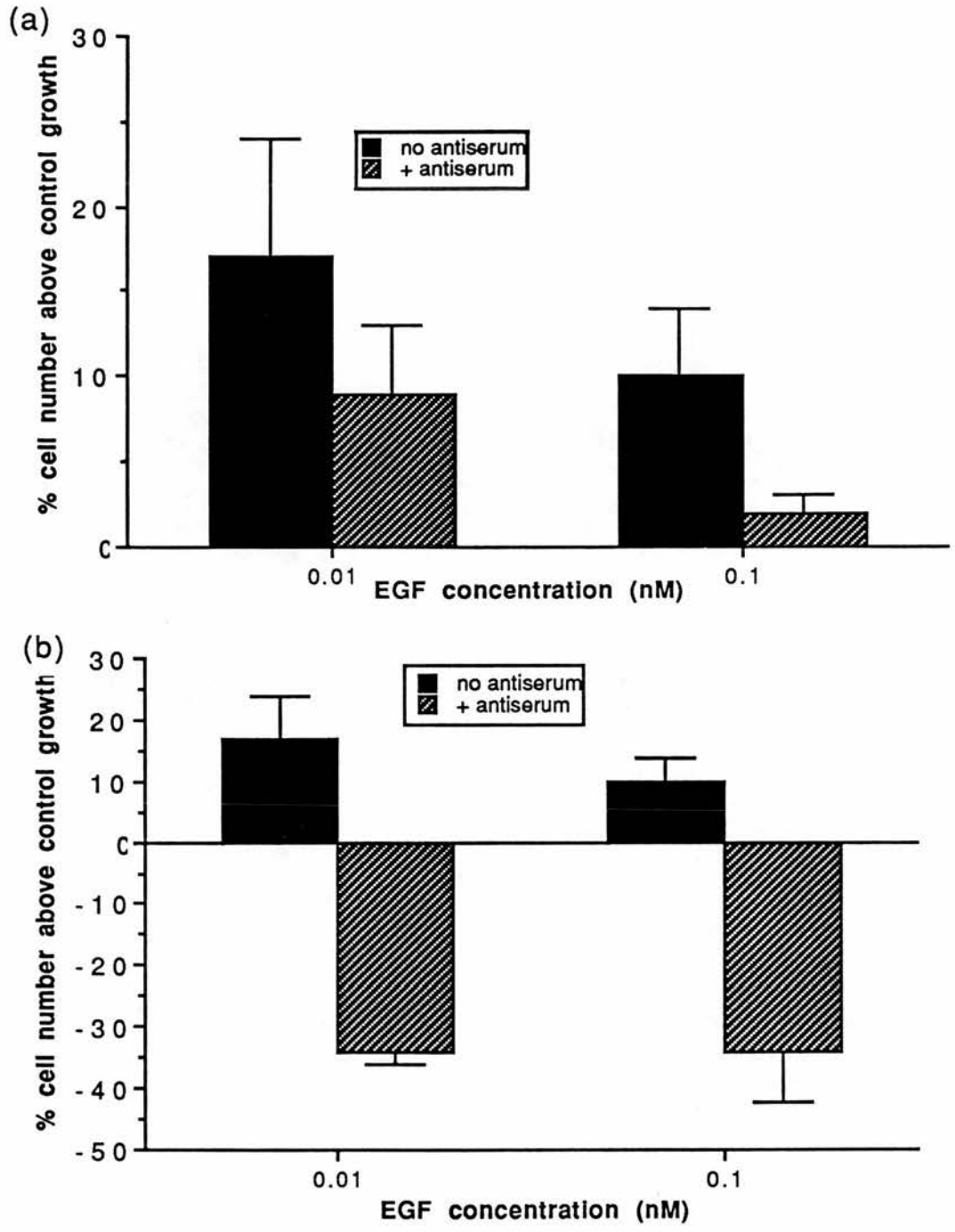

Figure 3.61. Effect of EGF antiserum on the EGF-stimulated growth in PEO14 cells. Cells were incubated in phenol red free RPMI 1640 containing 5% dcs-FCS plus EGF (0.01 or 0.1 nM), in the presence and absence of anti-EGF antiserum, diluted (a) 10^4 or (b) 10^3, for 4 days. Cells were harvested by trypsinisation and counted. The figures show mean ± S.E. for 2 separate experiments. Each value represents the mean of quadruplicate counts. Results are expressed as % of cell number above control growth, where controls represent untreated cells.

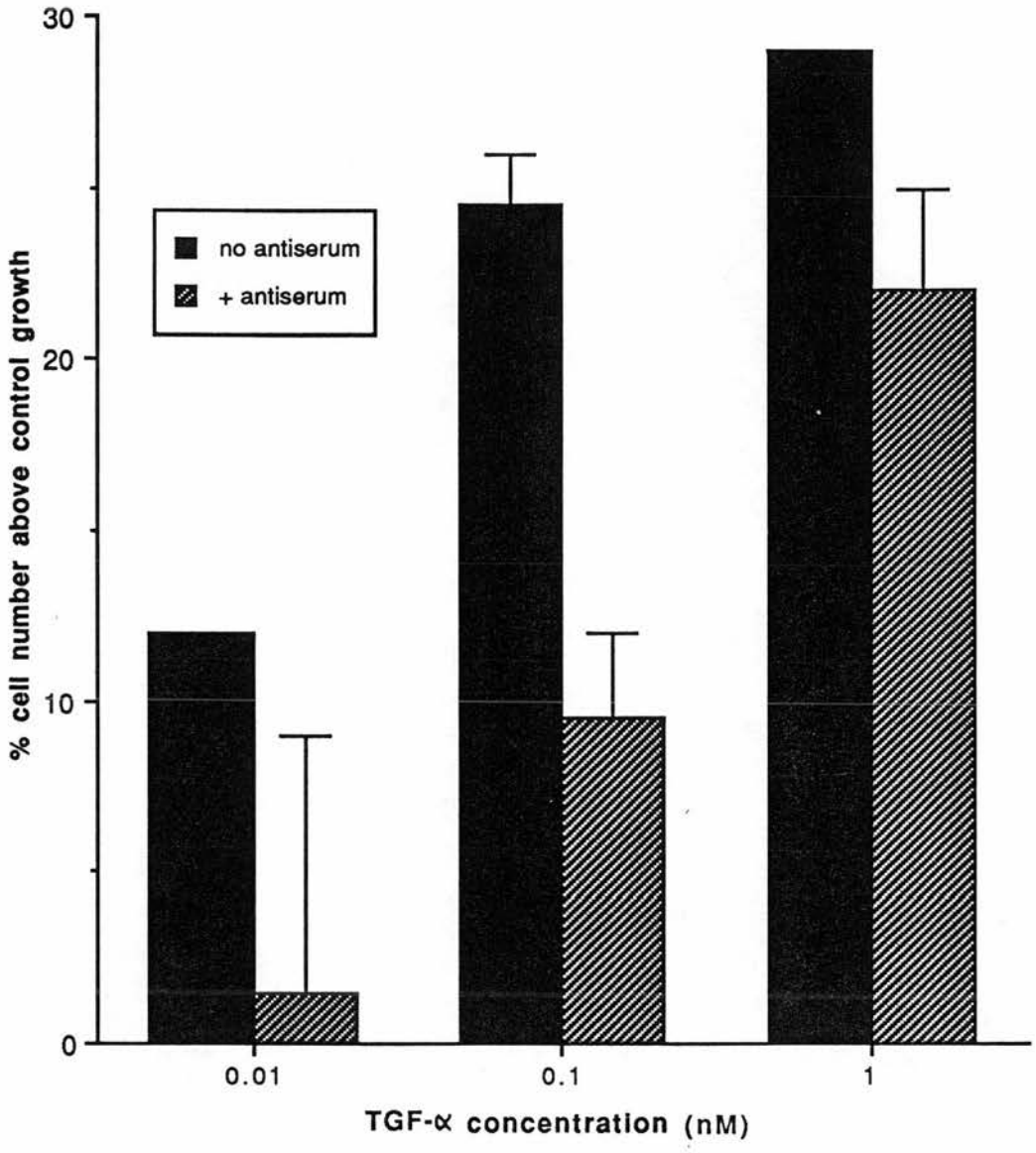

Figure 3.62. Effect of TGF-α antiserum on the TGF-α-stimulated growth in PEO4 cells. Cells were incubated in phenol red free RPMI 1640 containing 5% dcs-FCS plus TGF-α (0.01, 0.1 or 1 nM) in the presence and absence of anti-TGF-α antiserum (diluted 1: 2000) for 4 days. Cells were harvested by trypsinisation and counted. The figure shows mean ± S.E. for 2 separate experiments. Each value represents the mean of quadruplicate values. Results are expressed as % of cell number above control growth, where controls represent untreated cells.

produced a decrease in the growth stimulation induced by all concentrations of TGF-α tested.

(iii) Effects of EGF antiserum on the basal rates of growth

The effects of the antiserum on the basal rates of growth of PEO4 and PEO14 cells were investigated. The EGF-antiserum was used at a dilution of 10^3. BSA at the same protein concentration as the antisera was used as a control, to determine if effects were due to non-specific addition of protein. No effects on growth were produced by BSA in any of the three cell lines (results not shown). The effects of incubation with EGF antiserum for four days on the two ovarian cell lines grown in RPMI 1640, containing 5% dcs-FCS, was determined on two separate occasions.

PEO4 cell line

The effects of the anti-EGF antibodies on the growth of PEO4 cells in the presence of 5% dcs-FCS are shown in figure 3.63 a. Growth of the cells was stimulated by the presence of the anti-EGF antibodies. The stimulation observed was of the order of that obtained with 0.01 nM EGF in previous experiments, approximately 20% above controls, and was significant when analysed by the Students t-test. The effect of the antiserum on the growth rate of PEO4 cells was also examined in RPMI 1640 plus HITS, to investigate whether residual factors in the dcs-FCS might influence the effects of the antiserum. These results are shown in figure 3.63 b. The presence of antiserum against EGF produced no significant changes in the

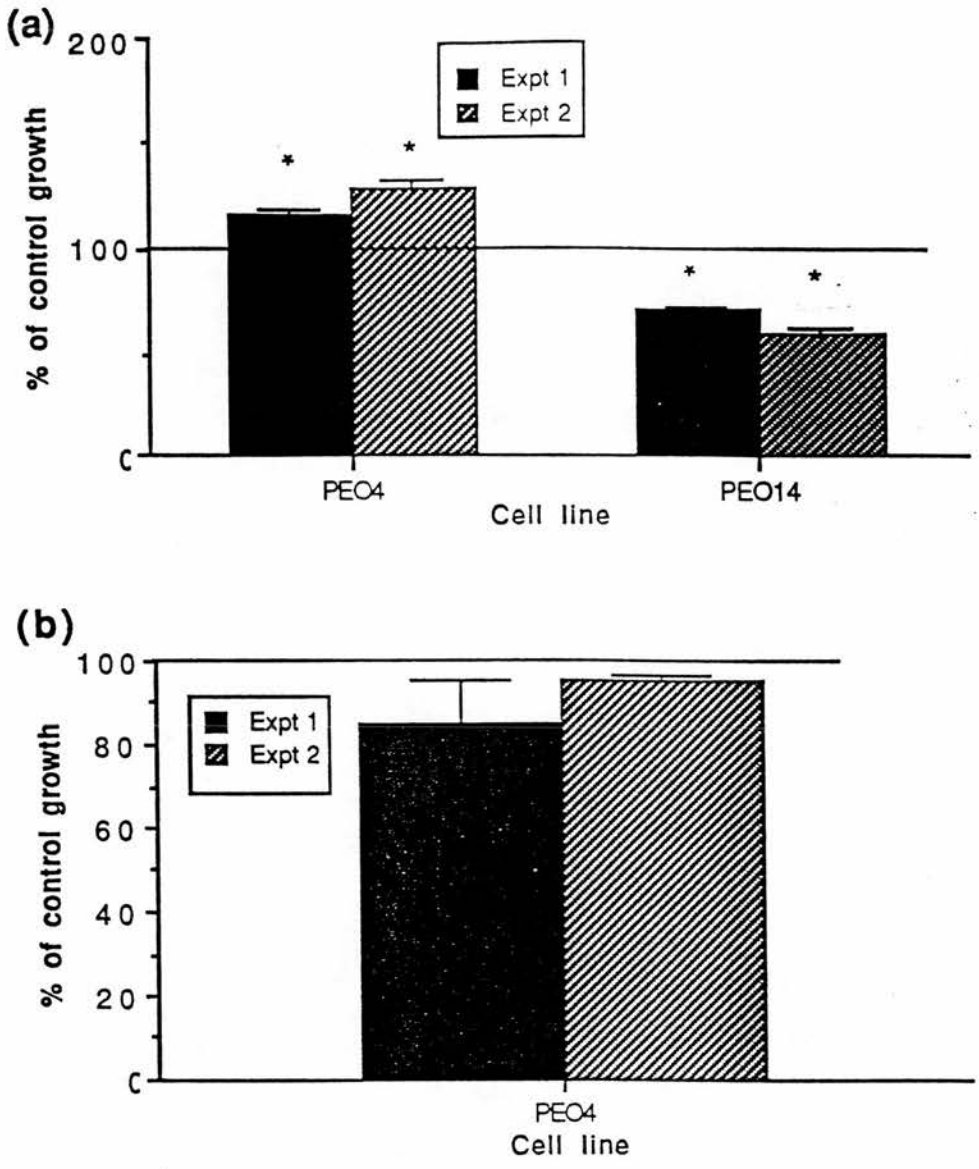

Figure 3.63. Effect of antiserum against EGF on the basal rate of growth of (a) PEO4 and PEO14 cells, grown in phenol red-free RPMI 1640 containing 5% dcs-FCS and (b) PEO4 cells, grown in phenol red-free RPMI 1640 containing HITS. Cells were incubated for 4 days in the presence and absence of the antisera (diluted 10^3), trypsinised and counted. Results are expressed as a % of control cell growth, where control represents untreated cells. The figure shows mean ± S. E. of 2 separate experiments. Values represent the mean of quadruplicate counts. * $P < 0.05$, according to Students' t-test.

growth rate of PEO4 cells compared with controls.

PEO14 cell line

These results are shown in figure 3.63 a. In the presence of the antiserum, the rate of growth of the PEO14 cell line was significantly decreased to approximately 60% of controls on both occasions.

(iv) Effects of TGF-α antiserum on the basal rates of growth

The effects of the antibodies raised against TGF-α were examined on the basal rates of growth of PEO1, PEO4 and PEO14 cells, in the presence and absence of 5% dcs-FCS. Results are shown in figure 3.64.

PEO1 cell line

The presence of antiserum in the culture media containing 5% dcs-FCS for 4 days significantly reduced the rate of growth of PEO1 cells by approximately 9%. In the absence of serum, the antiserum produced negligible effects on growth.

PEO4 cell line

The effect of the antiserum in the presence of serum on the PEO4 cell line was investigated four times. However, as the S.E. for the PEO4 cell line results are large, the interpretation of the data is difficult. The results from the individual experiments are shown in figure 3.65. On one occasion a slight increase in growth above control was noted, whereas in another

experiment a large inhibition of around 70% of growth was noted. On two further occasions, only very slight decreases in the basal rates of growth were seen.

In the serum free containing media with HITS, the presence of the antiserum stimulated the growth of the PEO4 cells by a small extent.

PEO14 cell line

The growth of the PEO14 cell line was unaffected by incubation with TGF-α antiserum in the presence of 5% dcs-FCS or HITS.

The effects of incubation with TGF-α antiserum in RPMI 1640 containing HITS were looked at in the three cell lines also, in an attempt to clarify the effects. The results in figure 3.64 show that in serum free media, the antiserum had no effect on the growth of PEO1 and PEO14 cell lines. However, the growth of PEO4 cells was significantly stimulated in the presence of the antiserum.

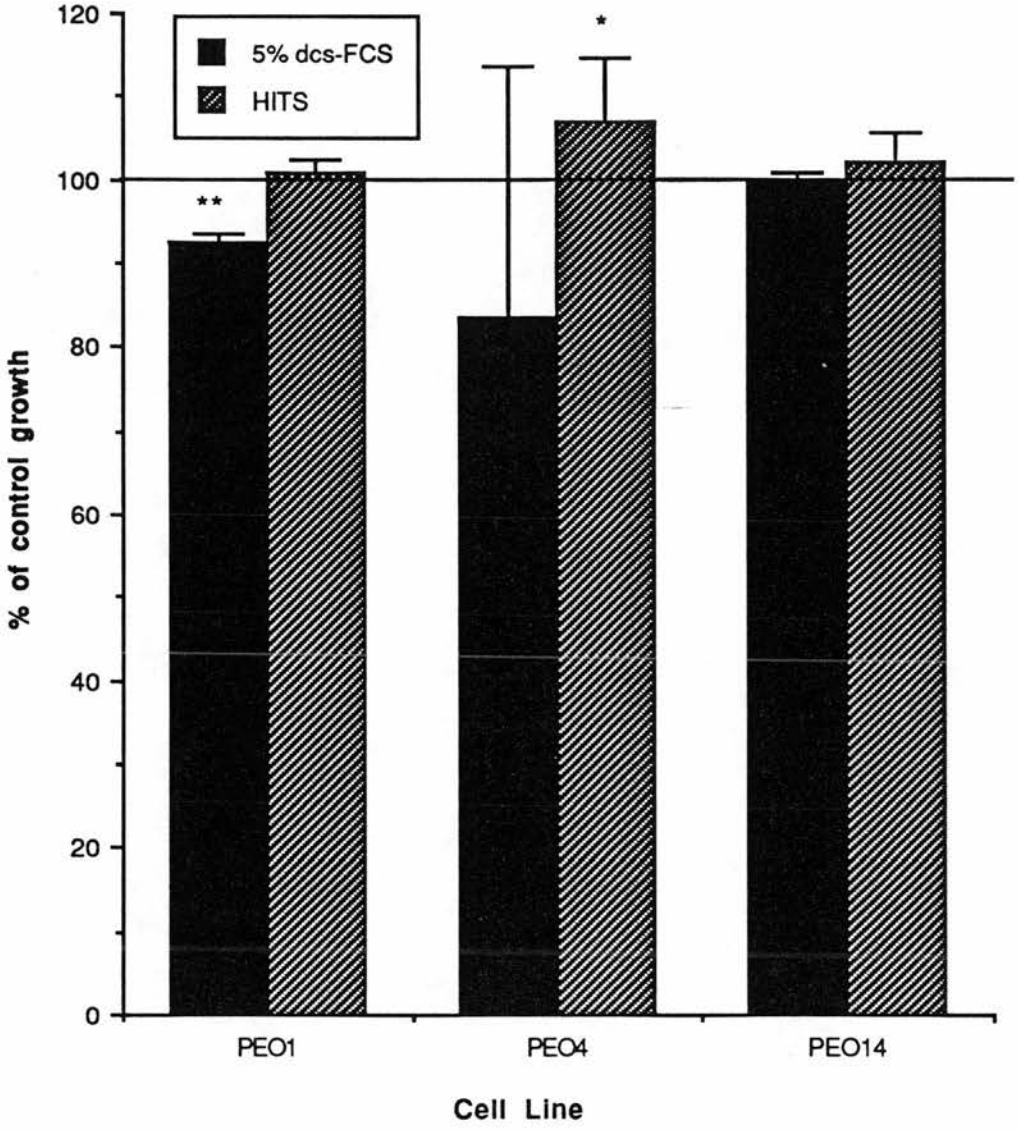

Figure 3.64. Effect of antiserum against TGF-α on the basal rate of growth of PEO1, PEO4 and PEO14 cells, grown in phenol red free RPMI 1640 containing 5% dcs-FCS or HITS. Cells were incubated for 4 days in the presence and absence of the antisera (1: 2000), trypsinised and counted. Results are expressed as a % of control cell growth, where control represents untreated cells. The figure shows mean ± S. E. of at least 3 separate experiments for the 5% dcs-FCS data and 2 experiments for the HITS data. * $P < 0.05$, according to Students' t-test.

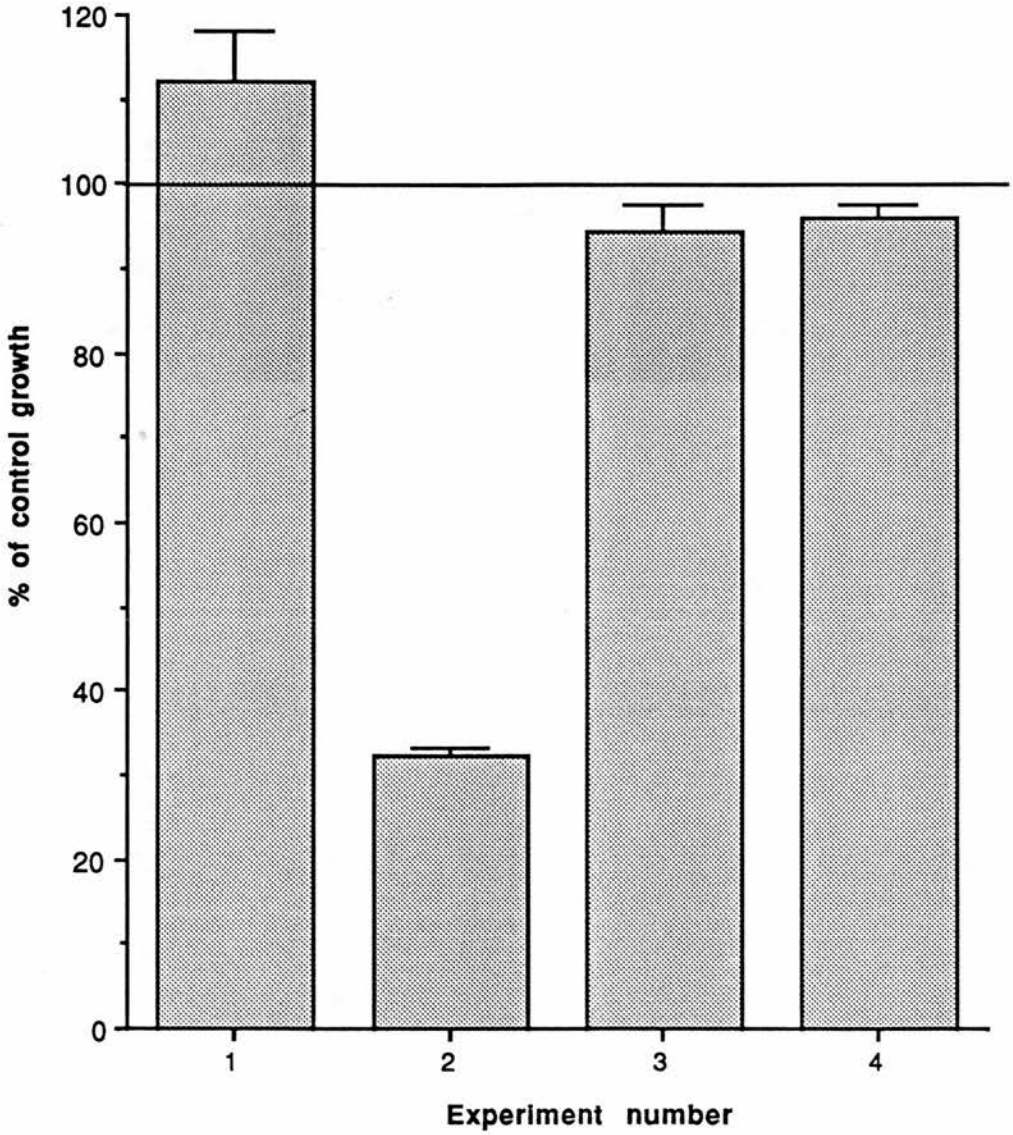

Figure 3.65. Effects of antiserum against TGF-α on the basal rate of growth of PEO4 cells grown in phenol red-free RPMI 1640 containing 5% dcs-FCS. Cells were incubated for 4 days in the presence or absence of the antiserum (diluted 1:2000), trypsinised and counted. Results are expressed as a % of control growth, where control represents untreated cells. The figure shows the mean ± S.E. of 4 separate experiments. The results for each experiment are from quadruplicate values.

CHAPTER 4

DISCUSSION

4.1 Effects of oestrogen/anti-oestrogens on growth

The effects of oestrogens and anti-oestrogens on the growth of breast cancer cells in culture have been well documented, and have enhanced understanding of the biology of the disease. However, similar understanding of ovarian cancer has been limited by the number of cell lines available for study and although recently a number of human ovarian cancer cell lines have been derived and characterised (Yamada, 1974; Disaia *et al*, 1975; Fogh *et al*, 1977; Sinna *et al*, 1979; Buick *et al*, 1985), the majority of studies have been concerned with their responses to cytotoxic agents.

Although there are a few reports of measurements of the steroid receptor status of ovarian carcinoma cell lines, many of the cell lines have been found to be oestrogen receptor (ER)-negative (Woods *et al*, 1979; Benard *et al*, 1985). However, two cell lines have been reported to express ER. The NIH:OVCAR-3 cell line was isolated from the ascites of a patient with a poorly differentiated ovarian adenocarcinoma (Hamilton *et al*, 1983) and expresses ER at a concentration of 28 fmol/mg cytosolic protein. Although this cell line exhibits the classical oestrogen-response end-effect of induction of progesterone receptors using an *in vivo* xenograft model (Hamilton *et al*, 1984), the clinically important end-effect measured by changes in tumour size does not occur in response to steroid. Furthermore, as would be expected from the lack of response to 17 β-oestradiol, tamoxifen treatment in nude mice which have been heterotransplanted with NIH:OVCAR-3 does not lead to an increased survival (Holt *et al*, 1987). A second ovarian carcinoma cell line BG-1, which was derived from the solid

tumour tisssue of a patient with a very poorly differentiated adenocarcinoma has an ER content of 23 fmol/mg protein (Geisinger *et al*, 1989). Although the growth of this cell line was stimulated by 17 β-oestradiol in a clonogenic assay (Geisinger *et al*, 1990) the level of ER expression can be considered to be low as it has been suggested that in breast cancer an ER value of greater than 30 fmol/mg protein is the critical value in determining response to endocrine manipulation in breast cancer (Jensen *et al*, 1976), ER-rich tumours being more likely to respond to endocrine therapy.

In this study, three ovarian serous adenocarcinoma cell lines which were derived and characterised in the Department of Clinical Oncology, Edinburgh from the ascitic fluid of two patients (Langdon *et al*, 1988) have been studied extensively. The PEO1 and PEO4 cell lines were obtained from the same patient with a poorly differentiated tumour, before and after drug resistance had developed. The third cell line, PEO14 was derived from a patient with a well-differentiated serous adenocarcinoma who had not received prior drug treatment. The PEO1 and PEO4 cells are novel in that they possess moderate-high levels of oestrogen receptor (ER) around 100 fmol/mg protein. These cell lines have been used as model systems of ovarian cancer to study the effects of oestrogens and anti-oestrogens on cell growth. In contrast to PEO1 and PEO4 cells, the third cell line which was derived and characterised, PEO14, is ER-negative and thus provides a negative control against which to make comparisons. In 1988, the PEO4 cell line was donated to Dr Ozols. Therefore,except for preliminary data on the PEO4 cell line reported by Nash *et al* (1989), no other results have been

presented on the hormone sensitivity of these cell lines, and the data in this thesis represents original observations on the potential growth control of ovarian cancer cells by oestrogen and anti-oestrogens.

In order to determine the effects on cell growth of the exogenous addition of oestrogen and anti-oestrogens, it was thought important to reduce the levels of endogenous oestrogen in the culture medium. Therefore, the experiments were performed in phenol red-free media supplemented with dextran-charcoal stripped serum. The pH indicator phenol red binds to the ER and acts as a weak oestrogen (Berthois *et al*, 1986). The removal of phenol red from the media has been shown to slow the growth rate and increase the responsiveness to oestrogen in MCF-7 human breast cancer cells in culture. In addition, the reduced rate of growth caused by incubation with anti-oestrogens previously seen in the presence of phenol red was abolished. Dextran-charcoal treatment of serum non-specifically removes small molecules, including oestrogen, which may be present in FCS at concentrations up to 10^{-8} M (Esbar *et al*, 1973). These two procedures therefore provide a system for cell culture in essentially oestrogen-free conditions.

Incubation with phenol red-free media and double-charcoal stripped (dcs) serum reduced the growth rates of all three ovarian cell lines compared with the growth in phenol red containing media supplemented with untreated FCS. As the removal of phenol red alone from the culture media did not

alter the growth rates (Dr S Langdon, personal communication), it is probable that dextran-charcoal treatment of serum removes some growth promoting activity. As the exogenous addition of 17 β-oestradiol to the two ER-positive cell lines stimulated growth to a rate comparable with or above that in the presence of phenol red and FCS, it is possible that at at least some of the growth promoting activity removed by dextran-charcoal treatment of serum is 17 β-oestradiol. However as the reduction in the growth rate of the ER-negative cell line, PEO14, which occurred when cultured in phenol red-free media and dcs-FCS was not reversed by the exogenous additon of oestrogen, it would seem that other factors important for growth of PEO14 cells are removed by dextran-charcoal treatment of the serum. It is worth considering the growth stimulatory effects of oestrogen in more detail.

Growth stimulation by 17 β-oestradiol of the two ER positive cell lines, PEO1 and PEO4 was seen over a wide range of concentrations. However, maximal stimulation was produced between 10^{-10} and 10^{-8} M, which are physiological concentrations (Esbar *et al*, 1973). These are also the concentrations which would be expected to saturate the ER with a Kd of between 6 and 7 x 10^{-10} M.

Similar results have been obtained in the PEO4 cell line following incubation with 17 β-oestradiol by Nash *et al* (1989), who reported that the growth was stimulated over a similar range of concentrations. Although maximum stimulation was noted in the same concentration range, the level

of stimulation varied between the studies. In the present study, growth stimulation up to 100% above control growth was observed, whereas only 50% stimulation was achieved by Nash *et al.* This difference may be due to different culture conditions as the media used by Nash *et al* was supplemented with insulin. Furthermore, in ER-positive breast cancer cell lines in culture, at least some of the stimulatory effects of oestrogen are thought to be mediated by the increased secretion of growth factors such as IGF-1 and the presence of insulin in the culture media reduces the sensitivity to 17 β-oestradiol (Huff *et al*, 1988). Therefore, assuming that 17 β-oestradiol may stimulate the growth of ovarian cancer cells in culture in a similar manner, the presence of insulin in the media may account for the reduced sensitivity. Furthermore, as the growth of the PEO4 cell line is stimulated by incubation with insulin (Dr J Bartlett, personal communication) it is possible that this contributes to the lesser stimulation observed by Nash *et al*, as there may be a limit to the rate of growth of the cells, which has been partially reached by the presence of insulin. In both sets of experiments, the serum was treated with dextran-coated charcoal to remove steroids. However, to ensure maximum removal, the serum used in the present study was treated twice. The presence of a smaller quantity of residual steroids in the serum may account for the increased sensitivity to oestrogen seen in our experiments.

In contrast to effects in PEO1 and PEO4 cells, incubation with 17 β-oestradiol at concentrations between 10^{-12} and 10^{-6} M produced negligible effects on the growth of the ER-negative cell line PEO14.

However, higher concentrations produced cytostatic and cytotoxic effects. Inhibitory effects of 17 β-oestradiol were also produced in the ER-positive ovarian carcinoma cell lines with high concentrations, although they differed in their sensitivies. The absence of an effect at 10^{-5} M 17 β-oestradiol in PEO1 cells which produced cytostatic effects in PEO4 cells cannot be explained by differences in the level of ER expression. At the highest concentration tested, 10^{-4} M, cytotoxic effects were produced in both cell lines.

A similar phenomenom has been observed in breast cancer cells. Inhibitory effects of oestrogens at high concentrations have been reported in ER-positive and negative breast cancer cells in culture (Reddel and Sutherland, 1987) at comparable concentrations to those in the present study although previous results have shown that the ER-negative breast cancer cell line MDA-MB-330 was less sensitive to high concentrations of oestrogens than ER-positive MCF-7 cells. This is therefore inconsistent with the results in the present study where the ER-negative and positive ovarian cancer cells were equally sensitive to high concentrations of oestrogen.

The growth stimulation by 17 β-oestradiol of the ovarian carcinoma cell lines was correlated with the presence of ER, which is consistent with the mechanism through which oestrogen is believed to exert its biological effects. Furthermore, this growth stimulation could be removed by co-incubation with anti-oestrogens which are believed to compete with the natural ligand for binding to the ER.

The stimulatory effects of 10^{-10} M 17 β-oestradiol in the ER-positive cell line, PEO4 were blocked by tamoxifen at a 10,000-fold higher concentration. Antagonism of the growth stimulation of oestrogen with an antioestrogen in PEO4 cells has also been demonstrated by Nash *et al*, (1989), although the studies differed as 10^{-8} M 4-hydroxytamoxifen was used in the other study. *In vivo*, tamoxifen is converted to a number of hydroxylated and methylated metatabolites (Borgna and Rochefort, 1981). The compound 4-hydroxytamoxifen is a quantitatively minor metabolite of tamoxifen (Etienne *et al*, 1989) but binds to the ER with greater affinity than tamoxifen. It has also been shown that 4-hydroxytamoxifen has a 100-fold greater potency than tamoxifen in inhibiting the growth of MCF-7 cells *in vitro* (Katzenellenbogen *et al*, 1984) consistent with the differences in binding affinities. This difference in potency correlates with the differences observed between Nash *et al* and the present studies in the excess concentration of anti-oestrogen required to remove the oestrogen stimulation.

In addition to the oestrogen antagonism properties, tamoxifen also possesses agonist activity depending on the species and target tissue being studied (Furr, 1988). Tamoxifen stimulates uterine growth of immature rats, but even when tested at high doses, the maximum stimulatory effect is below that achieved with 17 β-oestradiol (Wakeling and Bowler, 1987). When animals were treated with 17 β-oestradiol and tamoxifen, the uterotrophic action of 17 β-oestradiol was only partially blocked. Therefore the antioestrogenic effects are limited by the oestrogenic activity of the compound.

Consistent with the studies which have shown that tamoxifen is an oestrogen antagonist and agonist, the growth of the two ER-positve cell lines was stimulated by incubation with tamoxifen at concentrations between 10^{-12} and 10^{-7} M, in reduced oestrogen conditions. The growth stimulation was less than that produced by 17 β-oestradiol. Similar concentrations of tamoxifen produced no effects on the ER-negative PEO14 cell line. These results are consistent with similar findings of several studies on MCF-7 and T47-D breast cancer cells *in vitro* where addition of tamoxifen at concentrations of 10^{-8} or 10^{-9} M to the culture media stimulated the growth of the cells (Reddel and Sutherland, 1984, Cormier and Jordan, 1989).

In the present study, incubation of the cell lines with 10^{-6} M tamoxifen had no effect on the growth of PEO1 cells, but tended to produce growth stimulation in the PEO4 cell line in oestrogen-free culture conditions. It has been reported previously that incubation of PEO4 cells with 10^{-7} M 4-hydroxytamoxifen has been shown to reduce the growth rate (Nash *et al,* 1989). This apparent discrepancy may be due to the varying efficiencies of the charcoal-stripping of the FCS, and therefore different levels of oestrogens in the culture media.

In the absence of oestrogen in the culture media, a high concentration of 10^{-5} M tamoxifen was cytotoxic to the two ER-positive cell lines. This is a concentration similar to that which has been previously reported to be cytotoxic to ER-positive MCF-7 breast cancer cells (Sutherland *et al*, 1983b). Cytostatic but not cytotoxic effects were produced by tamoxifen at this

concentration in the ER-negative cell line, PEO14. Therefore, the toxic effects may be governed by the concentration of tamoxifen which is taken into the cell which may be facillitated by the expression of ER. The effects on the ER-negative cell line may due to other pharmacological properties of tamoxifen such as effects on the androgen receptor or anti-oestrogen binding site, direct cytolytic actions or effects on prostaglandin synthetase (reviewed in Furr, 1988). Other properties such as antagonism of intracellular calcium and calmodulin effects at the histamine receptor have also been reported. These type of effects may also be responsible for the remission of 12% of ER-negative breast tumours following tamoxifen therapy (Patterson *et al*, 1982). The stimulation of killer cell activity by tamoxifen (Berry *et al*, 1987) which has been described, may also have a role in the tumour response *in vivo*.

As reported in other systems, this data has shown that tamoxifen is a partial oestrogen antagonist, by blocking the growth stimulatory effects of oestrogen, but in the absence of oestrogen producing a small growth stimulation. The net oestrogen antagonism of tamoxifen is therefore a balance between the agonist and antagonist activity in a particular tissue. The potential of the development of "pure" anti-oestrogens which have no oestrogen agonist activity may be to increase the benefit to patients with breast or ovarian cancer, by the complete removal of oestrogens. ICI 164,384 is one of a group of novel antioestrogens developed by ICI. In culture, it has been shown to inhibit the oestrogen-stimulated growth of ZR-75-1 breast cancer cells (Wakeling and Bowler, 1987), and it can also

inhibit the oestrogen and tamoxifen induced increase in uterine weight in immature rats (Wakeling and Bowler, 1988).

Although ICI 164,384 binds to the human uterine ER with an affinity which is similar to 17 β-oestradiol (kd= 0.44 nM and 0.69 nM respectively [Weatherill *et al,* 1988]), in the present study, preliminary experiments have suggested that the growth stimulatory effects of 10^{-10} M 17 β - oestradiol in the ER-positive cell lines could be completely blocked by incubation with 10^{-6} M ICI 164,384 in PEO4 cells, but at lower concentrations of 10^{-7} and 10^{-8} M the anti-oestrogen only partially blocked the effects of oestrogen. This excess of ICI 164,384 is consistent with the previously reported 50-100-fold molar excess which was required in order to achieve half maximal inhibition of $2x10^{-10}$ M 17 β-oestradiol action in the MCF-7 breast cancer cell line (Wiseman *et al*, 1989). This may be the result of a decreased ability to enter cells compared with 17 β-oestradiol or a higher affinity for non-receptor proteins. At similar concentations, tamoxifen is unable to reduce the oestrogen stimulation in the PEO4 cell line (Dr S Langdon, personal communication). This difference in activity is supported by studies comparing the growth inhibitory actions of the two antioestrogens on ER-positive MCF-7 breast cancer cells in culture in which the potency of ICI 164,384 was at least 100-fold greater than that of tamoxifen (Wakeling and Bowler, 1988). Furthermore, competitive binding studies using ER preparations from the uterus of either rat (Wakeling and Bowler, 1988), human or porcine sources (Weatherhill *et al*, 1988) have shown that the affinity of binding of ICI 164,384 for the ER is approximately 10-fold greater

than that of tamoxifen.

Unlike other anti-oestrogens previously tested, ICI 164,384 does not show uterotrophic effects in immature rats (Wakeling and Bowler, 1988). In addition, ICI 164,384 does not induce the expression of any of six oestrogen stimulated RNAs which have been identified in MCF-7 cells *in vitro* (May and Westley, 1986; 1987, Westley and May, 1987), whereas tamoxifen induces their expression to varying degrees (Wiseman *et al*, 1989). Tamoxifen acts in a manner similar to oestrogen in that it binds to the ER and promotes DNA binding by the receptor/ligand complex. As described in the Introduction, section 1.22, the ER contains two distinct transcriptional activational factors (TAF) which are both active in the presence of oestrogen. However, following the binding of tamoxifen to the ER, TAF-1 but not TAF-2 is active (Tora *et al*, 1989). It is therefore possible that the agonist activity of tamoxifen may result from TAF-1, whereas the antagonistic activity may be a result of the lack of TAF-2 activity. The variation which is observed in the agonist activity of tamoxifen between species and tissues may be explained by a difference in the activity of TAF-1 and the nature of the target gene. The target site specificity of tamoxifen has been shown in an experiment in which nude mice were transplanted with human endometrial carcinoma (EnCa101) and breast cancer (MCF-7) cell lines. In mice which were treated with oestrogen and tamoxifen, the growth of the MCF-7 tumour was controlled whereas the EnCa101 tumour grew more rapidly compared with growth in mice treated with oestrogen alone (Gottardis *et al*, 1988). Although the "pure" anti-oestrogen ICI 164,384 also binds to the ER, the

ligand/receptor complex fails to activate either TAF-1 or TAF-2. This appears to be due to the prevention of the formation of receptor dimers as antibodies to the receptor, which are capable of restoring the DNA binding activity to mutant receptors with a defective dimerisation domain, are also capable of reversing the inhibitory effects of ICI 164,384 (Fawell *et al*, 1990). In the present study, preliminary data has shown that ICI 164,384 produced growth stimulatory effects at low concentrations in the two ER-positive ovarian cancer cell lines, PEO1 and PEO4. It is therefore possible that ICI 164,384 produces a ligand/receptor complex which possesses biological activity in the absence of dimerisation, or is capable of acting on the cell by means other than via the ER. However the lack of response to culture with ICI 164,384 at similar concentrations by the ER-negative PEO14 cell line lends support to the hypothesis that the effect is mediated via the ER. It is interesting that the maximum stimulatory effects of ICI 164,384 are present at 100-1000 fold lower concentrations than those observed for tamoxifen in the PEO1 cell line. This correlates with the difference between tamoxifen and ICI 164,384 in potency of growth inhibition of MCF-7 breast cancer cells (Wakeling and Bowler, 1988). The data obtained is preliminary, and it is important that further experiments be performed to clarify the effects of low concentrations of ICI 164,384 on the ovarian cell lines and other ER-positive cell culture systems other than MCF-7 and ZR-75-1 cells.

As observed with tamoxifen, incubation with high concentrations of 10^{-6} and 10^{-5} M ICI 164,384 produced inhibitory effects in the ER-positive and negative ovarian carcinoma cell lines. The effects produced in the

ER-positive cell lines were not as dramatic as those observed for an equivalent concentration of tamoxifen. The reason for this difference in cytotoxicity is unknown. As inhibitory effects were also observed in the ER-negative cell line, entry of ICI 164,384 into the cell is not dependent on the presence of the ER and it is possible that ICI 164,384 has other effects which are not mediated via the ER, as has been suggested for tamoxifen (Furr, 1988).

The results obtained in this study have shown that the growth of the two ER-positive ovarian carcinoma cell lines is stimulated by oestrogen and that this stimulation can be reduced by anti-oestrogens. In breast cancer, in which 60-70 % of tumours express ER (Hawkins *et al*, 1980), tamoxifen has probably been more widely used in the treatment of advanced breast cancer than any other agent. Between 50-60% of the patients with ER-positive tumours respond to therapy (Miller, 1990). These findings in conjunction with the fact that 63% of ovarian tumours express ER (Slotman and Rao, 1988), suggest that a proportion of patients with ovarian cancer would benefit from anti-oestrogen therapy. However, relatively few clinical trials have looked at the role of tamoxifen therapy in ovarian cancer and the studies which have been performed, have reported low response rates (Slotman and Rao, 1988). There are several possible reasons why poor responses to tamoxifen have been reported. Firstly, in the majority of studies the patients in the trial have failed chemotherapy previously, and may therefore have a more biologically aggressive tumour. In addition, patients have not been selectively recruited to enter the trials on the basis of the ER content of their

tumours and finally as the majority of ovarian cancers are not diagnosed until the disease is in an advanced stage, the disease burden may be too great to achieve good results with tamoxifen therapy. Clinical trials in which tamoxifen is used as the primary form of therapy in ovarian cancer or in early stages of the disease may yield better responses.

Further evidence which suggests that tamoxifen may be effective in controlling the growth of a proportion of ovarian tumours is provided by studies which have shown that tamoxifen inhibits the growth of some ovarian tumour explants in soft agar clonogenic assays (Gronroos *et al*,1984, Runge *et al*, 1986). However, no consistency between response and presence of ER was found. Consistent results in these types of studies are often difficult to obtain as tumour material contains many cell types, and various proportions of dying cells. In addition, the tumour may contain endogenous steroids which may affect the response. In contrast, a correlation between the expression of ER and PgR at levels greater than 30 fmol/mg protein in ovarian carcinomas and the greatest decrease observed in colony formation in soft agar in response to tamoxifen has been reported (Lazo *et al*, 1984).

Although tamoxifen produced growth stimulatory effects in the two ER-positive ovarian carcinoma cell lines, this was in oestrogen free conditions. In the presence of oestrogen, tamoxifen acted as an anti-oestrogen. Similar concentrations of 17 β-oestradiol to those which stimulate the growth of the cell lines are present in the premenopausal woman (Grodin *et al*, 1973) and in addition, in postmenopausal women the

conversion of adrenal androgen precursors to oestrogen may maintain the circulating oestrogen levels at stimulatory concentrations. Therefore the growth of ER-positive tumours *in vivo* may be partially under the control of oestrogens, and tamoxifen would be more likely to act as an oestrogen antagonist.

The complete removal of the oestrogen stimulus may provide greater benefit to patients with ER-positive ovarian tumours. This could be achieved potentially with the clinical use of "pure" antioestrogens. However, the observation from initial studies that the PEO1 and PEO4 cell lines are stimulated by low concentrations of ICI 164,384 merits further study before a clinical trial in ovarian cancer is initiated. The study suggests that in the PEO1 cell line, stimulatory effects of ICI 164,384 are produced at lower concentrations that tamoxifen. If this was reflected *in vivo*, it would be easier to achieve higher concentrations of the pure anti-oestrogen which are not growth stimulatory than the concentrations of tamoxifen which would be required, and would therefore offer an advantage over tamoxifen. However, as some of the effects of partial agonists may be beneficial such as protection from bone loss and blood biochemistry disorders, caution should be taken as to the effects of complete removal of oestrogen.

4.2 Effects of EGF/TGF-α on growth

A variety of growth factors have been suggested to play a role in growth control of many malignancies. In ovarian cancer, there is evidence to

suggest that EGF and TGF-α may be involved as it has been shown that between 40-77 % of ovarian tumours express EGF receptors (Bauknecht *et al*, 1989b; Battaglia *et al*, 1989; Owens *et al*, 1991; Berchuck *et al*, 1991). Furthermore, TGF-α has been identified to be present in up to 90% of ovarian tumours and EGF in 38% (Owens and Leake, 1989; Leake and Owens, 1990). In addition, specific cytoplasmic staining for TGF-α has been detected in the majority of tumours in the epithelial cells (Kommoss *et al*, 1990). It was therefore of interest to investigate the effects of EGF and TGF-α on the growth of the three ovarian cell lines.

The growth effects of the factors were studied in phenol-red free RPMI 1640 containing either 5% or 0.5% dcs-FCS or in serum free medium containing hydrocortisone, insulin, transferrin and sodium selenite (HITS). The experiments were performed in phenol red free media with dextran-charcoal treated serum as it is known that in the oestrogen sensitive MCF-7 breast cancer cell line, oestrogen increases the amount of TGF-α which is secreted (Dickson *et al*, 1986). Therefore, the presence of oestrogen may influence the sensitivity of the ovarian cell lines to EGF and TGF-α. The effects of the growth factors were also tested in media with a reduced percentage of dextran-charcoal treated serum as it is possible that serum contains similar factors, which may reduced the sensitivity to their exogenous addition. A small concentration of serum was included in the media as EGF and TGF-α may require co-factors present in the serum to be active. This possibility was addressed by investigating effects in the absence of serum, in defined

media.

Although the growth of PEO1, PEO4 and PEO14 cells was stimulated by EGF and TGF-α in a dose-dependent manner, some differences in the sensitivity to the growth factors was evident between the cell lines. The PEO14 cell line was less sensitive to EGF and TGF-α as stimulation was not observed at picomolar concentrations, and the stimulation produced by the higher concentrations of EGF and TGF-α was less marked than that in the PEO1 and PEO4 cell lines. This may be a reflection of the longer doubling time of the PEO14 cell line. Similar concentrations of EGF have also been shown to stimulate the growth of human breast cancer cells in culture (Osborne *et al*, 1980; Fitzpatrick *et al*, 1984b) In the PEO14 cell line there was also evidence that the effects of EGF and TGF-α were not equivalent. In the presence of 5% dcs-FCS, EGF induced growth stimulation at lower concentrations than TGF-α. EGF and TGF-α are equipotent in many systems such as the ability to induce premature eyelid opening of new-born mice (Smith, 1985), but differences in other systems have been reported. For example, TGF-α is more efficient at stimulating bone resorption (Ibbotson *et al*, 1985) and in stimulating arterial blood flow (Gan *et al,* 1987).

Stimulatory effects of EGF and TGF-α also occurred in the presence of a lower concentration of serum and also in the absence of serum. Thus, EGF and TGF-α do not require factors which may be present in serum to be

mitogenic.

In the present studies the growth stimulatory effects of EGF and TGF-α are accompanied by rapid changes in the cell cycle distributions. As decreases in the percentage of cells in the G0/G1 phase associated with increased proportions in S and G2/M occurred following the addition of growth factors, it is possible that EGF and TGF-α may stimulate growth by recruitment of cells into the cell cycle from the G0/G1 phase. This is consistent with the previous reports that EGF exerts its action during G1 (reviewed in Pardee, 1989). EGF has been shown to stimulate the incorporation of thymidine into MCF-7 breast cancer cells after 12-18 hours (Osborne e*t al,* 1980) which is consistent with the timing of the effects on the distribution of cells in the S phase in the present study. Although 10 nM EGF and TGF-α did not produce greater growth stimulation than 0.1nM EGF and TGF-α in PEO1 and PEO4 cells, the effects on the cell cycle were consistently more pronounced in the presence of the higher concentration of growth factor. It is therefore possible that the mitogenic growth response is not a direct reflection of the changes which occur in the cell cycle.

The *in vitro* growth of a wide variety of primary tumour cells, including ovarian carcinomas have been shown previously to be stimulated by EGF and TGF-α at concentrations similar to those found to stimulate the three ovarian cell lines (Hamburger *et al*, 1981; Singletary *et al*, 1987), although large variations in the responses were obtained between tumours. Studies using primary tumours frequently have problems with reproducability due to

heterogeneity of the tissue. In addition, the presence of varying concentrations of endogenous growth factors, such as platelet derived growth factor (PDGF) or TGF-β, which have been reported to modulate the actions of EGF and TGF-α (Rozengurt, 1986) may interfere with the responses to these growth factors. Cell lines therefore represent a model in which to systematically study the effects of EGF and TGF-α under controlled conditions. There are a few reports of the effects of EGF on other ovarian carcinoma cell lines, although EGF has been shown to stimulate the growth of two of four ovarian carcinoma cells lines studied measured by thymidine incorporation (Berchuck *et al*, 1990b). Stimulatory concentrations correlated with those in the present study.

4.3 Expression of EGF receptors

Three different techniques were employed to measure EGF receptors, to determine the level and number of components of binding of receptors in fmol/mg protein which would enable comparison with other systems, to obtain information about the specific distribution of receptors, and also to investigate the degree of heterogeneity of EGF receptor expression in the cell lines. Thus, the ligand binding assay was quantitative but did not provide information on the proportion of cells within each cell line which expressed EGF receptors. This was assessed using immunohistochemical techniques. Two monoclonal antibodies, EGFR1 directed against the external domain of the EGF receptor and F4 directed against the cytoplasmic domain of the EGF receptor were employed in an indirect immunoperoxidase staining technique, providing semi-quantitative data.

Information regarding the heterogeneity of staining obtained from this technique is subjective. Therefore, the EGFR1 antibody was also used to analyse the percentage of cells expressing EGF receptors using flow cytometry.

EGF receptors have been measured in various subcellular fractions, obtained by differential centrifugation, using a ligand binding assay (Fitzpatrick *et al*, 1984a, Perez *et al,* 1984; Sainsbury *et al*; 1985; Owens *et al*, 1991). The present study differs as the concentration of receptors have been measured in the total particulate fraction of the cell for ease of tissue preparation and to avoid loss of binding activity as EGF receptor binding activity was found in all subfractions of the cell (Hawkins *et al*, 1991). The level of EGF receptors was consistently higher in the PEO1 cell line compared with PEO4 and PEO14 which expressed similar levels of binding. Scatchard analysis revealed two components of binding in all three ovarian carcinoma cell lines, representative of two classes of receptor with high and low binding affinities. The affinities for the high and low sites are similar to those previously reported for epithelial cell lines (Fitzpatrick *et al*, 1984b). The biological significance of the presence of high affinity and low affinity binding sites is unknown, although various theories have been put forward. Briefly, the high affinity sites have been proposed to represent the dimeric form of the EGF receptor and the lower affinity sites the monomeric form (Schlessinger, 1988). Secondly, the affinity of a proportion of the EGF receptors may be altered by "transmodulation". PDGF is thought to decrease the affinity of the EGF receptor by this mechanism, by activating

protein kinase C (Rozengurt, 1986). It may also be possible that EGF or TGF-α present in the serum, or produced by the cells has bound to a proportion of the receptors and altered the affinity. However, as culture of the ovarian cells in the absence of serum had no significant effect on the level or affinity of the EGF receptors in the ovarian cell lines there is no evidence that dextran-charcoal treated serum contains factors that interact directly or indirectly with the EGF receptor.

In primary ovarian tumours, there are some reports that Scatchard analysis demonstrated only one component of binding (Bauknecht *et al*, 1984; 1989a), with binding affinities ranging between 2 and 10 nM which is consistent with the range of binding affinities present in the lower binding component of the ovarian cell lines. Other studies (Owens *et al*, 1991) have found the presence of both high and low affinity receptors in a proportion of ovarian tumours, which have binding affinities comparable with the cell lines in the present study. It therefore seems that there is variation in the number of types of binding sites which are present *in vivo*.

The distribution of EGF receptors on the cell lines was initially examined using an indirect immunoperoxidase technique employing two monoclonal antibodies, EGFR1 and F4. The results with F4 correlated qualitatively with the ligand binding assay results as greater than 90% of the cells in the three cell lines were stained positively. However, no quantitative difference in the intensity of staining between the lines was visible, which probably reflects the sensitivity of the assay. Using the EGFR1 antibody only PEO1 and PEO4

were stained positively. The percentage and intensity of staining correlated with that obtained with F4. In light of the results obtained with the ligand binding and the F4 antibody, the absence of staining of PEO14 cells with EGFR1 is difficult to explain.

As the measurement of the heterogeneity and level of staining is subjective in the indirect immunoperoxidase technique, EGF receptors were also measured by staining the cells with the monoclonal antibody EGFR1 followed by analysis by flow cytometry, providing an objective measurement. Two separate pieces of data are obtained, the percentage of cells in the population which express the EGF receptor and the intenstity of staining which represents the level of expression. Both the intensity and percentage of cells obtained increased with EGFR1 concentration. However, the concentration of antibody which gave the highest values (1:2) could not be used due to a limited supply. Therefore, the actual values obtained for the percentage and intensity of cell stained may be underestimates, but they do provide accurate information of the relative values between cell lines. In order for the percentage of cells stained and the intensity of staining to be analysed accurately by flow cytometry, the cells must be in a single cell suspension. Due to concern about the integrity of the EGF receptors, various methods of cell harvesting were tested, resulting in trypsinisation being chosen. The results obtained with the flow cytometric analysis correlated with those obtained by ligand binding as PEO1 cells showed a consistently higher percentage of cells stained and intensity of staining compared with PEO4 and PEO14 cells which showed similar staining. The positive staining

with PEO14 cells contrasts with results obtained from the static immunocytochemistry, as no positive staining was present using the same monoclonal antibody EGFR1. Repeated flow cytometric measurements of the EGF receptor expression of the three ovarian cell lines showed that within each cell line a large variation in the values for percentage and intensity of staining were obtained. This may be due to cells being harvested during varying phases of growth (eg lag versus logarithmic) or at different cell densities. The variation of the staining for EGF receptors is unlikely to be caused by cell cycle influences as there was no difference in the levels of EGF receptors expressed by cells in the different stages of the cell cycle (G0/G1, S and G2/M) in any of the three cell lines.

The presence of EGF receptors on all three cell lines is consistent with the growth stimulatory effects produced by EGF and TGF-α. Although the PEO1 cell line tended to express higher levels of EGF receptors compared with the PEO4 cell line, this difference was not reflected by a difference in the sensitivity to exogenous addition of EGF and TGF-α. Conversely, the observed decreased sensitivity of PEO14 cells to EGF and TGF-α compared with PEO4 cells cannot be explained by different levels of expression of EGF receptors. There have been previous reports of the lack of correlation of response to EGF measured by clonogenic growth of primary ovarian tumour cells and EGF receptor number (Pathak *et al*, 1982).

4.4 Modulation of EGF receptors by EGF

As the biological effects of EGF and TGF-α are mediated via the EGF

receptors it was of interest to investigate factors which modulate the EGF receptor expression. Using the ligand binding assay a decrease in the level, but not the disappearance of EGF receptors was detected after incubation with EGF. EGF has been well documented to "down-regulate" the expression of its own receptor. Following binding of EGF to the receptor, the ligand/receptor complexes cluster in coated pits and are then removed from the cell surface (reviewed in Pastan and Willingham, 1983). This is a rapid process as complexes have been visualised by immunofluorescence to be arranged around the nucleus approximately 30 min after binding of EGF to the receptor (Beguinot *et al*, 1984). However, not all of the EGF receptors are removed from the cell surface by down-regulation (Das and Fox, 1978) which supports the results obtained from ligand binding with PEO1 cells. The proportion of EGF receptors remaining at the cell surface may be due to an inherrent unknown difference or may be due to the synthesis of the EGF receptor being stimulated in the presence of EGF (Clark *et al*, 1985, Kudlow *et al*, 1986). This decrease in the level of EGF receptors on PEO1 cells may not reflect internalisation, but may be due to the inability of ^{125}I to bind to the receptors which may already be occupied by unlabelled EGF which was present in the culture media. However, a decrease in EGF receptors following culture in the presence of EGF was also measured by flow cytometry utilising the EGFR1 monoclonal antibody. As this monoclonal antibody is directed against a determinant in the extracellular portion of the EGF receptor which is discrete from the binding domain, it recognises occupied as well as unoccupied receptors (Waterfield *et al*, 1982; Green and Couchman, 1985). Therefore, the decrease in measurement of EGF

receptors on PEO1 cells is probably due to their removal from the cell surface. Using flow cytometric analysis, internalisation of the EGF receptors in PEO4 and PEO14 cells also occurred following incubation with EGF. As incubation with TGF-α decreased the level of receptors by a similar level as EGF it is probable that TGF-α is internalised and processed in the same manner.

The time course of effects on the EGF receptor level on PEO1, PEO4 and PEO14 cells following incubation with EGF were also examined. Maximum decreases in the receptor levels, measured by the percentage and intensity of staining with EGFR1, occured after 1-2 days incubation with EGF. This time course is consistent with results from studies in MDA 468 breast cancer cells (Kudlow *et al*, 1986) in which levels of EGF receptors then increased after this period, although remained beneath that of control cells. EGF has been shown to stimulate the synthesis of its receptor (Clark *et al*, 1985; Earp *et al*; 1986, Kudlow *et al*, 1986). Levels of mRNA for EGF receptors are maximally increased between 4-8 hours following addition of EGF. Changes in the levels of the EGF receptors can probably be explained by the changing rates of EGF receptor degradation compared with synthesis. As the levels of receptors decreased initially rapidly, the rate of internalisation exceeded the rate of synthesis of new receptors, whereas after 2 days, the rate of synthesis increased. Steady levels of EGF receptors were due to the two opposing rates becoming constant, but as the EGF receptor level remained less than control cells, the overall rate of internalisation always exceeded the synthesis. It has been suggested that the function of the

remaining receptors is to enable a constant supply of ligand/receptor complexes to be internalised which may be necessary for the mitogenic signal.

4.5 Secretion of EGF-like factors

The levels of EGF detected in the media conditioned by the growth of the three cells lines by a RIA are within the range of concentrations of EGF which have been shown to stimulate the growth of the cell lines. It is therefore probable that the secreted EGF plays a role in the maintenance of growth of the cell lines The levels secreted are also within the range of concentrations which have been reported to be present in primary ovarian tumours (Owens and Leake, 1989; Leake and Owens, 1990; Bauknecht *et al,* 1989b) and are therefore of relevance to the situation *in vivo*. In addition to the RIA, a less specific RRA was used to determine whether other growth factors such as TGF-α were secreted also into the media. No displacement of ^{125}I EGF binding above what was present in the unconditioned medium was detected. In theory, this assay should detect EGF, TGF-α, amphiregulin or gp30 which all bind to the EGF receptor (Shoyab *et al*, 1989; Lupu *et al*, 1990). However, the presence of concentrated conditioned media may have impaired the ability of the factors to bind to the receptors. It is known that the PEO1 and PEO4 cell lines express mRNA for TGF-α which has not been detected in PEO14 cells (Dr J Bartlett, personal communication),but whether this is translated and secreted as mature TGF-α is not known.

Without considering the possibility that TGF-α may be secreted into the

culture media, there is an inverse correlation between the level of expression of EGF receptors and the concentration of EGF which is secreted and measured by the RIA. PEO1 has consistently higher levels of receptors, but secretes the least EGF-like factors, whereas PEO4 cells tend to express the lowest levels of EGF receptors and produces the greatest quantity of EGF-like factor. A similar inverse correlation between EGF production and EGF receptors has been detected in primary ovarian carcinomas (Bauknecht *et al*, 1989b). As has been previously discussed EGF causes down-regulation of the EGF receptor. It is therefore possible that the lower EGF receptor levels on PEO4 cells are due to a higher concentration of EGF being secreted which leads to internalisation of the receptors.

The presence of receptors for EGF, growth responses to EGF and TGF-α, and secretion of EGF into the media or expression of TGF-α mRNA suggest that EGF and TGF-α may control growth in an autocrine or paracrine mechanisms in the three ovarian cell lines. In order to provide direct evidence that the EGF and TGF-α produced contributed to growth control of the cells, antibodies against EGF and TGF-α were used in an attempt to modulate the growth rates. The experiments were only performed on a limited number of occasions due to a very restricted supply of the antisera. Therefore, in some experiments it was not possible to draw meaningful conclusions. However, the rate of growth of PEO14 cells was decreased repeatably in the presence of the antiserum directed against EGF, which would be consistent with EGF being an autocrine factor in this cell type. In the presence of serum, the antiserum against EGF produced growth

stimulation in the PEO4 cells, whereas in serum free medium the growth was unaffected. Although the results obtained in the presence of serum are paradoxical, there may be several explanations. As it has been shown that aggregation of EGF/receptor complexes is an important step in the EGF-stimulated synthesis of DNA (Schechter *et al*, 1979), it is possible that the antibodies crosslink EGF receptors which have bound EGF and have not yet been internalised, thus stimulating mitogenesis. However, this hypothesis would not explain why the PEO1 and PEO4 cell lines do not respond similarly. Alternatively the growth stimulation may be due to the PEO4 cells responding to the withdrawal of EGF by producing another growth factor, perhaps TGF-α, which stimulates the growth above the normal rate. This production of growth factor may require factors that are present in the serum to be synthesised. Experiments in which the cells were incubated with antibodies against EGF and the EGF receptor simultaneously would test this hypothesis. If the increase in growth was due to TGF-α, the effect would be abolished in the presence of an anti-EGF receptor antibody.

In comparison with the effects of the EGF antiserum, the antiserum directed against TGF-α does not effect the growth of PEO14 cells in the presence or absence of serum which is consistent with the inability to detect mRNA in this cell line. It is therefore possible that TGF-α is not synthesised in PEO14 cells. The growth of PEO1 cells is reduced in the presence but not in the absence of serum. As the growth of the PEO4 and PEO14 cells lines was also unaffected by the TGF-α antiserum in serum free media it is possible that TGF-α production is dependent on the presence of serum. To examine this

possiblity it would be necessary to look for the production of TGF-α mRNA in cells grown in serum free media. The secretion of TGF-α by the cell lines in various culture conditions could also be examined directly by RIA. The results obtained for the effects of the antiserum against TGF-α on PEO4 cells in serum-containing conditions are equivocal because although the experiment was performed on 4 separate occasions, a clear reduction in the growth rate was present on only one occasion. One reason why the results obtained from this type of experiment vary is possibly the dependence of the concentration of EGF and TGF-α in the medium on the cell numbers. The production of growth factors will be proportional to the cell number. If an unlimited supply of antisera had been available, it would have been interesting to determine whether the level of oestrogen-growth stimulation could have been decreased in their presence, as previous experiments have provided indirect evidence that an enhanced secretion EGF or TGF-α produced by oestrogen may contribute to the stimulatory activity.

The results from these studies have shown that the growth of the three ovarian cell lines is stimulated by the exogenous addition of EGF and TGF-α, and that the presence of EGF receptors is consistent with their mode of action. In addition EGF-like material is secreted by the three lines. Therefore, the elements which are required for an EGF/TGF-α autocrine growth regulation pathway are present. In two of the cell lines, the basal rate of growth can be decreased by the addition of antisera raised against EGF or TGF-α providing direct evidence that such a growth regulation

pathway exists.

4.6 Modulation of EGF/TGF-α growth regulation by oestrogen and tamoxifen

The exact mechanism by which oestrogen induces growth stimulation is unknown, but it is known that transcriptional induction of several genes occurs. Studies on MCF-7 breast cancer cells *in vitro* have shown that oestrogen increases the secreted levels of stimulatory growth factors such as TGF-α and IGFs (Dickson *et al*, 1986, Huff *et al*, 1986) and decreases levels of the growth inhibitor, TGF-β (Knabbe *et al*, 1988). In addition, the oestrogen-stimulated growth of MCF-7 cells was significantly inhibited by monoclonal antibodies to the EGF receptor (Eppstein *et al*, 1989) providing further evidence that oestrogen-stimulation is at least in part mediated by an autocrine or paracrine loop involving EGF or TGF-α. If a similar mechanism of growth stimulation exists in the ovarian cell lines, it is possible that the EGF receptor level would be modified.

The effects of oestrogen on the levels of the EGF receptors were measured by ligand binding in the PEO1, PEO4 and PEO14 cell lines. Levels in the ER-positive cell lines PEO1 and PEO4 were decreased following 5 days in culture with oestrogen. This is consistent with an effect that is mediated via the ER as oestrogen failed to affect the level of EGF receptors in the ER-negative cell line PEO14. The affinity of the low affinity binding component was increased in both cell lines, whereas the affinity of the higher affinty binding sites was not altered. The absence of an effect of

oestrogen on the affinity of EGF receptors has been reported previously (Mukku and Stancel, 1985, Berthois *et al*, 1989). A corresponding decrease in the percentage of cells stained and the intensity of staining with EGFR1 detected by flow cytometry was present only in the PEO4 cell line, and was absent in PEO1 cells. The reason for the discrepancy in results from the two techniques is not known. Compared with the effects of EGF on EGF receptor level, the effects produced by oestrogen measured by flow cytometry were quantitatively less and were delayed. Therefore, the combined results of these studies suggest that it is possible that oestrogen may induce the *de novo* synthesisn of a growth factor which binds to the EGF receptor and causes its' internalisation. It has been proven that EGF and TGF-α decrease EGF receptors by this mechanism in the three cell lines, therefore it is possible that it is their secretion which is being regulated by oestrogen in the ER-positive cell lines. Other growth factors have also been identified which can bind to the EGF receptor. Amphiregulin, which was initially identified in the conditioned medium of a stimulated breast cancer cell line (Shoyab *et al*, 1989) binds most strongly to the c-*erb*B-3 receptor, but also binds weakly to the EGF receptor (Shoyab *et al*, 1990). In addition, a recently identified 30 Kd growth factor which is secreted by some breast cancer cells (Lupu *et al*, 1990) is also able to bind to the EGF receptor with low affinity. It is therefore possible that these growth factors may also be secreted and their levels modulated by oestrogen. In order to determine if growth factors are modulated by oestrogen, further studies need to be conducted to investigate the identity and level of secretion of their secretion. This could be determined by assaying for mRNA of the growth factors or hormones, or by

detection of the specific peptides using radioimmunoassays. The effect of short-term culture with oestrogen are in contrast to those previously reported for MCF-7 breast cancer cells (Berthois *et al*, 1989) in which incubation with oestrogen was shown to intially reduce the EGF receptor level, but following 5 days in culture, the EGF receptor level was increased by 200%.

The anti-oestrogen, tamoxifen is also thought to exert part of its biological action by changing the levels of growth factors which are secreted by cells. For example, in MCF-7 breast cancer cells in culture tamoxifen has been reported to increase the level of TGF-β which is secreted into the media and to decrease the levels of TGF-α and IGF-1 (Dickson and Lippman, 1987, Knabbe *et al,* 1987). In addition, the effects of anti-oestrogens on T47-D breast cancer cells in culture can be partially reversed by incubation with EGF (Koga and Sutherland, 1987). The reversal of growth inhibition is observed at concentrations of EGF which do not produce growth stimulatory effects when added to the culture media alone. This provides further evidence that tamoxifen may alter the production of growth factors including EGF or TGF-α. In the present study the decrease in the EGF receptor level in the PEO4 cell line produced by incubation with tamoxifen is therefore not consistent with this hypothesis. However, in our culture conditions, tamoxifen appears to have oestrogen agonist activity on PEO4 cells. Although on average the effect was small, in certain individual experiments, clear growth stimulation of the cells occured. The time course of tamoxifen effects on EGF receptors follows a similar pattern to that of oestrogen, although the effects are less marked. This also suggests the concept that

tamoxifen may be acting as an oestrogen agonist. The less marked agonist activity compared with oestrogen is characteristic of a partial agonist. This has been demonstrated by the stimulation of uterine growth of immature rats. The maximum stimulatory effect of tamoxifen is less than that acheived with oestrogen even when tested at high doses (Wakeling and Bowler, 1987).

4.7 Conclusions

The data presented in this thesis has shown that the growth of three ovarian cell lines may be controlled by an autocrine or paracrine regulation loop involving EGF and TGF-α. Furthermore, this regulatory pathway may be under hormonal control. A schematic diagram of this potential control mechanism is shown in figure 4.1.

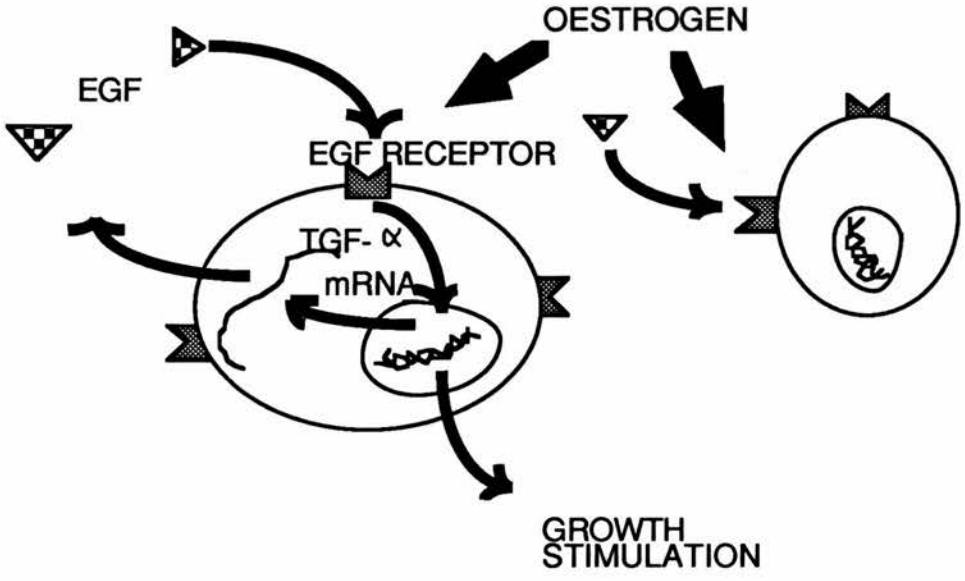

Figure 4.1. Schematic diagram showing potential growth regulation pathways in the ovarian carcinoma cell lines.

As the majority of ovarian tumours express ER (Slotman and Rao, 1988), and a large proportion express EGF receptors (Bauknecht *et al* , 1989a; Battaglia *et al,* 1989; Berchuck *et al*, 1991; Owens *et al* 1991) it is possible that these results may be reflected *in vivo*. Thus, anti-oestrogen therapy may potentially interfere with growth of a proportion of ER-positive tumours by competing with binding to the ER and also by regulating the effects of EGF and TGF-α. The sensitivity of the three cell lines to EGF and TGF-α suggests that therapies which interfere with the effects of these growth factors may also be of therapeutic benefit to ovarian cancer patients.

4.8 Future directions

As oestrogen, EGF and TGF-α have been identified as important regulators of growth of ovarian cancer cells *in vitro*, it is necessary to determine whether these effects are reproduced *in vivo*. The effects of these factors on the growth of xenografts in animal models propogated from the cell lines, and the potential to interrupt this growth control should be investigated. In addition,it is important to determine that the potential for similar mechanisms exists in primary ovarian tumours.

CHAPTER 5

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Appendix (Publications)

Data from this thesis has been presented in the following abstracts and papers:

Bartlett, J.M.S., Langdon, S.P., Crew, A.J., Scott, W.N. and Miller, W.R. (1991). Differential transforming growth factor mRNA expression in ovarian carcinoma cell lines. Br J Cancer. 63:supplement XIII (P143) 61.

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