REGULATION OF PROLIFERATION OF BREAST CANCER CELLS BY GROWTH FACTORS

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DEDICATION

For the weans - irritating, unintentionally angelic, amusing, occasionally frightening but above all, mine.

DECLARATION

I state that all the work in this thesis was performed personally unless otherwise acknowledged.

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ABBREVIATIONS

Bovine Serum Albumin	BSA
Bromodeoxyuridine	BUdR
Deoxyadenosine triphosphate	dATP
Deoxycytidine triphosphate	dCTP
Deoxyguanosine triphosphate	dGTP
Deoxyribonucleic acid	DNA
Deoxythymidine triphosphate	dTTP
Diethylpyrocarbonate	DEPC
Dissociation Constant	Kd
Epidermal Growth Factor	EGF
Epidermal Growth Factor Receptor	EGF-r
Foetal Calf Serum	FCS
High Pressure Liquid Chromatography	HPLC
Hydrochloric Acid	HCl
Insulin Like Growth Factor I	IGF-I
α- Interferon	α-IF
Long Terminal Repeats	LTR
Messenger Ribonucleic Acid	mRNA
Normal Rat Kidney	NRK
Oestrogen Receptor	ER
Platelet Derived Growth Factor	PDGF
Sodium Chloride	NaCl
Sodium Dodecyl Sulphate	SDS
Transforming Growth Factor -α	TGF-α
Transforming Growth Factor - β	TGF-β
Transforming Growth Factor - β Receptor	TGF-βr

SUMMARY

Since Beatson demonstrated in 1896 that castration resulted in shrinkage of breast tumours in approximately 30% of women thus treated, it has been shown that oestradiol is a trophic hormone for certain types of mammary cancer. Recent evidence suggests that the mitogenic effects of oestradiol are mediated by the stimulatory growth factors IGF-I and TGF-α, acting in a way like second messengers. This is consistent with the autocrine growth hypothesis proposed by Sporn and Todaro, in which tumour cells produce mitogenic polypeptides to which they can respond via specific cell surface receptors. Additional evidence indicates that the cytostatic effects of the anti-oestrogenic compound tamoxifen may be mediated by secretion of TGF-B by ER positive human breast cancer cells, in an autoinhibitory loop.

In this thesis it has been shown that TGF-β (50 pM) has a significant cytostatic effect on ER positive human breast carcinoma cells (the ZR-75 cell line). Similarly, treatment with α-IF (500iu /ml) has an anti-proliferative effect. The duration of the cytostatic effect for TGF-β and α-IF was related to the duration of exposure to each of these agents.

When they were removed from the cell culture medium, the breast cancer cells started to regrow after a lag period of approximately 24 hours, parallel to control cultures. α-IF reduced expression of ER protein to 40% of control, untreated values, and again this effect was maintained for the duration of exposure to α-IF. After a lag phase of 24 hours, α-IF induced a rise in TGF-β mRNA by some 8 fold relative to untreated cells and a reduction in ER mRNA to approximately 50% of control. The kinetics of induction of TGF-β mRNA were similar to the antiproliferative effect of α-IF in that there was an initial lag phase, the duration of cytostasis was proportional to the duration of exposure of α-IF and when α-IF was removed from the medium, the cells started to regrow and TGF-β mRNA levels return to unstimulated values after a short delay. Nuclear run on assays showed that the increase in TGF-β mRNA concentration was due to an increase in its transcriptional rate.

Using a bioassay for TGF-β, which was related to its dose-dependent inhibition of ^{3}H-thymidine incorporation by mink lung epithelial cells, it was shown that a commercially available anti-TGF-B polyclonal antibody could neutralise the effects of TGF-B (50μg of antibody neutralised 1ng of TGF-β in the assay.) Coincubation of ZR-75 cells with α-IF and the anti-TGF-β antibody reduced the cytostatic effect of α-IF by 60%.

Cell binding studies with radio-iodinated TGF-β revealed that the ZR-75 cells have 5,800 high affinity binding sites with a dissociation constant of 70 pM.

Oncogene transfection of c-*myc* and activated H-*ras* oncogenes into mink lung epithelial cells revealed that this altered the response of the cells to exogenous growth factors. C-*myc* transfection reduced the response (^{3}H-thymidine incorporation) to TGF-β but enhanced the normal stimulatory response to EGF. Activated H-*ras* transfection abolished the response of the epithelial cells to both TGF-β and EGF.

Taking these results in concert, it would appear that a significant proportion of the cytostatic effect of α-IF is mediated by TGF-β in this human breast cancer cell line. Growth factor responsiveness can be altered by oncogene transfection and the presence of other growth factors in the microenvironment. This could be relevant to the process of promotion during epithelial carcinogenesis.

CHAPTER I

INTRODUCTION

SECTION 1.1 EPIDEMIOLOGY OF BREAST CANCER

Breast cancer is the most common form of all malignant diseases in women, with an annual incidence of 21,000 new cases in England and Wales and 100,000 new cases in the United States. It is therefore a relatively common cause of mortality and morbidity in the Western world.

There are a number of known aetiological factors (1). Linked, reproductive variables which increase the risk of developing the disease include early menarche and late menopause. First childbirth over the age of 30 years increases breast cancer risk threefold, relative to women who have their first child before the age of twenty. Pre-existing benign breast disease such as hyperplastic cystic mastopathy contributes to breast cancer risk. There is also a familial component in that first degree relatives of breast cancer patients are 2-3 times more likely to develop the disease compared to controls. Obese women, probably through increased aromatisation of steroid hormones in adipose tissue, have a small but significantly increased risk of breast cancer.

In view of the endocrine implications of these risk factors, there is a vast literature attempting to correlate plasma and tissue hormone concentrations to the risk of developing breast cancer.

Total plasma oestradiol levels do not predict breast cancer risk in pre or post menopausal women (2). However, recent reports from a large, prospective epidemiological trial based in Guernsey have indicated that if corrections are made for the extent of oestradiol bound to sex hormone binding globulin, the concentration of free oestradiol was significantly elevated in those patients who subsequently developed interval breast cancers (3). These data are consistent with a model of breast carcinogenesis which postulates that cancer risk is a function of "biological breast age" which integrates the duration and degree of exposure of mammary tissue to "active oestrogen" (4).

It is possible to use the framework of epidemiological data to generate a testable, stochastic laboratory model of mammary carcinogenesis. According to current hypotheses, carcinogenesis is a multi-stage process; the first stage, initiation, is induced by mutagens (e.g. carcinogenic chemicals,radiation and viruses) and is irreversible; the second stage, promotion, can be brought about by agents such as hormones (steroids and polypeptides), phorbol esters and some viruses; the third stage, progression, is characterised by the development of heterogeneity within tumours. Again, the literature in the field of mammary carcinogenesis is vast, but in summary, laboratory studies in vitro and in vivo in mice suggest that oestrogen (in its various forms) is a weak genotoxic agent at physiological concentrations.

Oestrogen is unlikely to be responsible for initiation (5). In contrast, promotion of human breast tumour cell growth is well-recognised to be oestrogen dependent in vitro (6) and in vivo (7) using murine and human tumour model systems.

The hormonal responsiveness of breast tumours is mediated primarily by the oestrogen receptor (ER), a 66-kilodalton protein consisting of a central DNA binding domain, a carboxy-terminal oestrogen-binding domain and an amino-terminal oestrogen "modulating" domain. Cloning of the ER and mutational analysis studies have allowed regional mapping and definition of the function of the various sectors of the molecule. Binding of oestrogen to its receptor, which resides predominately within the nucleus of target cells (8), increases the affinity of the hormone-receptor complex for the hormone responsive genes which are the targets for activated receptor. Electron microscopy has suggested that DNA-bound receptor dimers undergo cooperative binding thereby linking these distant hormone responsive elements to form DNA loops which may then participate in interactions between other trans-acting transcriptional enhancers and gene promoters (9,10). The mRNA species which are induced by hormone-receptor-DNA interaction are incompletely characterised and the function of their protein products is obscure, but is an active area of current research.

Estimation of ER concentration in breast tumour specimens gives clinically useful information. Much of the early work was bedevilled by interpretative problems due to interlaboratory variation in assay procedure, but with international agreement on standards and improved protocols for specimen handling and processing, these problems have been largely overcome. The traditional assay for ER estimation is based on radioligand binding of ^{3}H-oestrodiol. However, this correlates well with newer methods such as immunocytochemical stains for ER,which gives an idea of heterogeneity of the ER tissue distribution (11).

Primary breast tumours are more commonly ER positive (i.e. greater than 10fmol receptor/mg protein) in post menopausal patients (60%) compared to premenopausal women (30%). ER positive tumours tend to be well differentiated, exhibit lower DNA ploidy and lower proliferative rates (as assessed by flow cytometric estimation of S-phase fraction), than ER negative tumours (12, 13, 14).

ER concentration gives valuable prognostic information in that there is an improved overall survival from ER positive breast cancer (15), which has a prediliction for metastasising to bone, and is inversely correlated with the development of disease in liver and brain (16).

ER status also helps therapeutic decisions; liver metastases tend to be unresponsive to hormonal manipulation regardless of ER status, while "relatively hormone responsive" sites of disease include bone, soft tissue and pleura; responses (i.e. tumour shrinkage) to hormonal manipulation are seen in up to 70% of ER positive patients (17).

Sir George Beatson was the first to show, in 1896, that removal of the ovaries could cause regression of breast cancer, in about 30% of all women thus treated. This was the original basis for the notion that oestrogens were trophic for certain breast tumours. A variety of hormonal therapies have been utilised over the past decades, but anti-oestrogens, aromatase inhibitors (which prevent synthesis of oestrogen and its metabolites) and progestational agents have emerged as the dominant therapeutic modalities (18). The molecular mechanism of action of those agents will be discussed later.

SECTION 1.2 CURRENT HYPOTHESES ON AUTOCRINE / PARACRINE CONTROL OF BREAST CANCER CELL PROLIFERATION

Growth of normal cells is controlled largely by the interplay between polypeptide hormones and hormone-like growth factors present in tissue fluids. In general, malignant cells require less of these exogenous growth factors than normal cells for optimal growth. Sporn and Todaro (19) postulated that the malignant transformation of cells may be mediated by the release of polypeptide growth factors. A growth-promoting transforming polypeptide should have the following properties; it should be a strong mitogen and cause loss of density-dependent inhibition of growth in culture; it should cause morphological transformation of cells; it should cause anchorage independent growth. Such factors have been isolated and Sporn and Todaro coined the terms of autocrine and paracrine growth control. Autocrine control (fig 1) implies that the tumour cell produces polypeptide growth factors which bind to cell surface receptors on the same cell, autostimulating the tumour cell to grow. Paracrine growth control (fig 1) is exerted by release of polypeptides into the local environment and can interact, again via cell surface receptors, with adjacent cells, perhaps mediating a tumour/stromal cell interaction. Several oestrogen dependent breast cancer cell lines such as MCF-7 and ZR-75 have been isolated from patients with metastatic breast cancer.

In vitro, the growth of these lines is stimulated by the addition of oestrogen and the formation of tumours following innoculation of MCF-7 cells into athymic, nude mice, is absolutely oestrogen dependent (20-24). Lippman and coworkers (25, 26) have presented evidence that "growth factor activities," some of which were oestrogen-induced, are released by these cell lines into serum-free conditioned medium. Subsequently they identified three of these factors as being transforming growth factor-α (TGF-α), insulin like growth factor I (IGF-I) and platelet derived growth factor (PDGF). MCF-7 cells have receptors for epidermal growth factor (EGF)(to which TGF-α binds) and IGF-I receptors but not PDGF-receptors (although these receptors have been demonstrated on breast stromal cells (27)). On the basis of this information these authors proposed autocrine growth stimulation of MCF-7 cells in vitro, with the polypeptides acting in a way like second messengers following exposure to oestrogen (summarised in fig 1).

Further studies were performed to determine if these oestrogen induced growth factors in conditioned medium were capable of replacing oestrogen itself in induction of tumour formation by MCF-7 cells in vivo in the nude mouse. Dickson et al (25), demonstrated that the oestrogen conditioned medium, administered by mini-pumps, stimulated the development of MCF-7 cell implants into tumours grown in ovariectomised athymic mice.

Conditioned medium from cells grown in the absence of oestrogen had no such effect. No stimulation of uterine wet weight was noted in either set of experiments which implies that the effect was independent of oestrogen. While early tumour development was indistinguishable under oestrogen and oestrogen conditioned medium stimulation, the conditioned medium stimulated tumours were not capable of growth beyond approximately 0.5 cm in diameter. MCF-7 cells secrete other proteins, including proteases and angiogenic substances into their microenvironment following oestrogen exposure. Clearly these factors could be important in promoting tumour growth in vivo, but could have been absent from the conditioned medium infused via the mini-pumps and therefore full tumourigenesis was not observed.

ER positive human breast cancer differs from ER negative disease in several biological and clinical parameters. ER negative breast cancer is usually hormone independent and tends to behave in a clinically more aggressive manner than ER positive breast cancer. Receptor negative tumours tend to be less well differentiated, more aneuploid and have a higher proliferative rate (measured indirectly using methods such as thymidine labelling index and the fraction of cells in S-phase estimated using flow cytometry of ethidium bromide stained, disaggregated tumour (12, 13)).

Recent biochemical studies in vitro suggest that ER negative breast cancer cells have a high constitutive secretion of certain growth factors including TGF-α and IGF-I (29). As the cancer cells have surface receptors to these peptide factors, autocrine growth loops could provide these cells with a proliferative advantage.

The main, purely paracrine growth factor studied thus far in breast cancer is PDGF, to which the breast cancer cells themselves are insensitive, but which has marked effects on the surrounding stroma. PDGF is a potent mitogen for vascular endothelial cells and fibroblasts and can elicit chemotactic responses in fibroblasts and smooth muscle cells (28-31). It is possible that PDGF secreted by breast cancer cells would have a primary role in maintaining the fibro-vascular stroma required for tumour growth via paracrine growth factor loops.

The work described thus far has originated from cell lines. There is some evidence to suggest that primary breast cancers express transcripts for TGF-α , IGF-II and epidermal growth factor receptor (EGF-r). Travers et al (32) isolated mRNA from samples of tissue in biopsy specimens from 52 malignant and 15 non-malignant tumours of the breast and 4 samples of normal breast.

Transcripts for both TGF-α and its receptor, EGF-r, were found more commonly in carcinomas that were ER negative (64% and 87% respectively) than in those that were ER positive (27% and 30% respectively). Insulin-like growth factor II mRNA was present in all 15 samples of non-malignant tissue but was found (in considerably lower amounts) in only 11 of 21 (52%) carcinomas. EGF-r was also found in all non-malignant breast tissues, compared with 19 of 45 (42%) carcinomas. PDGF mRNA transcripts coexisted in all normal and benign tissues and carcinomas.

Regardless of the mechanism of production, activation and delivery of growth factors, they all elicit biological events from the cell surface. The target cell must possess the specific cell surface receptor to effectively bind the growth factor. There is a substantial clinical data base for EGF-r, which binds both EGF and TGF -α, in primary breast cancer specimens.

In a prospective series conducted by Sainsbury et al (33), 135 patients with operable breast cancer underwent either lumpectomy with axillary sampling (25 patients) or simple mastectomy (110 patients). EGF-r concentration was measured by a radioligand binding assay in each tumour specimen. The authors found that there was a significant inverse relation between EGF-r and ER status and a significant association with tumour size and poor differentiation.

The relapse-free survival and overall survival were significantly worse for patients with EGF-r positive tumours compared with EGF-r negative tumours. Multivariate analysis of the parameters associated with prognosis showed that EGF-r status was the most important variable in predicting relapse-free and overall survival in lymph node negative patients and the second most important variable in lymph node positive patients. It would appear therefore that measurement of EGF-r concentration is of some clinical utility and might define a subgroup of node negative patients who would benefit from adjuvant chemotherapy.

It has become increasingly clear that there is a close relationship between growth factors, their receptors and oncogenes (Table 1.1). Oncogenes are mutated forms of proto-oncogenes, which are genes involved in cell growth and differentiation. Both types of genes have been cloned, studied in detail and the basis of their "activation" in some tumours has been determined. The role of individual oncogenes in multistage carcinogenesis is not completely understood and their stage-specific action, in terms of initiation and promotion in mammary carcinogenesis, is not clear. Nevertheless, there are clear links between steroid hormones, growth factors and oncogenes in determining the behaviour of established human breast cancer.

The membrane receptor for TGF-α (EGF-r) is coded for by a gene that has 2 related genes which have the properties of oncogenes. The *erb*-B gene is homologous to EGF-r gene except that its protein product lacks an extracellular domain (34). The second, closely related component is the *neu* oncogene, detected initially in a chemically induced neuroblastoma (35). The protein product of this oncogene has an extracellular domain, but it binds neither TGF-α nor EGF.

In a series of 189 human primary breast cancers , evaluation of alterations in the HER-2/*neu* gene revealed that amplification occurred relatively frequently and was found to be a significant predictor of disease-free and overall survival. An initial survey involved tissue samples from 103 primary breast tumours. DNA was digested with EcoRI and subjected to Southern blot analysis with a ^{32}P labelled HER-2/*neu* probe which detects a 13kb hybridising band in human DNA. Nineteen tumours (18%) showed evidence of HER-2/*neu* amplification. When all tumours were categorised according to whether they contained one, 2-5, 5-20 or >20 copies, no association was seen between any of these groups and oestrogen or progesterone receptor status, tumour size, or age at diagnosis. However, a trend was noted for nodal status in that 11% of node-negative patients, 10% of those with 1-3 involved nodes and 32% with >3 involved nodes had gene amplification.

For >3 positive nodes, the increase in amplification was significant ($P < 0.05$) compared with 0-3 positive nodes (36).

A second study was therefore conducted on 86 breast cancers from node-positive patients for whom a median follow-up of 46 months provided relapse and survival information. After mastectomy, 49% of these patients received adjuvant systemic therapy alone, 17% radiotherapy alone and 19% received both. At analysis, 35 patients had relapsed and 29 had died with median times of 62 and 69 months, respectively. Amplification of the HER-2/*neu* gene was found in 40% of this series and correlated significantly not only with number of involved nodes ($P = 0.002$), but also with oestrogen receptor status and tumour size. Univariate analysis revealed a highly significant correlation between degree of amplification and both time to relapse ($P<0.001$) and overall survival ($P = 0.0011$). Moreover, patients with >5 copies had significantly shorter disease free and overall survival times ($P = 0.015$ and $P = 0.06$, respectively). In predicting these end points, amplification was found to be superior to all other prognostic factors except nodal status which it equalled. In multivariate analyses, amplification remained a strong independent prognostic factor providing additional information even when other factors were taken into account (36).

In the whole series of 189 breast cancers, rearrangements of the HER-2/*neu* gene were rare (3 cases). Moreover, rearrangements or amplification of the EGFr gene occurred in only one and four cases, respectively. The phenomenon of gene amplification is therefore not general for tyrosine kinase-specific receptors in breast cancer. The data indicate that HER-2/*neu* may play a role in the biological behaviour and/or pathogenesis of human breast cancer.

The avian erythroblastosis virus is an acutely transforming retrovirus containing two oncogenes, v-*erb*B and V-*erb*A. Previous work from Waterfield and collaborators (37,38) showed that V-*erb*B is probably a truncated form of the chicken EGF receptor gene, but the origin of v-*erb*A has remained a puzzle. Construction of viruses that only expressed v-*erb*A suggested that this gene is not capable of inducing neoplastic transformation on its own but co-operates with tyrosine kinase oncogenes, such as v-*erb*B or v-*src*, to produce transformed erythroid cells with a high capacity for self renewal and a low capacity to differentiate. Recent data on the cloning and structure of human glucocorticoid receptors suggested that the c-*erb*A gene might also encode a steroid hormone receptor. It appears that there are at least two families of c-*erb*A genes located on chromosomes 3 and 17 and that these genes encode proteins capable of binding thyroid hormones (T3 and T4) with high affinity (39).

Thus, the c-*erb*A gene appears to encode receptors for thyroid hormones. Thyroid hormone receptors are expressed at low levels in many different tissue types and it is possible that alterations to c-*erb*A could be involved in many different types of tumour. The evidence suggests that the v-*erb*A protein is a constitutively activated form of the receptor that does not bind hormone with high affinity. Activation appears to result from point mutations, some of which occur in a putative DNA-binding domain. The viral protein may therefore bind to DNA in the absence of the hormone ligand and thus activate or suppress gene expression (39).

The role of *erb*-A, *erb*B, and *neu* oncogenes in mammary carcinogenesis is unclear, but as mentioned, preliminary clinical studies have demonstrated their usefulness in assessing prognosis of primary breast cancer.

Activation of host cell oncogenes by insertion of proviral DNA is a common mechanism of tumourigenesis by retroviruses used sucessfully to identify proto-oncogenes by transposon tagging. Two proto-oncogenes, *int*-1 and *int*-2, have been implicated in control of development of murine mammary glands (40-42). Although the *int* proteins are unrelated to each other, they both have a structure characteristic of signal transmitting molecules.

The *int*-1 gene is the mouse homologue of the Drosophila segment polarity gene wingless and *int*-2 encodes a member of the fibroblast growth factor family.

In transgenic mice, expression of *int*-1 linked to a murine mammary tumour virus long terminal repeat as a transcriptional enhancer has dramatic effects on the growth of the mammary gland. Benign mammary hyperplasias develop in both male and female animals and mammary tumours appear after long periods of latency, in mice of both sexes. Nevertheless, the authors comment that although all mammary epithelial cells express high levels of *int*-1, the number of mammary tumours which develop is small, implying that there are other factors which contribute to what is presumably a multistage process (43).

A similar study with similar results has been performed with enhanced expression of c-*myc* in transgenic mice. Several strains of transgenic mice bearing the murine c-*myc* gene fused to the long terminal repeat of the mouse mammary tumour virus have been bred (44). A high level of expression of the *myc* transgene was observed only in the mammary gland with minor expression in the salivary gland and small intestine. Their results suggested that deregulated *myc* expression was necessary, but not sufficient in isolation, to ensure mammary tumour production.

Compared with colon or lung carcinomas where mutant *ras* genes are present in 30% or more of tumours, in breast cancer, *ras* gene mutation and activation is of low incidence (10% or less). A recent survey has shown that about 30% of primary breast tumours contain c-*myc* amplification but this does not correlate with clinical behaviour or other indices of biological behaviour (45). Therefore, no clear picture has emerged for the biological role of these oncogenes. If, as seems likely, multiple genetic changes are required during carcinogenesis, it will be difficult to separate important changes from epiphenomena. The studies on murine mammary carcinogenesis show that oncogenes are important in the early stages of tumour induction and that no one model will provide a single, adequate answer. More work is required to elucidate the nature of the secondary or cooperating events during mammary carcinogenesis in these murine model systems and then apply these to the molecular events occuring in the clinical setting.

Another group of genes involved in carcinogenesis, termed onco-suppressor genes, shares the property that their expression suppresses the oncogenic phenotype of cancer cells (46). The study of phenotypic reversion of transformed cells, cell fusion experiments with normal/ malignant cells and ultimately transfer of chromosomes or cloned DNA sequences, has given great insight into the function of onco-suppressor genes.

Following early epidemiological studies, Knudson developed a 2 hit theory which proposed that the development of the malignancy required 2 mutational events (47). He recognised that retinoblastoma had a hereditary predisposition and proposed that in bilateral reinoblastoma a mutant gene was passed in the germline such that all cells in the body contained a mutation. A second mutation in the other allele of the same gene in the embryonic retina would lead to tumour formation. For the unilateral or sporadic form of the disease which has no familial predisposition, he suggested that somatic mutations affecting both alleles occurred in the same retinoblast. The retinoblastoma gene, located on chromosome 13q14 has been isolated and cloned.

Cytogenetic studies in breast cancer indicate that a number of chromosomal deletions have been described involving: 11p14; chromosome 13; chromosome 17p. Several cancers seem to involve defects in the same chromosomal location as breast cancer (eg colorectal carcinoma, bladder carcinoma, osteosarcoma, small cell lung cancer and Wilm's tumour).

The mechanism by which the protein products of onco-suppressor genes suppress the malignant phenotype is not known, but some preliminary evidence suggests that the 105 kDa product of the retinoblastoma gene interacts with the human adenovirus 5 1 EIA protein, which is synthesised early in viral infection and together with the protein EIB is required for transformation of cells. The regions of the EIA that are required for transformation are precisely those that interact with the retinoblastoma gene product (48).

The situation in breast cancer is less clear but as mentioned, a significant number of relevant chromosomal deletions has been described. It is likely that there will be significant interactions between oncogenes and onco-suppressor genes implying that the malignant phenotype will be maintained by the interplay of a range of genetic factors.

SECTION 1.3 REVIEW OF BIOLOGICAL FUNCTIONS OF TGF-β AND THEIR RELEVANCE IN BREAST CANCER

TGF-β, a polypeptide consisting of 2 subunits each of 112 amino acids, was first identified by its ability to cause phenotypic transformation of rat fibroblasts using a soft agar colony forming assay (49-51) and was cloned in 1985 (52). It is now known to comprise a super gene family with 4 closely related members (TGF-β, TGF-β_2, TGF-β_3, and TGF-β_4) identified by gene cloning and sequencing techniques, which have similar and overlapping biological functions (53). It is clear that there is a high degree of conservation of amino acid sequence of TGF-β on comparing TGF-β's from different species which implies that it is a molecule which is likely to be important to the cell's economy in evolutionary terms. This is borne out by the range of regulatory activities which TGF-B has in a large number of diverse cell types, both normal and neoplastic.

A series of independent findings has revealed other factors which are structurally and functionally related to TGF-β. They include activins (54), inhibins (55) and the Mullerian inhibitory substances (56) in mammals, as well as the product of the decapentaplegic gene complex of Drosophila (57).

The homologies of these proteins with each other reside mainly in the C-terminal domains of their precursors which are generally cleaved to form active dimeric proteins. The conservation of multiple cysteines involved in intrachain and interchain disulphide bond formation is marked and presumably underlies the structural importance of these moieties.

TGF-β is a dimer (molecular weght 25,000) which can be reduced to two subunits which are not individually biologically active. It is stable under acidic conditions, and this forms the basis for its purification from platelets (51). The gene has been cloned (52) and it is apparent that the monomer of 112 amino acids is derived by proteolytic cleavage from a precursor protein of 391 amino acids, of which the monomer is cleaved from the C-terminal end of the protein. The precursor is rapidly cleaved to active monomer under acidic conditions, but the physiological mechanism whereby TGF-β is activated is unclear (58). This is obviously an important potential site for the regulation of TGF-β's activity and more research is required in this area.

TGF-β binds to specific, high affinity cell surface receptors, which are fairly ubiquitously expressed (58). Massague and coworkers (59) have used an affinity labelling method with radio-iodinated ligand to identify TGF-β receptors. They have shown that there are 3 cell surface glycoprotein receptors for TGF-β.

Two of the three cell-surface receptors displayed by many cells, glycoproteins of 65 kd and 85 kd have an affinity for TGF-β_1 that is 10-fold higher than for TGF-β_2; the functions of these 2 receptors nevertheless remain unknown. The third receptor type is a 280 kd glycoprotein which does not discriminate between the various TGF-β molecules, but has been implicated in the mediation of a range of cellular effects of TGF-β. The post receptor signal transduction pathways which are activated by binding of TGF-β to its receptor have not been fully characterised but there is some evidence to suggest that it stimulates an increase in cellular inositol triphosphate levels which is maintained for several hours (59).

TGF-B has numerous effects on cell function which are relevant to mammary carcinogenesis. The original studies describing the discovery of TGF-β measured its ability to stimulate proliferation of NRK fibroblasts in soft agar.

In a cell of mesenchymal origin, whether TGF-β stimulates or inhibits cell proliferation is a function of the entire set of growth factors which are operant within the microenvironment of the cell. For example, TGF-β stimulates the growth of c-*myc* transfected fibroblasts in the presence of PDGF (60), whereas similar concentrations of TGF-β inhibit fibroblast proliferation in the presence of EGF (61).

TGF-β stimulates mesechymal cells to synthesise and secrete components of intercellular matrix such as laminin, fibronectin and collagen (62).

TGF-β is one of the most potent inhibitors of epithelial cell proliferation yet discovered. Thus, it has been shown to inhibit the proliferation of normal mammary epithelium, breast carcinoma cell lines, bronchial epithelial cells, keratinocytes, colonic mucosal cells and other malignant epithelial cells in culture (reviewed in 63).

Although TGF-β has a direct anti-proliferative effect on endothelial cells in vitro (64), it is an angiogenic substance in the intact animal due to its chemotactic effect on macrophages and other migratory cell types which themselves release strongly angiogenic substances (65).

From the point of view of tumour progression, TGF-β could be considered "anti-oncogenic" in that it has been shown that some malignant cells derived from TGF-β sensitive epithelial cells have lost their ability to be inhibited by the peptide (66). A related phenomenon has been reported for the A549 human lung alveolar carcinoma as it has been shown to secrete TGF-β in its precursor, latent form, suggesting that the cells have lost the ability to proteolytically activate the peptide (63).

Taking this information in concert, one could propose that the ideal tumour cell would synthesise and secrete TGF-β, which would help to support a fibro-vascular stroma required for tumour growth via paracrine effects on fibroblasts and angiogenic substance secreting cells, but not respond to the antiproliferative properties of TGF-β by either receptor deletion or loss of the TGF-β activating mechanism.

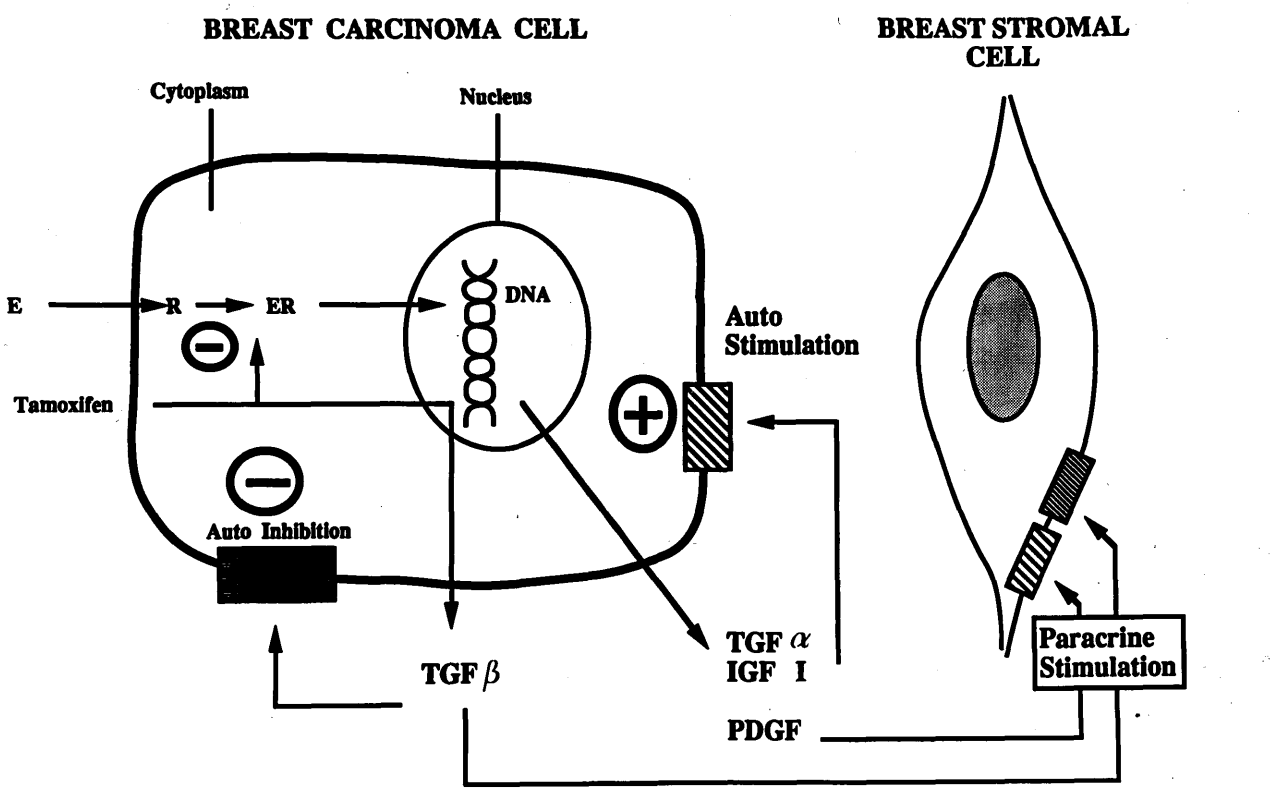


FIGURE 1

Role of TGF-β in regulation of cell proliferation (autoinhibitory loop) and epithelial/ stromal cell interaction (paracrine loops).

SECTION 1.4 THE BIOLOGICAL EFFECTS OF INTERFERONS IN BREAST CANCER

There are 3 major classes of interferons - leukocyte or alpha interferon (α-IF); fibroblast or beta interferon; and immune or gamma interferon. New insights have been gained into their biochemistry, molecular biology and clinical mechanism of action because large quantities of purified interferons are available for study through the application of recombinant DNA technology.

The interferons have a huge range of biological effects (for review see 67) including antiviral properties and immmunological effects such as stimulation of the cytotoxic activities of lymphocytes, macrophages and natural killer cell activity. The interferons have also been shown to alter cell surface expression of class I and class II major histocompatability antigens and modulate cellular differentiation. These and other observations indicate that many of the effects of the interferons can be dissociated and are due to different molecular mechanisms.

The direct antiproliferative effect of interferon was first reported by Paucker (68). Despite preliminary studies on the distribution and affinity of interferon receptors, there are no general rules to predict which cells will be inhibited and which will not.

In general, empirical results of antiproliferative and antitumour activity have been employed to use interferon as an antitumour agent although thee mechanism underlying the direct cytostatic effects of the interferons is unknown.

In breast cancer, α-IF has significant antiproliferative effects in a range of mainly ER positive cell lines (69). This prompted a series of phase II clinical studies using interferon as single agent in the treatment of advanced breast cancer. These initial trials recruited relatively small numbers of patients, but it did appear that α-IF did have useful clinical activity. However, more recent randomised prospective trials have failed to confirm its early promise and most experts feel that on balance, IF has only limited activity in advanced breast cancer and is less efficacious than conventional hormone and cytotoxic therapy (70).

Fibroblast interferon has been administered to patients with advanced breast cancer and induced a reduction in the size and number of skin metastases. The number of patients treated was small but it is interesting to note that the investigators biopsied skin metastases in 6 patients and found that interferon increased expression of ER in these metastatic nodules (71).

There is interest in the potential for a combination of α-IF and conventional horome treatment for breast cancer in an aduvant setting as there is some evidence to suggest that the interferons are more active in clinical situations with minimally low cancer burdens.

SECTION 1.5 AIMS OF THESIS

The aims of this thesis are:

1. To determine the role of TGF-β in controlling proliferation of human breast cancer cells in vitro.

2. To determine whether α-IF has a significant cytostatic effect on human breast cancer cells in vitro.

3. To determine the mechanism of action of α-IF's induction of cytostasis.

4. To determine the effect of oncogene transfection (c-*myc* and H-*ras*) on epithelial response to exogenous growth factors.

TABLE 1

GROWTH FACTORS AND THEIR RECEPTORS DEMONSTRATED ON HUMAN BREAST CANCER CELLS.

GROWTH FACTOR			RECEPTOR	
AGENT	RELATED ONCOGENE PRODUCT	BREAST CELLS	RELATED ONCOGENE PRODUCT	MODE OF ACTION
PDGF	*sis*	absent	none	paracrine
TGF-α	none	present	*neu*/*erb*-B	autocrine/ paracrine
TGF-β	none	present	none	autocrine/ paracrine
IGF-I	none	present	none	autocrine/ paracrine
Oestradiol	none	present	*erb*-A	endocrine

CHAPTER II

MATERIALS AND METHODS

SECTION 2.1.1 MATERIALS

All chemicals used were supplied by BDH Chemicals Ltd., Poole, Dorset unless otherwise stated.

REAGENT	SUPPLIER
Acrylamide	Sigma Chemical Co., PO Box 14508, St Louis, MO 63178, USA
Agar	GIBCO Laboratories, Paisley, Scotland
Anti TGF - β Antibody	R & D Systems Inc., 614 M^{c}Kinley Place, Minneapolis, MN 55413, USA
Bio-Gel P-60 column packing	Pharmacia Ltd. (Great Britain) Prince Regent Rd., Middlesex
Bio-Rad Protein Assay Kit	Bio-Rad Laboratories, 27 Holmesdale Rd., Bromley, Kent
BSA	Sigma Chemical Co.

DMEM Medium (10x)	Flow Laboratories, PO Box 17 Second Avenue, Irvine Industrial Estate, Ayrshire
Ecoscint	National Diagnostics Ltd., Manville, New Jersey, USA
EGF	R & D Systems Inc.
Foetal Calf Serum	Flow Laboratories
Geneticin	Schering Corporation, Kenilworth, NJ 07033, USA
Glutamine (200mM)	Flow Laboratories
Hank's Balanced Salts Medium	Flow Laboratories
HEPES	Flow Laboratories
α-interferon	Schering Corporation

Isotopes: ^{3}H - Thymidine	The Radiochemical Centre
^{32}P - dCTP	Amersham, Bucks.
^{125}I - Sodium iodide	
Mercaptoethanol	Sigma Chemical Co.
Nitrocellulose Membranes	Gene Screen Hybridisation, NEN Research Products, Boston, USA
Pepstatin	Sigma Chemicals Co.
Phenylmethylsulphonyl-fluoride	Sigma Chemicals Co.
Random Primed DNA Labeling Kit	Boehringer Mannheim Corp., Bell Lane, East Sussex
Sephadex G-50	Pharmacia Ltd.
Tissue Culture Flasks etc	GIBCO Laboratories
Trypsin	Flow Laboratories

SECTION 2.1.2 TISSUE CULTURE AND CELL LINES

The cells were all maintained in HEPES buffered Dulbecco's DMEM supplemented with 10% FCS (deionised distilled water, 400ml; DMEM (10x), 45ml; 1M HEPES pH 7.3, 9 ml; sodium bicarbonate 7.5%, 2.5 ml; adjust to pH 7.4 with sodium hydroxide; glutamine (200mM), 5 ml; FCS, 50 ml). The cells were subcultured every week, when in use, whilst in the late exponential phase of growth, following a brief exposure to 0.25% trypsin. The cell lines used in this thesis are described below.

ZR-75 cell line: a human, ER positive breast carcinoma cell line derived from the pleural effusion of a patient with metastic breast cancer obtained from the European Collection of Animal Cell Cultures, Porton Down, Salisbury, England.

MDA-41 cell line: a human ER negative breast carcinoma cell line obtained from the European Collection of Animal Cell Cultures.

NRK-49 F cell line: a normal rat kidney fibroblast cell line obtained from the European Collection of Animal Cell Cultures.

CCl-64 cell line: a mink lung epithelial cell line obtained from the European Collection of Animal Cell Cultures

SECTION 2.2 PURIFICATION OF TGF-B :

SECTION 2.2.1 PLATELET EXTRACTION

Rationale : This section deals with the methods developed to purify TGF-B for subsequent experimental use.

Platelet concentrates (30-40 units, 2-5 days old) were obtained by courtesy of the Blood Transfusion Service (Law Hospital, Scotland) and centrifuged (3200 x g, 30 min., $0^{o}C$) to remove remaining plasma proteins. The platelets were washed twice by resuspension in 500ml portions of Tris-HCl/citrate buffer and centrifuged as described. (Washing buffer: 9 vol of 17mM Tris-HCl, pH 7.5/0.15M NaCl/0.1% glucose and 1 vol of acid/citrate/ dextrose buffer - this was prepared according to National Institutes of Health formula A: 8g citric acid monohydrate/22g of dextrose (anhydrous)/26g of sodium citrate (dihydrate) made up to 1 litre with distilled water). Washed platelets (30-40g weight) were added to a solution of acidic ethanol (375ml of 95% (vol/vol) ethanol and 7.5ml of concentrated HCl, plus 33mg of phenylmethyl sulphonyl fluoride and 1.9mg of pepstatin as protease inhibitors) and immediately extracted in a Dounce homogeniser (4ml of ethanol/g of platelets).

After incubation overnight at 4^0 C, precipitated proteins were removed by centrifugation (10,000 x g, 10 min, 0^0 C) and the resulting supernatant adjusted to pH 3 by addition of concentrated ammonia. Proteins and TGF-β activity were precipitated from the solution (overnight at 4^0 C) by addition of ethanol (2 vol, 0^0 C) and ethyl ether (4 vol, 0^0 C). The precipitate was collected by centrifugation and suspended in 1M acetic acid. TGF-β activity was solubilised by an overnight extraction at 4^0C. Centrifugation clarified the solution; the supernatant was freeze dried or subjected directly to gel filtration. The amount of protein in the extract was determined by reaction with Coomassie blue (Bio-Rad Protein Assay) using bovine serum albumin as reference. In summary, 200μl of Bio-Rad protein assay solution was aded to 775μl of distilled water and 25μl of the test protein solution. Appropriate standards were prepared with BSA and standard curves were made plotting reference protein concentration against optical density at 595nm (Beckman-Dickinson Spectrophotometer, High Wycombe, Bucks). Protein concentrations in the unknown samples were derived by extrapolation from the standard curves.

SECTION 2.2.2 PURIFICATION OF PLATELET DERIVED TGF-B:

The solubilised platelet extract (10ml in 1M acetic acid) was gel filtered at a flow rate of 20ml/hr on a column (4.4 x 115cm) of Bio-Gel P-60 equilibrated in 1M acetic acid. Fractions containing 5ml were collected. The elution position of TGF-β was determined by bioassay (as described below) and the fractions containing the peak of activity were pooled and freeze dried. The amount of protein in the pool was determined as above. The freeze dried residue was solubilised in 50mM acetic acid and injected onto a Mono-Q anion exchange column (4 x 40cm; Pharmacia Ltd), equilibrated in 50mM acetic acid with buffer flow rate of 60ml/hr. A linear gradient 0-1M NaCl was introduced and 2ml fractions were collected. Individual fractions were assayed for TGF-β activity. Fractions (2ml) containing the peak of TGF-β activity were pooled and freeze dried. The residue was dissolved in 4mM HCl and prepared for a final HPLC clean up. 1ml samples were run on a C-18 microbondapack column equilibrated in 0.05% trifluoroacetic acid. The flow rate was 20ml/hr and a stepped gradient (15-40%) of acetonitrile was introduced. The 1ml samples corresponding to the peak of biological activity were freeze dried. Selected amounts of purified TGF-β were dried under vaccuum and analysed by SDS-polyacrylamide Gel (0.75mm, 12.5% acrylamide) run at 125V and stained with silver (Bio-Rad Diagnostics) under reducing (5% B-mercaptoethanol in the sample buffer) and non-reducing conditions.

SECTION 2.3.1 BIOASSAYS FOR TGF-β:

SOFT AGAR COLONY FORMATION.

Rationale: This section describes the bioassays which were developed for TGF-β. They were used to determine eluted biological activity during TGF-B purification.

This bioassay for TGF-β determines the ability of the polypeptide to induce anchorage-independent growth of non-neoplastic NRK-49 fibroblasts by measuring the formation of colonies of cells in soft agar (72). Agar underlays (0.6% in 0.5ml of tissue culture medium) were poured in 35mm petri dishes and allowed to set.
EGF (2.5ng per dish) and sterilised samples which had been freeze dried following column chromatography were added in 10μl of 4mM HCl. NRK-49 fibroblasts (5×10^5) were added in 0.3% agar in culture medium to the petri dishes. The plates were then incubated at 37^0C for 2 weeks in a humidified 5% CO_2 atmosphere without further feeding. The number of colonies was counted, unfixed and unstained at two weeks using phase contrast microscopy. Control plates with known quantities of commercially available TGF-β were also set up to determine a dose response curve.

One unit of TGF-β activity is defined as that biological response resulting in 50% maximal colony formation (colony size $\geq$ 3000 m^2) in the presence of EGF (2.5ng/ml). The maximal response of the assay is about 2500-3000 colonies/dish.

SECTION 2.3.2 BIOASSAY FOR TGF-β: INHIBITION OF INCORPORATION OF ^{3}H-THYMIDINE

This bioassay for TGF-β depends on its ability of inhibit the incorporation of ^{3}H-thymidine by mink lung epithelial cells in a dose dependent manner (73).

The mink lung epithelial cells (CCL-64 line) were seeded into 24 well plates at initial density of 5×10^{4} cells and incubated at 37^{0} C in a 5% CO atmosphere. After 1 hour the column samples to be tested were added and the cells incubated overnight for 22 hours. After 22 hours, 0.5μCi ^{3}H-thymidine (80Ci/mmol) was added to each well and incubated at 37^{0} C for 2 hours. The cells were fixed in 1ml of methanol/acetic acid (3:1 vol/vol) per well, for 1 hour at room temperature. The cells were then washed twice with 80% methanol and allowed to dry. 0.5ml of 0.25% trypsin solution was added to every well. After 1 hour, 0.5ml of 1% SDS was added and 5 minutes later the resulting mixture transferred to vials for liquid scintillation counting (Beckman Dickinson Scintillation Counter). Results were expressed as the % inhibition of incorporation of ^{3}H-thymidine relative to control (untreated) cultures. Known quantities of TGF-β were added to prepare a dose response curve.

SECTION 2.4 THE EFFECT OF α-IF AND TGF-β ON PROLIFERATION OF BREAST CANCER CELLS

Rationale: Basic experiments were performed to determine if α-IF and TGF-B have direct antiproliferative effects on ER positive and ER negative breast cancer cell lines.

Human breast carcinoma cells (ZR-75 and MDA-41 cell lines) were seeded at initial density of 5×10^3 cells/well in 24 well tissue culture plates in standard tissue culture medium. They were incubated at 37^0C in a 5% CO_2 atmosphere. The cells were exposed to α-IF (500iu/ml) or TGF-β (50pM) continuously, with a medium change (plus α-IF and TGF-β) every 2 days. Cells were harvested by a brief exposure to 0.25% trypsin, every 2 days and cell number counted using an electronic cell counter (Coulter Electronics Ltd., Hialeah, FL33010, USA). Control, untreated cultures were set up also to determine the normal growth characteristics of each cell line.

SECTION 2.5 ESTIMATION OF CELLULAR OESTROGEN RECEPTOR CONTENT

Rationale: There is some ambivalent experimental evidence suggesting that α-IF can influence phenotypic expression of ER, both in vivo and in vitro. These experiments were undertaken to clarify this situation and to extend the observations on the mechanism of direct cytostasis induced by the interferons. As oestradiol is an important trophic hormone for ER positive breast cancer cells, reduction in expression of ER could lead to reduction in the cell's ability to respond to the mitogenic oestradiol.

Measurement of ER concentration was made in late exponential phase for ZR-75 cells. The cells were grown in standard culture medium in 25cm^2 tissue culture flasks. The ZR-75 cells were exposed to α-IF (500iu/ml) or TGF-β (50pM) for varying periods of time (up to 96 hours), washed with ice cold phosphate buffered saline and harvested by scraping with a rubber policeman prior to ER estimation. Six flasks were treated at each duration of exposure to α-IF.

The ER assays were performed by Dr R E Leake, Department of Biochemistry, University of Glasgow and were based on standard Scatchard analysis of a ^{3}H-oestradiol competitive binding assay using 100-fold excess, unlabelled diethylstilboestrol to eliminate non-specific binding.

The results were expressed as fmol ER protein per mg cellular DNA. The inter and intraassay coefficients of variation were less than 10%.

SECTION 2.6 STUDIES WITH AN ANTI-TGF-β ANTIBODY

Rationale: These experiments were undertaken to test the hypothesis that TGF-β secretion mediates the cytostatic effect of α-IF. If this hypothesis is correct, co-incubation with α-IF and an antibody which will biologically neutralise TGF-β should reduce the antiproliferative effects of α-IF.

A commercially available polyclonal antibody against TGF-β was tested for its ability to neutralise the biological effects of TGF-B in the mink lung epithelial bioassays (section 2.3.2). TGF-β (1ng) was incubated with increasing concentrations of the antibody (1-50μg) for 2 hours at room temperature prior to addition to the CCl-64 cells with subsequent processing as previously described.

In subsequent experiments, ZR-75 cells were treated with anti-TGF-β antibody (50μg/ml) and α-IF (500iu/ml) for 4 days and cell number determined at intervals as described in section 2.4.

SECTION 2.7 ASSAY FOR TGF-β RECEPTORS

Rationale: These experiments were performed to estimate the number and binding affinity of TGF-β receptors on the ZR-75 cell surface.

The chloramine T method was used for iodination as the biological activity of the radioiodinated protein tends to be maintained with this procedure. 5μg of TGF-β was placed in a siliconised Eppendorf tube. 10μl of 1.5M potassium phosphate (pH 7.4) was added, followed by 0.75mCi sodium iodide^{125}. Then 3 x 5μl aliquots of chloramine T (0.1mg/ml), in 50mM sodium phosphate buffer (pH 7.4) was added, separated by 2 minutes to allow reaction to occur. Then 20μl of 50mM N-acetyl tyrosine was added, mixed and allowed to stand at room temperature for 2 minutes. This was followed by 200μl of 60mM potassium iodide and 200μl of 8M urea (ph 3.2). The reaction mixture was transferred to a Pharmacia disposable column (20 x 0.7cm) loaded with sephadex G-50 and equilibrated in column buffer (4mM HCl, 75mM NaCl, 0.1% BSA). The ^{125}I-TGF-β was eluted from the column and its biological and specific activity was determined (58).

The general procedure for the determination of cellular receptors for TGF-β depends on a competitive radiological assay using the radio-iodinated polypeptide.

ZR-75 cells were seeded in standard culture medium at 1.5-1.8 x 10^5 cells per ml (with 1 ml per well) in a 24 well plate, 24 hours prior to the binding experiment.

A series of samples were prepared containing "cold" and "radioactive" TGF-β.

Unlabelled TGF-β : 10ng of TGF-B stock solution in binding buffer. (8 doubling dilutions of stock solution was made.)
Radioactive TGF-B: ^{125}I-TGF-β stock was diluted to a final concentration of 100pM. ^{125}I-TGF-β (200μl) was added to each of the 8 dilutions of unlabelled TGF-β (200μl).

Twenty four hours after seeding, the cells were washed twice with 1 ml of binding buffer at room temperature. 200μl of mixture (i.e. ^{125}I-TGF-β + diluted unlabelled TGF-β) was added to each well. Duplicate samples for each dilution of unlabelled TGF-β were performed. The cells were incubated with the TGF-β for 2 hours at room temperature and were then washed with 1ml of Hanks/BSA (500ml of Hanks salts plus 500mg BSA) at 0^0 C.

Following this, the cells were solubilised by adding 750μl of prewarmed triton/glycerol/Hepes (2ml 1M Hepes; 1ml triton X 100; 10ml glycerol; 87ml water) to each well. They were placed in an incubator and held at 37^0C for 30 minutes. The solution (600μl) was then removed from each well and transferred to vials for counting by liquid scintillation (Beckman Dickinson Scintillation Counter). Appropriate controls (addition of binding buffer only) were present on each plate.

The data were analysed by the method of Scatchard using an in house computer programme based on the Marquhardt algor ythm.

SECTION 2.8.1 ISOLATION OF RNA AND NORTHERN BLOTTING

Rationale: α-IF has been shown to influence the rate of transcription and mRNA stability for a range of genes. This series of experiments was performed to determine the influence of α-IF on rates of transcription and steady state mRNA pools for genes of interest (ER and TGF-β) in breast carcinoma cells. This should allow definition of the molecular site of action of α-IF.

At varying intervals following treatment with α-IF (500iu/ml), subconfluent cultures of ZR-75 cells (approximately 10^7) were washed twice with ice cold phosphate buffered saline, trypsinised and collected by centrifugation (2,000 rpm for 5 minutes, 0^0 C). RNA was prepared from the cell pellet. 10ml of phenol equilibrated with 0.3M sodium acetate were added to the cell pellet, followed by a solution comprising 0.3M sodium acetate; 0.5% SDS; 5mM EDTA. The mixture was shaken in a rotary stirrer for 5 minutes and 10ml of chloroform was added and shaken for a further 5 minutes. The cellular extract was centrifuged at 3,000 rpm for 5 minutes at 4^0 C and the upper aqueous phase retained. This was re-extracted with 5ml of chloroform, shaken and centrifuged again. The aqueous phase was transferred and 2 volumes of ice cold ethanol were added and the resulting solution held at -20^0 C for 2-4 hours.

This was then centrifuged at 3,000 rpm for 60 minutes and the resulting pellet resuspended in 5ml DEPC treated water. An equal volume of 4M lithium chloride was added and kept at 4^0C overnight. The resulting solution was then centrifuged at 10,000 rpm at 4^0C for 30 minutes. The RNA pellet was resuspended in 250μl of 5mM magnesium chloride. DNA was degraded by the addition of DNA'se I (1μl of 20μg/ml) for 20 minutes at room temperature. The concentration of RNA in the sample was determined spectrophotometrically at OD_{260nm}.

The Northern blots were run according to Maniatis (73). The gels were prepared by melting 1g agarose in distilled water, cooled to 60^0 C and 10x concentrated gel running buffer (0.2M MOPS, pH 7; 50mM sodium acetate; 1mM EDTA, pH8) and formaldehyde were added to give a respective final concentration in the gel of 1x and 2.2M. The RNA samples were prepared by adding 20μg of RNA (in 4.5μl of water) to 2μl of 10x gel running buffer, 3.5μl formaldehyde and 10μl formamide. This mixture was heated at 55^0 C for 15 minutes. Two μl of sterile loading buffer (50% glycerol, 1mM EDTA, 0.4% bromophenol blue, 0.4% xylene cyanol) were added and the samples layered on to the agarose gel.

50ng of restriction fragments of DNA were used as molecular weight markers. The gel was run submerged in 1x gel running buffer at 1 V/cm at 4^{0}C overnight. The buffer was constantly recirculated using a peristaltic pump. The gel was washed in water and the RNA transferred to the nitrocellulose filter. The filter was wet in water, soaked for 5 minutes in 20 x SSC (3M NaCl and 0.3M trisodium citrate) and then placed in contact with the gel. The blotting buffer was 20 x SSC and RNA transfer was complete in 15-24 hours. The blotting procedure was as described in Maniatis (73). When RNA transfer was complete, the blot was dried at room temperature and baked in an oven at 80^{0}C for 2 hours.

For the RNA dot hybridisation assay, a stock solution of RNA in water (500μg/ml) was made. Eight doubling dilutions of 20μl of stock RNA solution were made in water. The samples were heated to 65^{0}C for 15 minutes, cooled on ice and condensation spun down in a microcentrifuge. Four μl samples were spotted onto a dry, nitrocellulose membrane (previously wet in water, soaked in 20 x SSC and air dried). After air drying, the membrane was baked at 80^0 C for 2 hours to immobilise the RNA.

cDNA probes for TGF-β (1.3 Kb insert from Sp65-C17N, supplied by R Derynck), ER (1.6Kb insert from OR3, supplied by P Chambon) and actin (Pst 1 insert cDNA of plasmid91, detecting alpha actin mRNA) were used. The DNA was labelled using a random primed DNA labelling kit (Boehringer Mannheim). The DNA sample was denatured by heating for 10 minutes at 95^0 C and subsequently cooled on ice. The following were added to an Eppendorf vial on ice:

25μg denatured cDNA

3μl dATP, dGTP, dTTP mixture

2μl hexanucleotide mixture in 10 x concentrated reaction buffer

5μl 50μCi [^{32}P] dCTP, 3000 Ci/mmol - made up to 19μl with sterile redistilled water

1μl Klenow enzyme

The mixture was mixed and incubated for 30 minutes at 37^0 C. The reaction was stopped by adding 2μl 0.2M EDTA (pH 8.0). The blots (Northern and dot blot) were sealed in polythene bags and prehybridised for 4 hours at 65^0 C in 10ml Church's buffer (0.5M Na_2HPO_4 (pH7.2); 7% SDS; 1mM EDTA; 1% BSA). The denatured, radioactive cDNA probes were added to the blots and incubated overnight at 65^0 C.

The blots had a succesive series of washes; 3 x SSC and 0.1%SDS, room temperature, 30 minutes; 1 x SSC and 0.1% SDS, 65^0 C, 1 hour.

The dried membranes were autoradiographed on XAR-5 film (Kodak Ltd) at - 80^0 C for 2 days. When required, the probes were eluted from the filters by washing in 0.1% SDS for 1 hour at 80^0 C. They were then reprobed with the appropriate cDNA for human actin which acted as a "house keeping" probe to determine the equivalency of amounts of mRNA added to the gels.

The autoradiographs were scanned using a densitometer (Elmer Perkins Ltd) and expressed with respect to hybridisation to the actin probe.

SECTION 2.8.2 NUCLEAR RUN ON ASSAY

Following treatment of ZR-75 cells in the late exponential phase of growth with α-IF (500iu/ml) for various periods of time (0, 0.5, 2, 12, 24 hours), the cells were washed 3 times with ice cold phosphate buffered saline. The cells (10^7) were harvested by a brief exposure to 0.25% trypsin and pelleted by centrifugation (3000rpm, 15 mins, 4^0 C). The cells were resuspended in lysis buffer (10mM Tris-HCl (pH 7.4); 10mM NaCl; 3mM $MgCl_2$; 0.5% (v/v) NP40) and held for 5 minutes on ice. The solution was spun at 500 x g for 5 minutes and the supernatant discarded. The nuclear pellet was washed by resuspension in 10ml of lysis buffer and spun again. The nuclear pellet was then resuspended in nuclear storage buffer (50mM Tris-HCl (pH 7.4); 5mM $MgCl_2$; 0.1mM EDTA; 40% (v/v) glycerol) in 100μl aliquots and stored at -80^0 C until used.

For the nuclear run off assay, each reaction consisted of 210μl of nuclei, 60μl of 5 x runoff buffer (5 x consists of 25mM Tris-HCl (pH 8.0); 12.5mM $MgCl_2$ 750mM KCl and 1.25mM triphosphates of adenosine, guanosine and cytosine). ^{32}P- labelled uridine triphosphate (6μl of 3000Ci/mM; 150μCi) was then added and the mixture was incubated at 30^0 C for 30 minutes, after which time 15μl of DNase I (5μg/ml) in 10mM $CaCl_2$ was added.

After 5 minutes at 30^0 C the reaction was made 1 x SET (1% SDS; 5mM EDTA; 10mM Tris-HCl 7.4)) and proteinase K was added to give a final concentration of 200μg/ml. After incubation at 37^0 C for 45 minutes, the solution was extracted with an equal volume of a mixture of phenol and chloroform and the interphase was again extracted with 100 l of 1 x SET. Ammonium acetate (10M) was added to the combined aqueous phases (original plus reextraction) to a final concentration of 2.3M and an equal volume of isopropyl alcohol was added and nucleic acid was precipitated (-70^0 C for 15 minutes). The precipitate was centrifuged in a microcentrifuge for 10 minutes and the pellet was resuspended in 100μl of TE (10mm Tris-HCl, 1mM EDTA) and centrifuged through a G-50 (medium) spin column. The eluate was made 0.2M in NaOH and after 10 minutes on ice, HEPES was added to give a final concentration of 0.24M. Two and a half volumes of ethanol were then added and the solution containing the precipitate held overnight at -20^0 C. After centrifugation in a microcentrifuge for 5 minutes, the pellet was resuspended in hybridisation buffer (10mM TES, pH 7.4, 0.2% SDS , 10mM EDTA, 0.3M NaCl, 1 x Denhardt's, and *Escherichia coli* RNA (250μg/ml)). Nitrocellulose filters containing plasmid DNA's were prepared with a Schleicher & Schuell Slot Blot Apparatus under conditions suggested by S and S, except that wells were washed with 10 x SSC. These filters were first hybridised in the hybridisation solution described above for a minimum of 2 hours at 65^0 C.

After this preliminary hybridisation, the filters were hybridised to the runoff products in hybridisation solution for 36 hours. A typical reaction contained 2 ml of hybridisation solution with 1×10^7 cpm/ml. After hybridisation, the filters were washed twice for 15 minutes in 0.1% SDS, 2 x SSC at room temperature and then washed at 60^0 C (0.1% SDS, 0.1 x SSC) for 30 minutes. Filters were then exposed to Kodak XAR film in cassettes at -70^0 for 2 days.

SECTION 2.9 TRANSFECTION OF MINK LUNG EPITHELIAL CELLS WITH ONCOGENE CONTAINING PLASMIDS.

Rationale: In view of the relationship between oncogene products and growth factor-receptor-signal transduction pathways, it was considered relevant to determine the effect of oncogene transfection of epithelial cell response to exogenous growth factors. Attempts were made to introduce the relevant oncogenes into ZR-75 cells but it proved difficult to select stable transformants whereas it did prove possible to derive stable oncogene transfectants of the mink lung epithelial cell line, which had previously been well characterised in terms of growth factor responsiveness. The cellular response to mitogenic or antiproliferative stimuli will be governed not only by its genotypic milieu, but also by the other growth factors operant in the cell's microenvironment. Therefore, further experiments were performed to determine ^{3}H-thymidine incorporation by the transfected cell lines following exposure to combinations of EGF and TGF-B.

The transfections described in this section were performed by Dr Demetrios Spandidos.

Using the calcium phosphate precipitation technique (74), recombinant DNA plasmids (figure 2) were introduced into the mink lung epithelial line (CCl-64), described in section 2.1.

The recombinant plasmids are shown in figure 2 and their construction has already been described (74-76). Basically, the plasmids consisted of the aminoglycoside phosphotransferase (aph) gene (which confers resistance to geneticin and therefore allows selection of geneticin resistant colonies in vitro), the proto-oncogene or oncogene of interest, flanked by transcriptional enhancers such as the long terminal repeat from the Moloney murine sarcoma virus or the enhancer from the SV40 virus. Following transfection, geneticin resistant colonies were grown after plating of 2×10^5 cells in soft agar (0.6% base coating , 0.3% cellular layer). Transfection efficiencies were expressed as the number of colonies grown per μg of plasmid DNA per 2×10^5 cells. Each derivative line was expanded from a single clone and named according to the plasmid with which they were transfected; HO6-N1 line transfected the normal Ha-*ras* 1 proto-oncogene; HO6-T1 line transfected with the human T24 activated Ha-ras 1 gene; MLMC1 line, transfected with the human c-*myc* proto-oncogene.

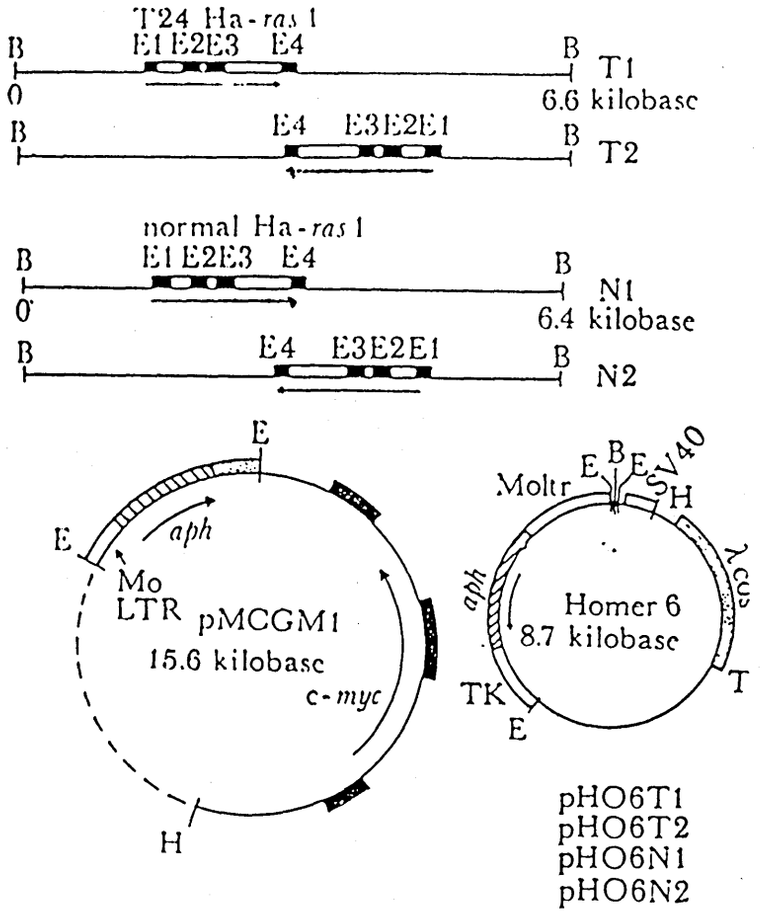


FIGURE 2

a) and b) Schematic representations of recombinant plasmids used in transfection experiments.

The derivative cell lines were characterised in the following ways:

Southern blotting and hybridization. Restriction enzyme cleaved DNA was transferred to Genescreen (NEN Research Products) hybridization membrane by vacuum blotting using the Vacugene (LKB Pharmacia) vacuum blotting unit. Hybridizations were performed according to Maniatis et al (73), using random primed ^{32}P-labelled probes at 42^0C in 50% formamide, 10% dextran sulphate, 0.1% SDS and 100μg/ml denatured salmon sperm DNA. Membranes were then washed at high stringency (0.1 x SSC, 0.1% SDS) at 65^0C for 1 hour with three changes of wash solution.

Growth curves were performed by seeding 1000 cells/ml in 1ml in the wells of 24 well plates, and counting by trypsinisation and electronic particle counting (Coulter Ltd) at daily intervals. The medium was changed at 3, 5, 7, 8 and 9 days.

Tumourigenicity was determined by injecting 1 x 10^6 cells subcutaneously in the flank of nunu immune-deficient mice. Lag time to the appearance of the first detectable lump was measured and the growth rate was estimated by caliper measurements at regular intervals.

Histology was performed on haematoxylin and eosin stained 10% formalin fixed 4 m sections.

Response of transfected cell lines to exogenous growth factors

The derivative cell lines were exposed to a range of concentrations of TGF-β (10-10,000pg) and EGF (10-10,000pg) in exactly the manner descibed in section 2.3.2. The results are expressed as the % ^{3}H-thymidine incorporation relative to untreated control cultures of the same cell line.

Additional experiments were performed with the parent CCl-64 cell line in which the cells were exposed to either a fixed concentration of EGF (1ng) and increasing concentrations of TGF-β(1-8ng) or a fixed concentration of TGF-β (1ng) and increasing concentrations of EGF (1-8ng). The cells were treated and processed as described in section 2.3.2.

CHAPTER 3

RESULTS

SECTION 3.1 PURIFICATION OF TRANSFORMING GROWTH FACTOR β

TGF-β was purified from the platelet extract by a three column procedure comprising gel filtration, ion exchange and HPLC. The crude acid-ethanolic extract of human platelets (about 100mg of protein from 25g of washed platelets) induced NRK fibroblasts to form colonies in soft agar. Typical colonies are shown in figure 3 and the dose response curve to purified, commercially available TGF-β in fig 4. These are highly refractile, by darkground microscopy, and contain > 40 cells. The crude extract elicited 1 unit of TGF-β activity at a protein concentration of 100ng/ml. Throughout TGF-β purification, protein concentrations were measured by the Bio-Rad colourimetric protein assay. Standard curves are shown in figure 5.

Gel filtration of the acid-ethanolic extract on Bio-Gel P60 in 1M acetic acid resulted in the profile in figure 6. The profile of eluted biological activity, assayed by NRK colony formation, is superimposed on figure 6. The biological activity of TGF-β eluted with an approximate molecular weight of 15000. Pooled TGF-β fractions (50-125 from figure 6) amounting to approximately 5mg total protein from the initial 100mg sample chromatographed, elicited 1 unit of biological activity at a protein concentration of 5-20ng/ml.

The pooled fractions were freeze dried, reconstituted in 50mM acetic acid and subjected to ion exchange chromatography on a Mono-Q column. The biologically active protein eluted in the void volume of the column (figure 7). The appropriate fractions were pooled and elicited 1 unit of activity at a protein concentration of 1ng/ml.

A final "cleaning up" procedure consisting of HPLC with a stepped gradient in trifluoroacetic acid/acetonitryl yielded a TGF-B preparation which elicited 1 unit of activity at a protein concentration of approximately 0.1ng/ml. The protein elution profile from the HPLC column is shown in figure 8. The relative concentration in TGF-β activity at each stage of its purification, is summarised in Table 2. Approximately 10ng of TGF-β was adjudged about 95% pure by silver staining of a SDS-polyacrylamide gel (figure 9).

The biological properties of purified, human platelet-derived TGF-β are summarised in Table 3. In the presence of EGF(2.5ng/ml), TGF-β elicits near maximal NRK colony forming activiity at 2ng/ml (figure 10). The biological activity of the growth factor was destroyed by reduction in mercaptoethanol; stimulation of NRK colony formation by an EGF/reduced TGF-β mixture was no greater than that of EGF alone.

TGF-β assayed in the absence of EGF was no better than 10% FCS alone. The purified TGF-B produced an identical dose-response curve (figure 11) to commercially available TGF-β by inhibiting incorporation of ^{3}H-thymidine by mink lung epithelial cells (the dose which produced 50% inhibition of ^{3}H-thymidine incorporation relative to control, ID_{50} = 120pg/ml).

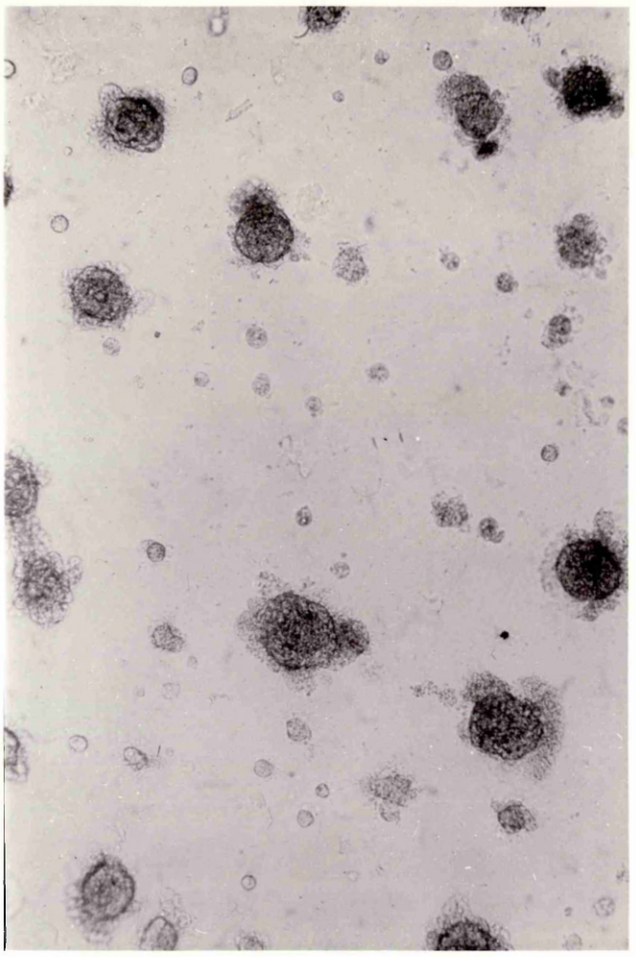


FIGURE 3

Photomicrograph of typical normal rat kidney (NRK) fibroblast colonies in soft agar (magnification, x 10).

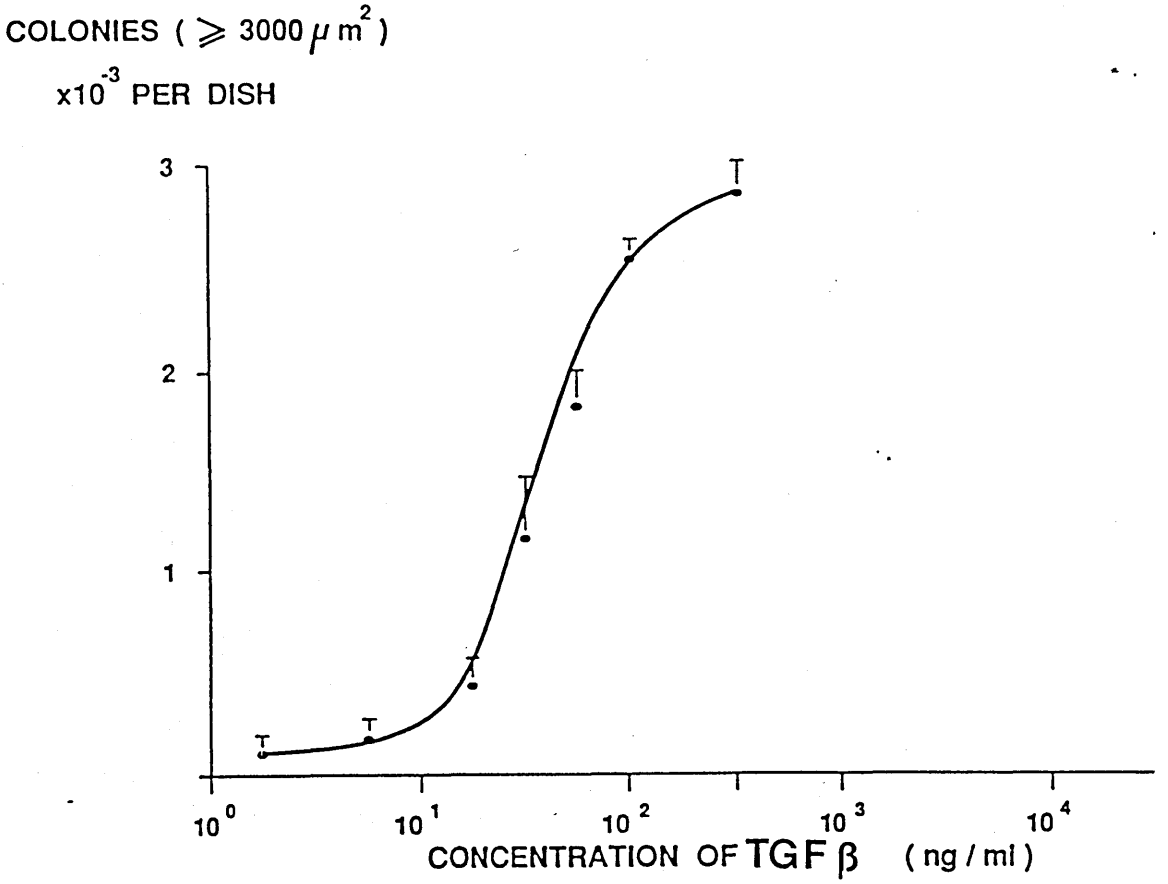


FIGURE 4

Dose response curve: number of NRK fibroblast colonies formed in response to exogenous, commercially purified TGF-β (in presence of EGF, 2.5ng/ml).

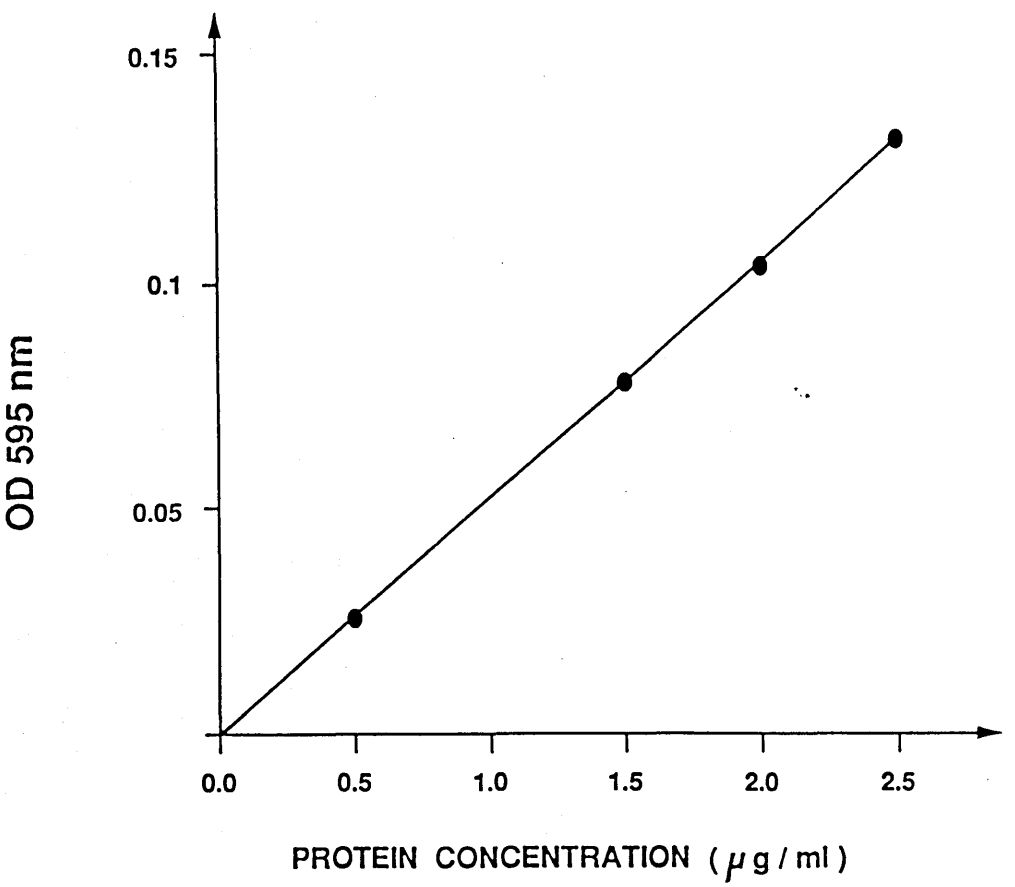


FIGURE 5

Standard curves for Bio-Rad protein assay.

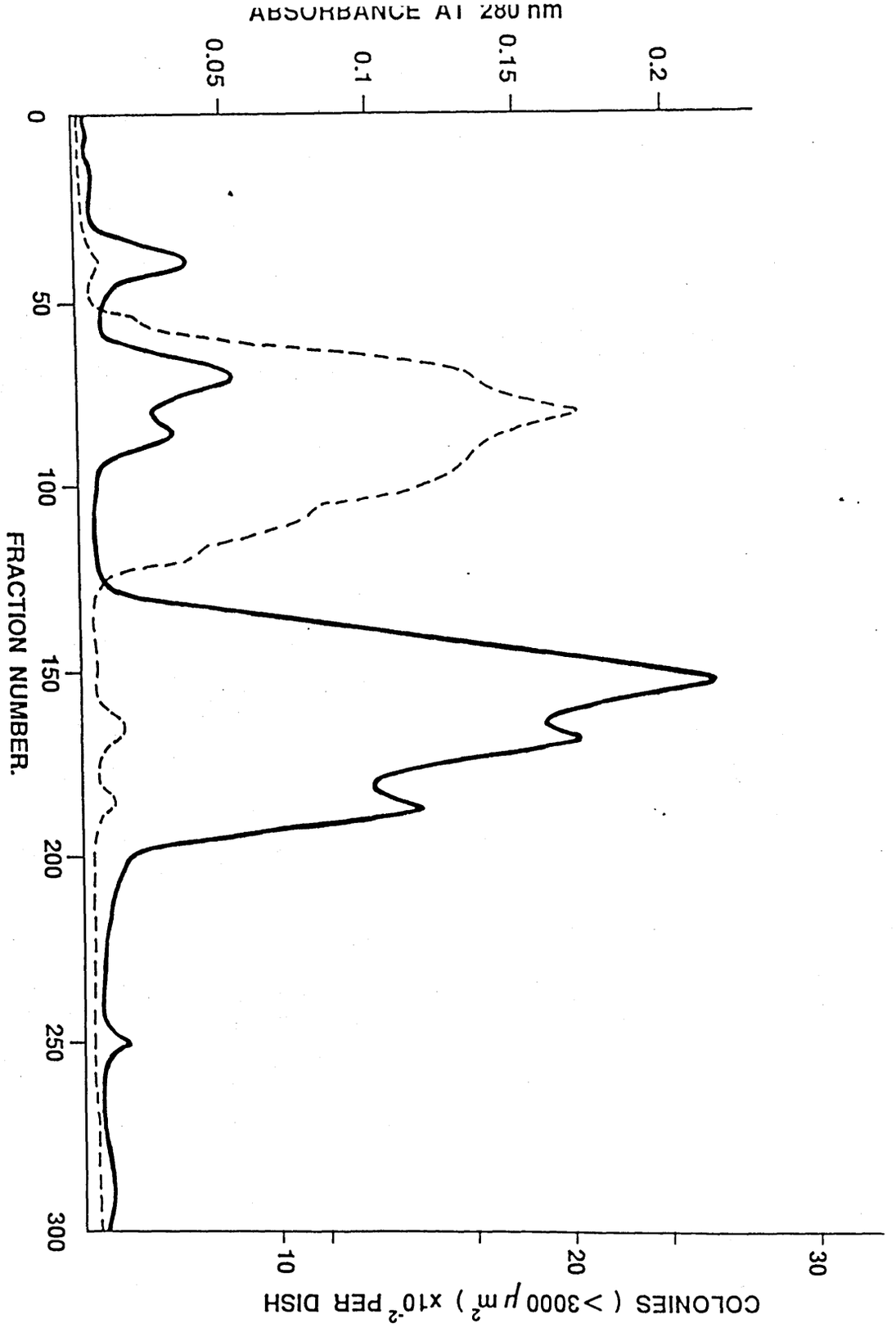


FIG 6

Protein elution profile from Bio-Gel P60 column (———). The number of colonies formed by addition of 50μg protein from each fraction is superimposed (- - - -).

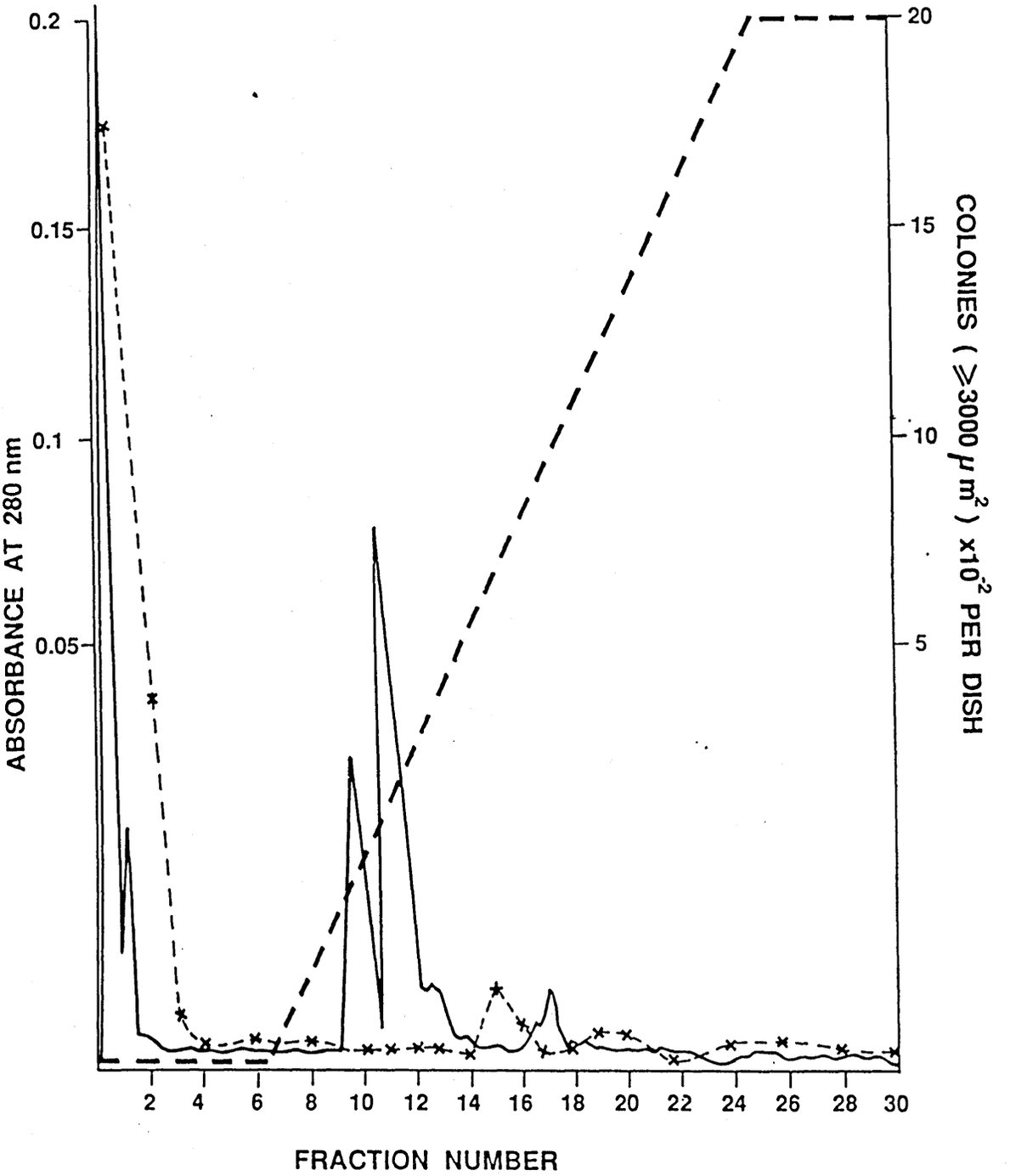


FIGURE 7

Protein elution profile from Mono-Q column (———).
The number of NRK colonies formed by addition of 50μg of protein from each fraction is superimposed (✳ - - ✳).

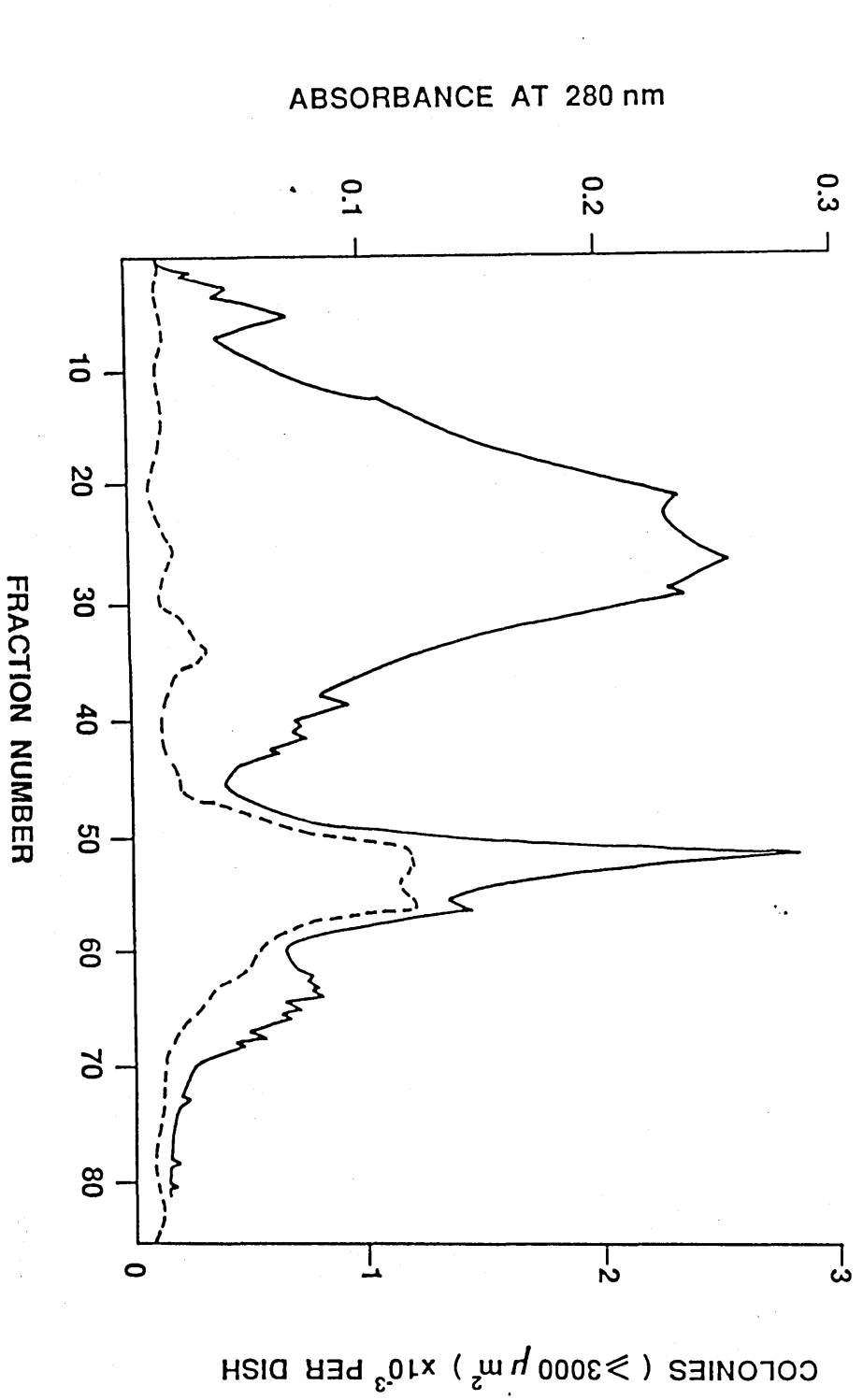


FIGURE 8

Protein elution profile from HPLC column (———). The number of NRK colonies formed by additon of 50 μg of protein from each fraction is superimposed (- - - -).

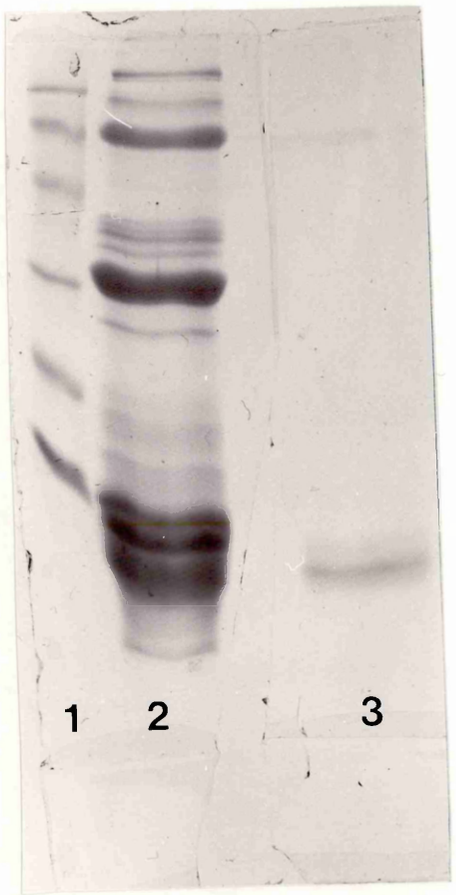


FIGURE 9

SDS-polyacrylamide gel electrophoresis of protein extracts at different stages.

Lane 1 Molecular weight markers

Lane 2 Pooled active fractions following bio-gel chromatography

Lane 3 Purified transforming growth factor-β (approximately 50ng)

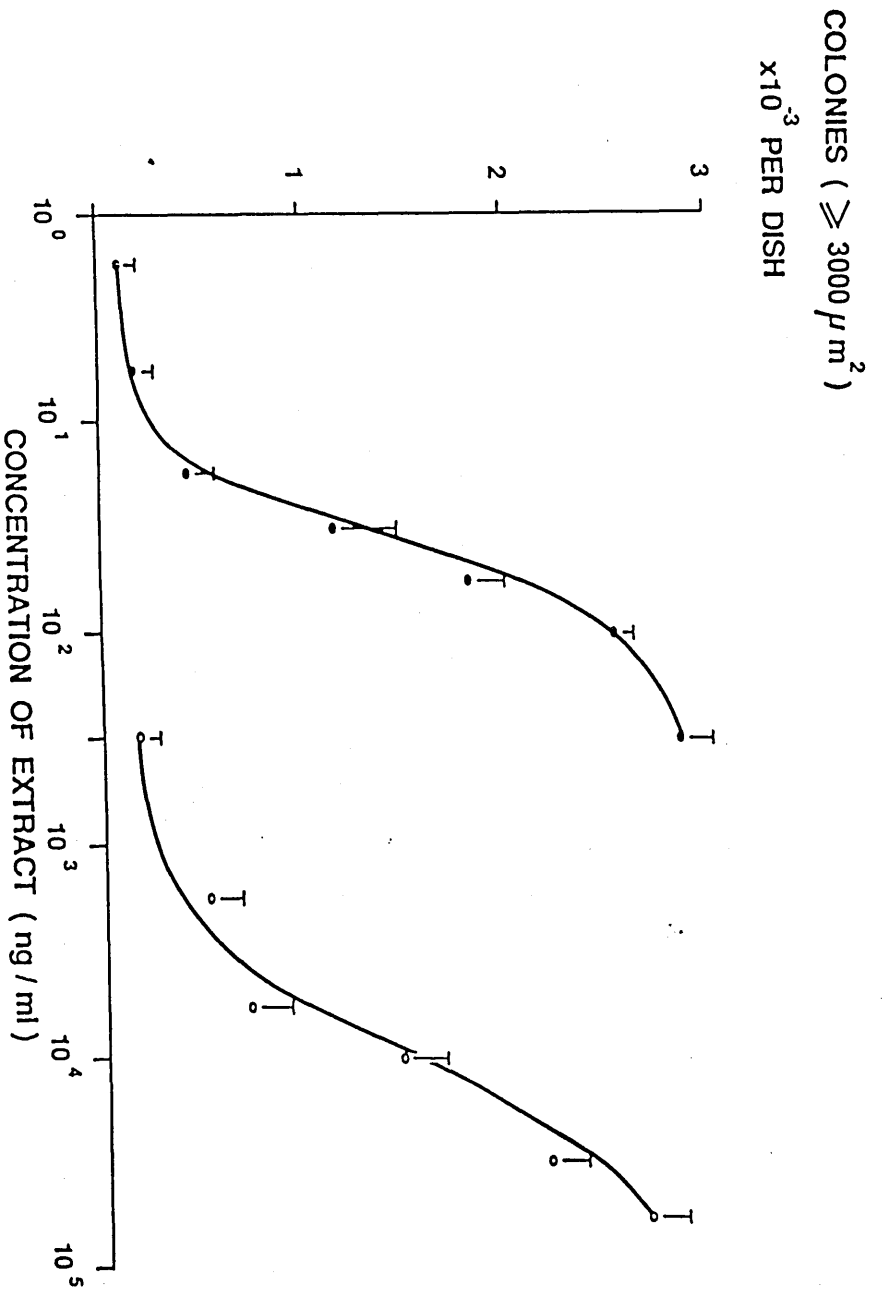


FIGURE 10

Dose response curve: number of NRK fibroblast colonies in soft agar formed in response to purified TGF-β alone(○) or in the presence of epidermal growth factor (2.5ng/ml) (●).

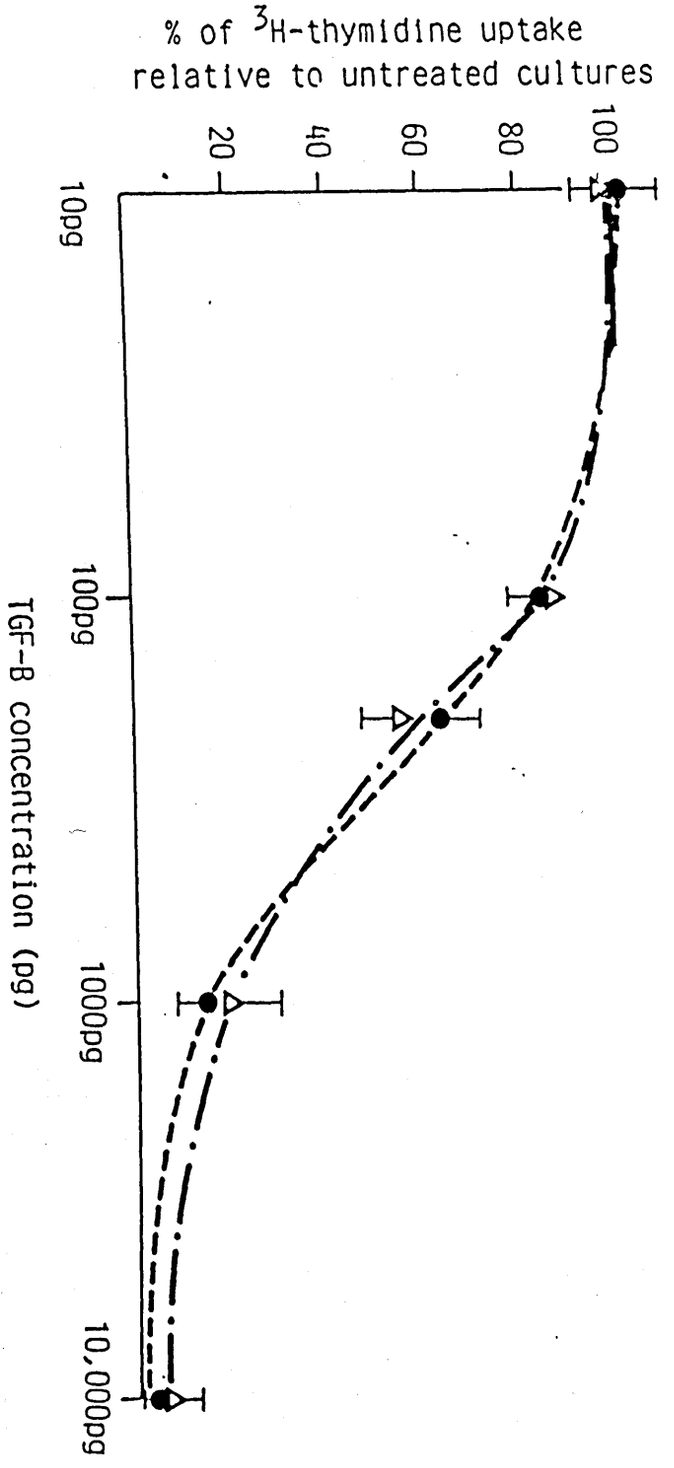


FIGURE 11

Dose response curve: % inhibition of ^{3}H-thymidine incorporation by CCl-64 cells in response to purified (●) and commercially available (△) TGF-β.

SECTION 3.2 THE EFFECT OF α-IF AND TGF-β ON THE PROLIFERATION OF BREAST CANCER CELLS

The ZR-75 cells grow exponentially with a doubling time of 28 ± 3 hours and MDA-41 cells with a doubling time of 26±4 hours.

α-IF (500iu/ml) has a significant cytostatic effect on ZR-75 cells (figure 12a). After an initial lag phase of about 12-18 hours, there is an anti-proliferative effect which persists during exposure to α-IF. When α-IF is removed from the medium, the cells start to regrow at a rate roughly parallel to control, after a lag phase of 18-24 hours.

TGF-β has a more profound cytostatic effect at a concentration of 50pM, but with similar kinetics. On removal of TGF-β from the medium, the cells start to regrow after a lag phase of 24-36 hours (figure 12a).

α-IF had no cytostatic effect on the ER negative MDA-41 breast cancer cell line. However, TGF-β (50pM) had a similar static effect as demonstrated in the ER positive line (figure 12b).

Similar experiments were performed with α-IF at a concentration of 10iu/ml, but no cytostatic effect was observed on either cell line.

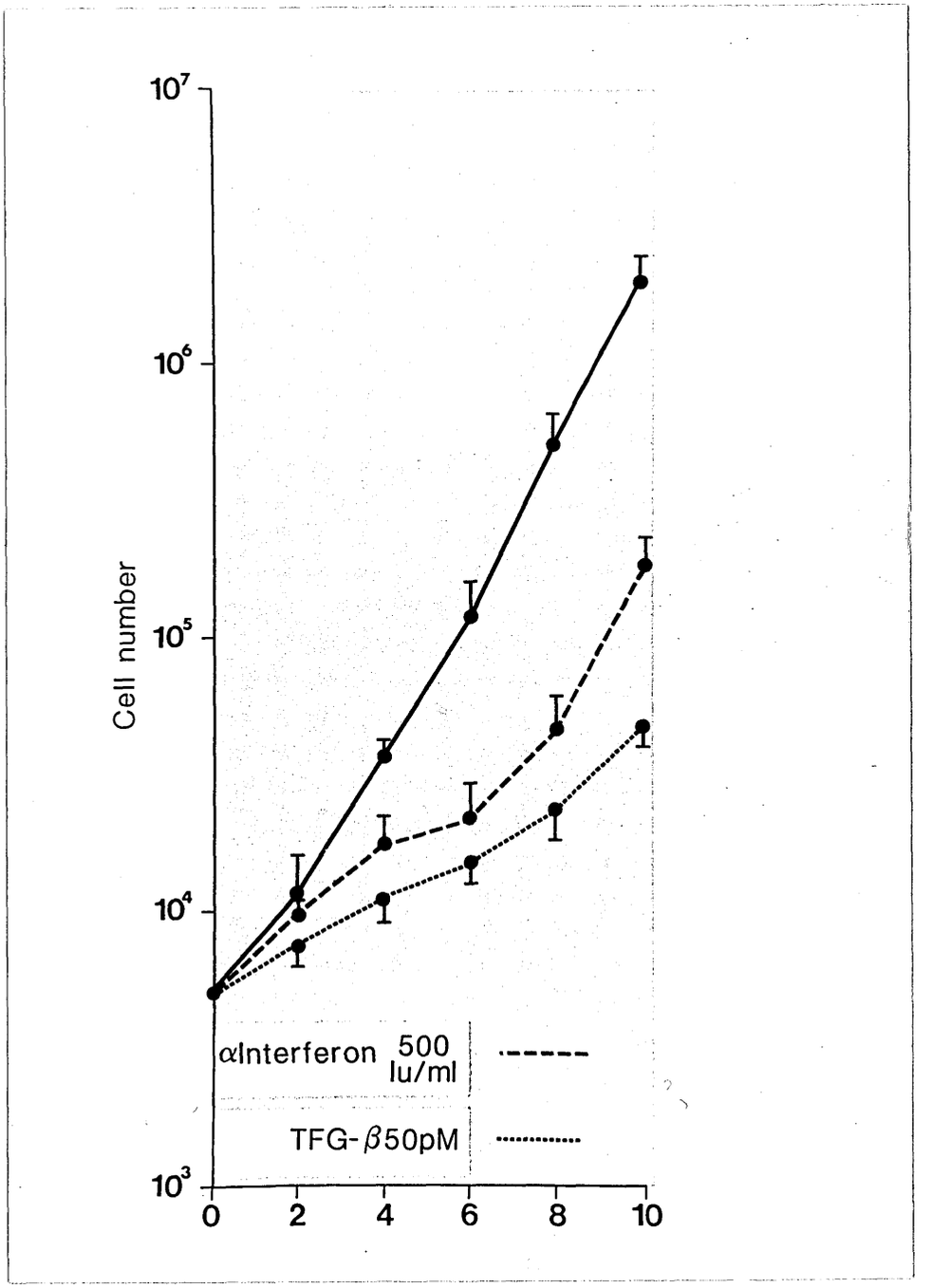


FIGURE 12(a)

The influence of α-interferon (α-IF) (500iu/ml) and TGF-β (50pM) on ZR-75 cell proliferation. The cells were exposed to each agent continuously for 6 days. Each point is the mean of 6 estimates and the vertical bar denotes 1 standard deviation.

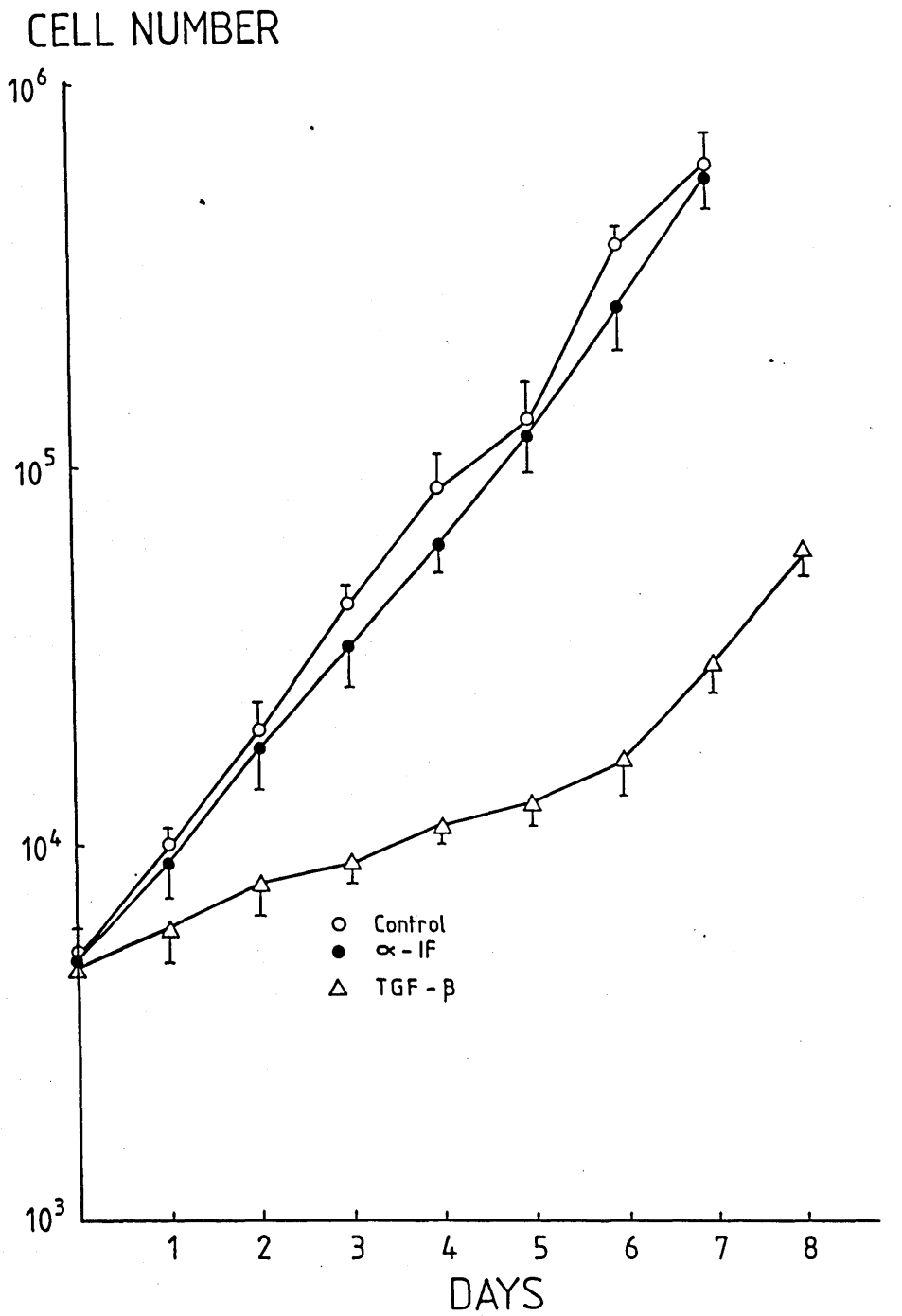


FIGURE 12(b)

The influence of α-interferon (α-IF) (500iu/ml) and TGF-β (50pM) on MDA-14 cell proliferation. The cells were exposed to each agent continuously for 6 days. Each point is the mean of 6 estimates and the vertical bar denotes 1 standard deviation.

SECTION 3.3 THE EFFECT OF α-INTERFERON ON CELLULAR OESTROGEN RECEPTOR CONCENTRATION

The mean cellular ER concentration was 105 ± 42 fmol/mg DNA.

Following treatment with α-IF, ER concentration fell to approximately 50% of control values by 24 hours and stayed at this reduced level for the duration of α-IF exposure (figure 13). When α-IF was removed from the medium, there was a lag period of 24 hours before ER concentrations rose to control values.

TGF-β (50pM) also decreased ER expression, but to a lesser extent than α-IF. ER concentration decreased to about 60% of control values by 24 hours after exposure to TGF-β, remained depressed but recovered to approximately control values (figure 14) when fresh medium without TGF-β was exchanged.

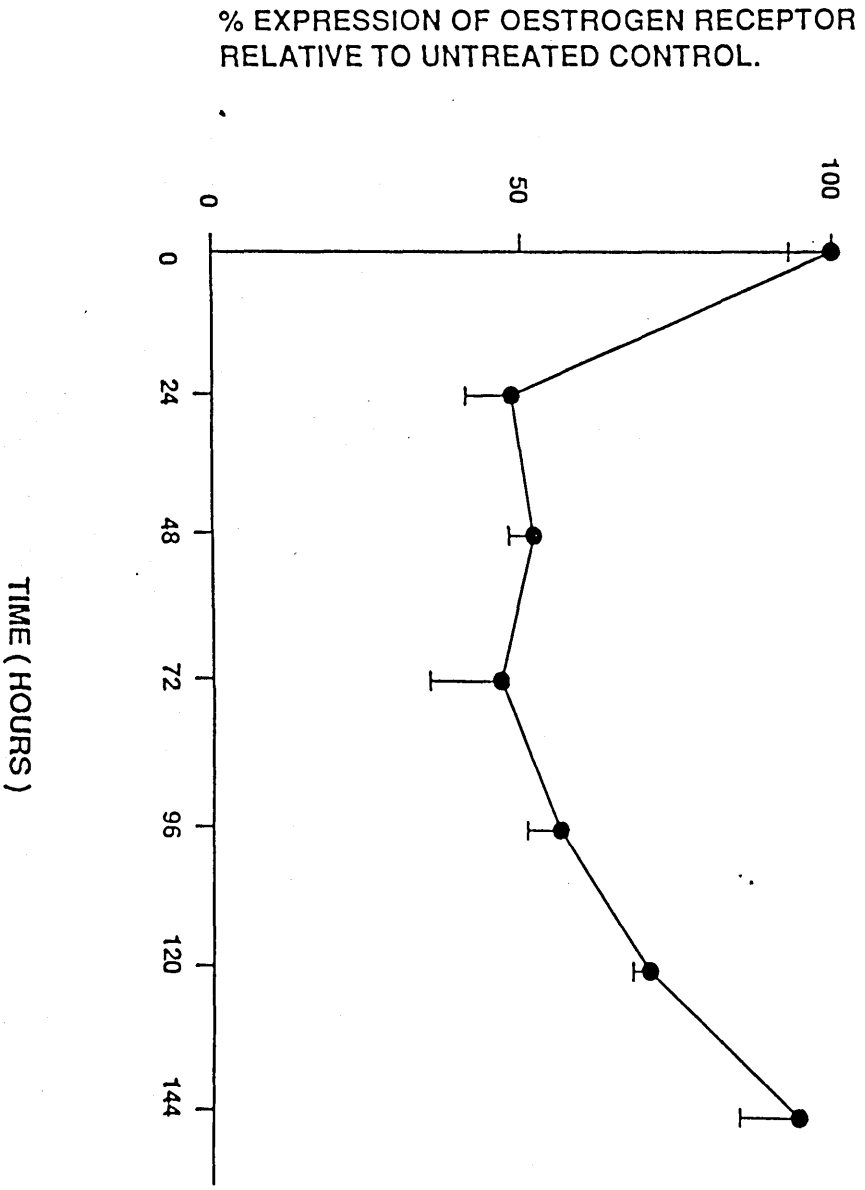


FIGURE 13

The influence of α-interferon (500iu/ml) on % oestrogen receptor (ER) protein concentration relative to control untreated cultures. The cells were exposed to α-IF for 96 hours.

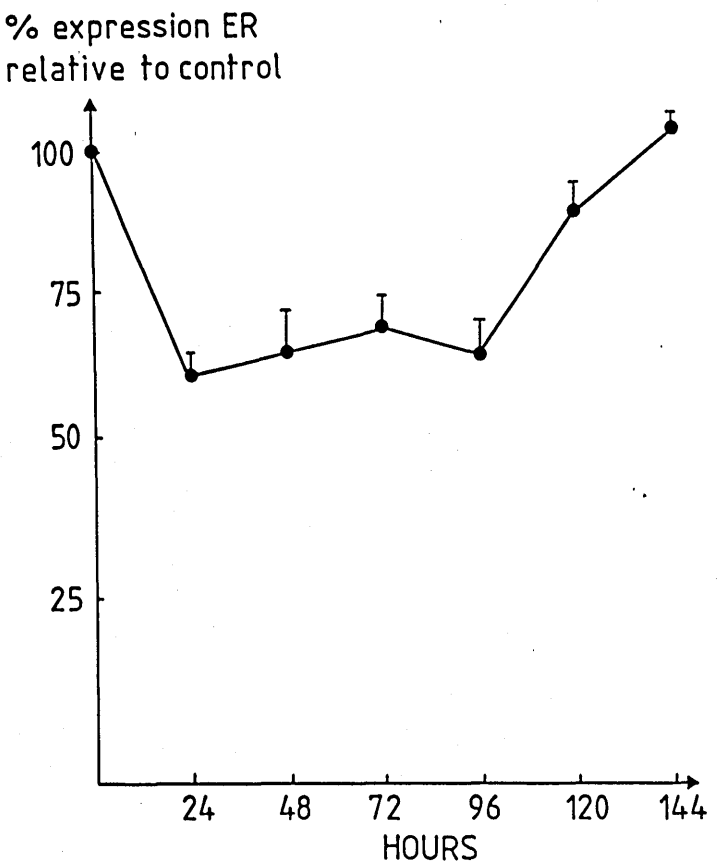


FIGURE 14

The influence of TGF-β (50pM) on ER protein concentration relative to untreated controls. The ZR-75 cells were exposed to TGF-β for 96 hours. Each point is the mean of 6 estimations and the vertical bar denotes 1 standard deviation.

SECTION 3.4 RADIO-IODINATION OF TGF-β AND CELL BINDING STUDIES

TGF-β was radio-iodinated as described to a specific activity of approximately 5μCi per μg. The protein was 95% pure, as adjudged by silver staining of SDS-acrylamide gels and maintained its biological activity (Table 3). The whole cell binding data was analysed and plotted according to the Scatchard method using an in-house computer programme based on the Marquhardt algorythm (figure 15). There was an average of 5800 binding sites per cell with a binding dissociation constant (Kd) of 70pM. The Kd is close to the concentration which was biologically active (section 3.2).

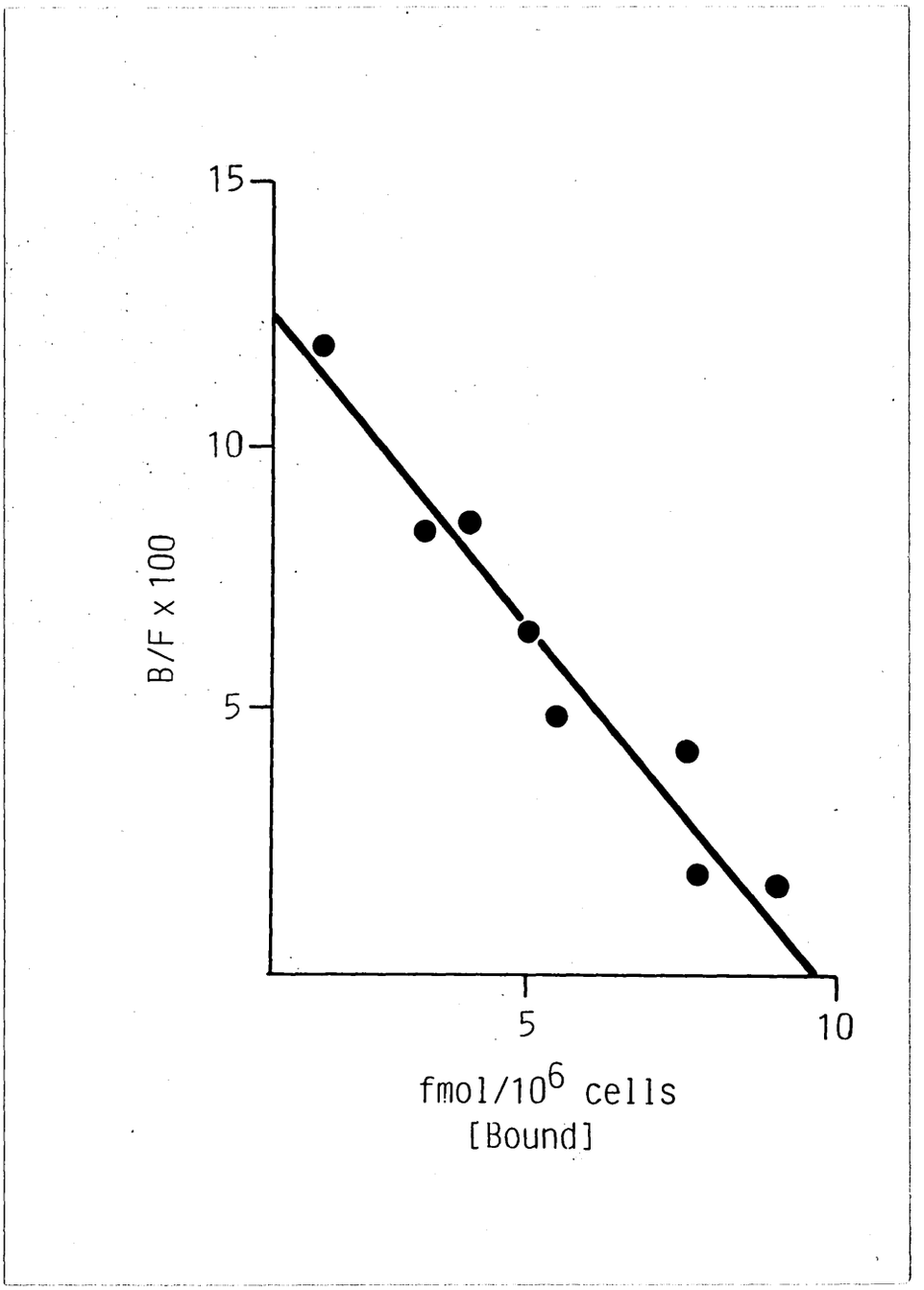


FIGURE 15

Scatchard plot of whole cell binding data of ^{131}I-TGF-β to ZR-75 cells.

SECTION 3.5 THE EFFECT OF α- INTERFERON ON TGF - β AND OESTROGEN RECEPTOR mRNA LEVELS

SECTION 3.5.1 TGF-β mRNA

Northern blot analysis revealed a single transcript for TGF-β at the expected size of 2.5 kb. Scanning densitometry of Northern blots (figure 16) revealed that there was significant induction of TGF-β mRNA steady state levels by 24 hours following treatment with α-IF (figure 17) by about 8 fold relative to control, untreated cells. Similar quantitative results were found with dot-blots using the same RNA isolated during and after α-IF treatment (figure 18). TGF-β mRNA concentrations were maintained at elevated values until α-IF was withdrawn from the medium on day 4 and then decreased rapidly to near control values 36 hours later. It is interesting to note that the kinetics of induction of TGF-β mRNA are similar to the α-IF effect on cellular proliferation. As cell division slows down, the concentration of TGF-β mRNA rises. The dual effects are maintained during exposure to α-IF and when fresh medium (without α-IF) is added, the cells begin to regrow after a lag phase during which TGF-β mRNA levels decline.

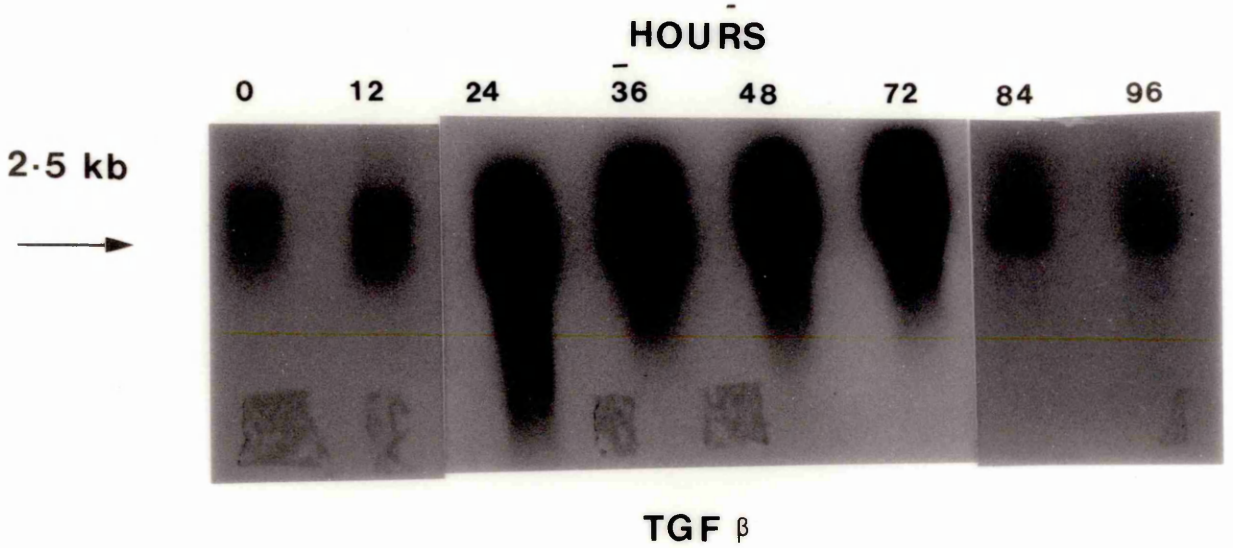


FIGURE 16

Northern blot of total cellular RNA probed with ^{32}P-cDNA for TGF-β following treatment of human breast cancer ZR-75 cells with α-IF for 72 hours.

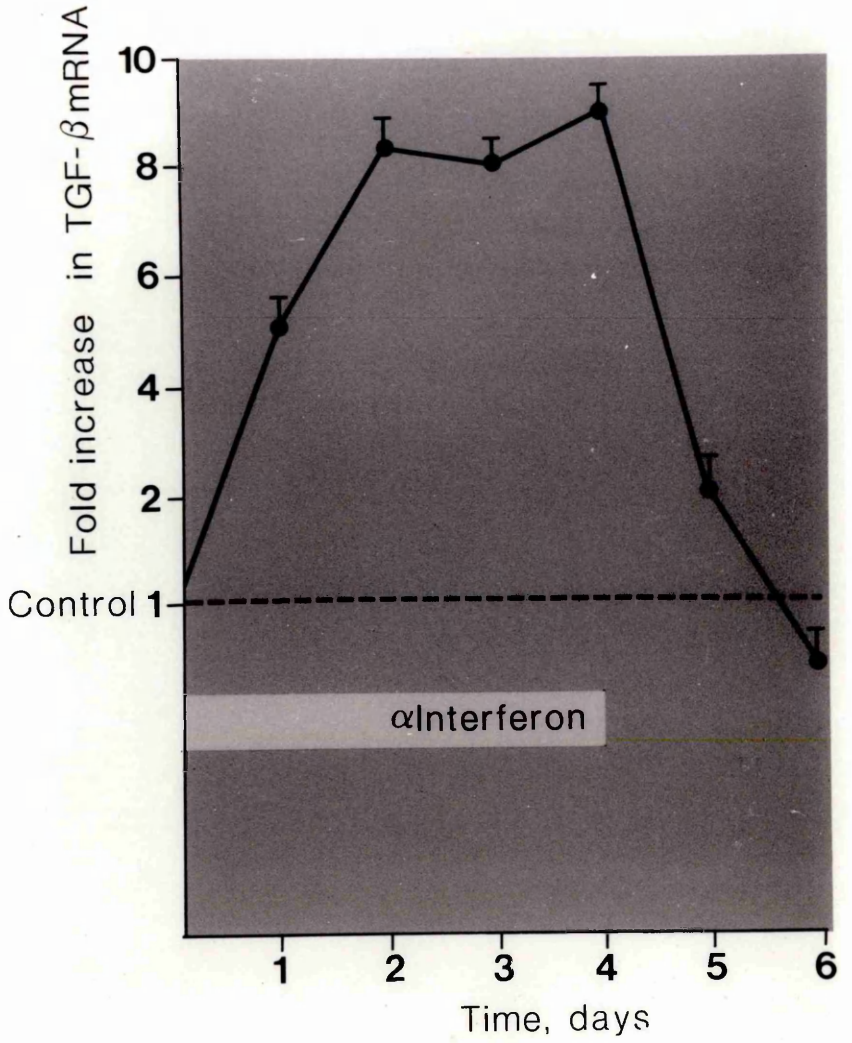


FIGURE 17

Fold increase (relative to control) in TGF-β mRNA in reponse to treatment with α-IF between days 0 and 4. TGF-β mRNA was quantatively assessed by densitometric scanning of Northern blots hybridised with ^{32}P-cDNA probe for TGF-β. Each point is the mean value of 4 distinct blots and the vertical bar denotes 1 standard deviation. The intra blot coefficient of variation for densitometric reading was always less than 5%.

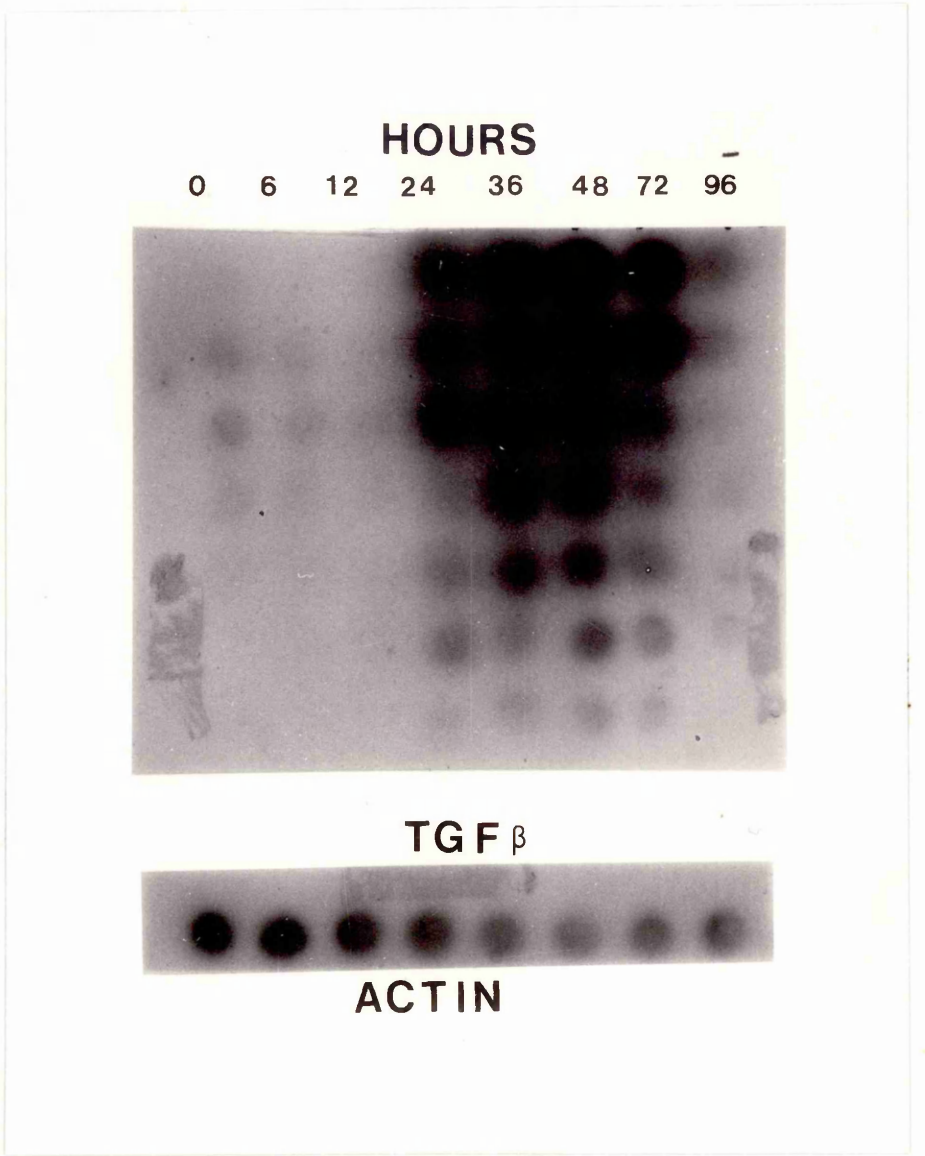


FIGURE 18

Dot blots of RNA from ZR-75 cells treated with α-IF for 72 hours was extracted and probed with ^{32}-cDNA for TGF-β. Eight, sucessive doubling dilutions of RNA were bound to the nitrocellulose filter in vertical columns. The size and intensity of the dot is related to the amount of specific RNA hybridised.

SECTION 3.5.2 ER mRNA LEVELS

Northern blots determined that the ER cDNA probe bound to a single mRNA species 6.3 kb in size, in agreement with published data (39). Dot-blot analysis revealed that ER mRNA levels (figure 19) tended to parallel ER protein levels (figure 13), with an initial early decrease to about 50% $\pm$ 4% of control, untreated cells, by 12 hours which was maintained at this reduced level during α-IF exposure. The maximum reduction in ER mRNA levels occurred before the maximal reduction in ER protein.

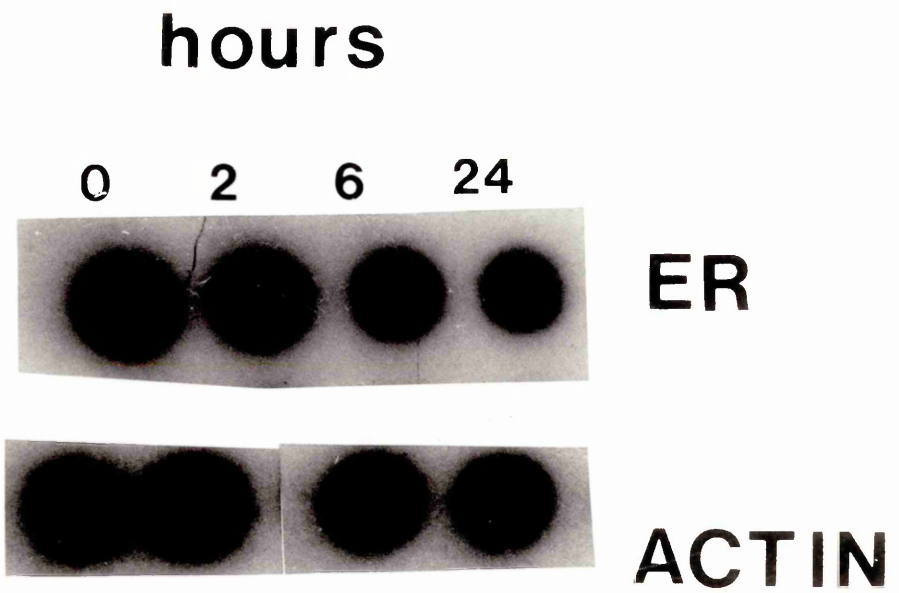


FIGURE 19

20μg of total RNA was extracted from ZR-75 cells at various stages during 24hour treatment with α-IF and hybridised with ^{32}P-cDNA for oestrogen receptor. The size and intensity of the dot is related to the amount of specific RNA hybridised.

SECTION 3.5.3 NUCLEAR RUN-ON ASSAY

The kinetics of mRNA induction were assessed using a nuclear run-on asay. Transcription of the TGF-β gene was induced following a delay of 24 hours and was maintained for the duration of exposure to α-IF (72 hours), returning to control values within 36 hours following withdrawal of α-IF (figure 20). The kinetics of the effect of α-IF on inducing transcription of the TGF-β gene are similar to those seen for steady state accumulation of TGF-β mRNA in section 3.5.1. B-actin transcription was used as control and was fairly constant throughout exposure to α-IF.

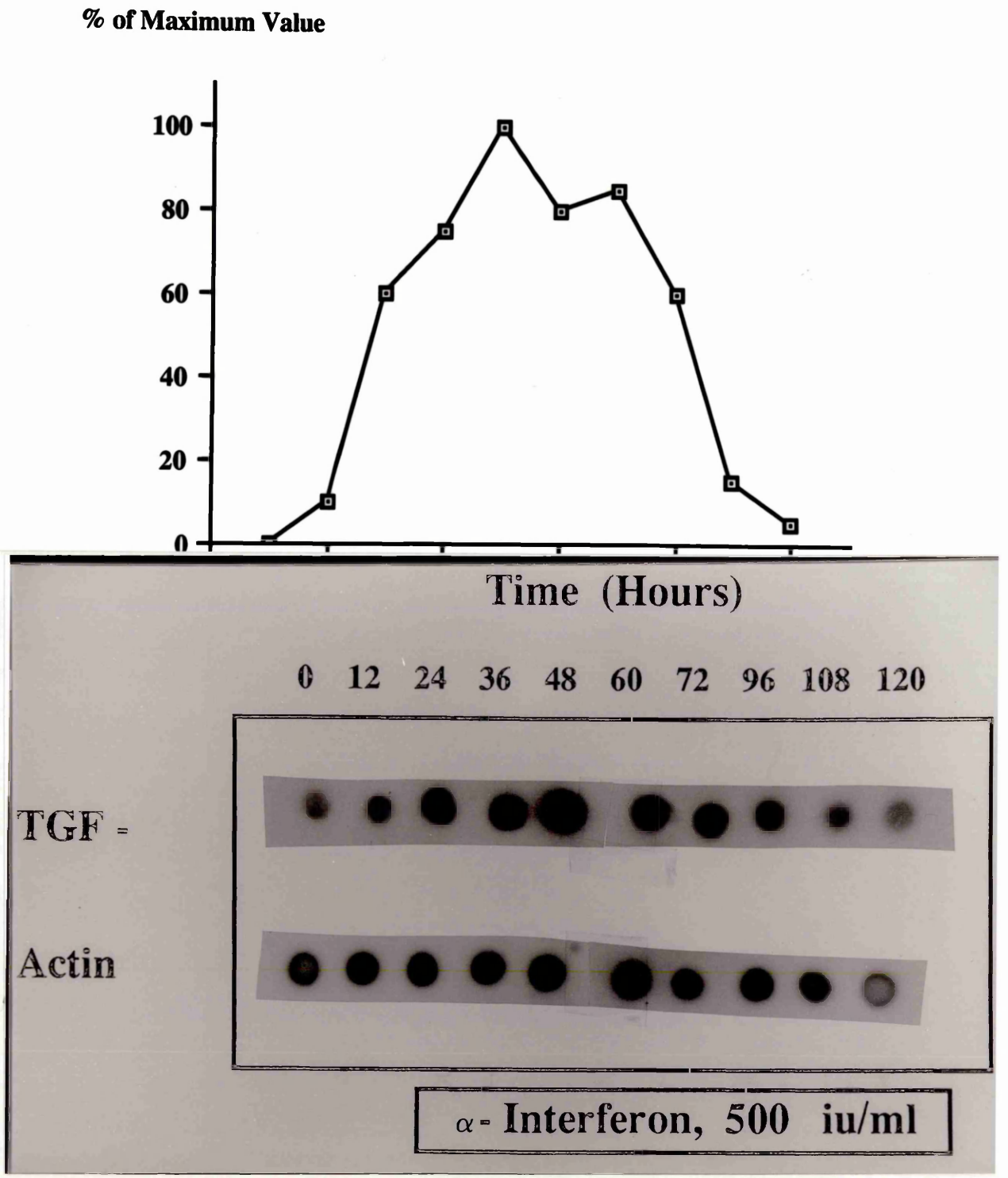


FIGURE 20

A - Slot blots of TGF-β mRNA induction in response to treatment with α-IF (500iu/ml) for 72 hours. B-actin cDNA was used for control purposes.

B - The graph shows the integrated densitometer tracings of the TGF-β autoradiograph in A.

SECTION 3.6 THE EFECT OF ANTI-TGF-β ANTIBODY ON THE CYTOSTATIC RESPONSE TO α- INTERFERON

TGF-β inhibits incorporation of ^{3}H-thymidine by mink lung epithelial cells in a dose-dependent fashion (figure 11) with an ID_{50} of 120pg/ml. By incubating a fixed concentration of TGF-β (1ng/ml) with varying concentrations of a commercially available anti-TGF-B polyclonal antibody, it was possible to demonstrate that the antibody could neutralise the effects of TGF-β in the bioassay in a dose dependent manner (figure 21). It is apparent from figure 21 that 50μg of antibody abolished the activity of TGF-β (1ng) in the mink lung assay.

In a series of subsequent experiments, ZR-75 cells were exposed to α-IF (500iu/ml) and 50μg of anti-TGF-β antibody for 4 days. As can be seen from figure 22, α-IF has a significant cytostatic effect (similar to figure 12a), but this was abrogated by the addition of the anti-TGF-β antibody. When α-IF and the antibody were removed from the medium, the cells started to regrow after a short lag phase. The suppliers of the antibody have performed experiments which indicate that the antibody does not bind to α-IF (personal communication, R & D, Minneapolis).

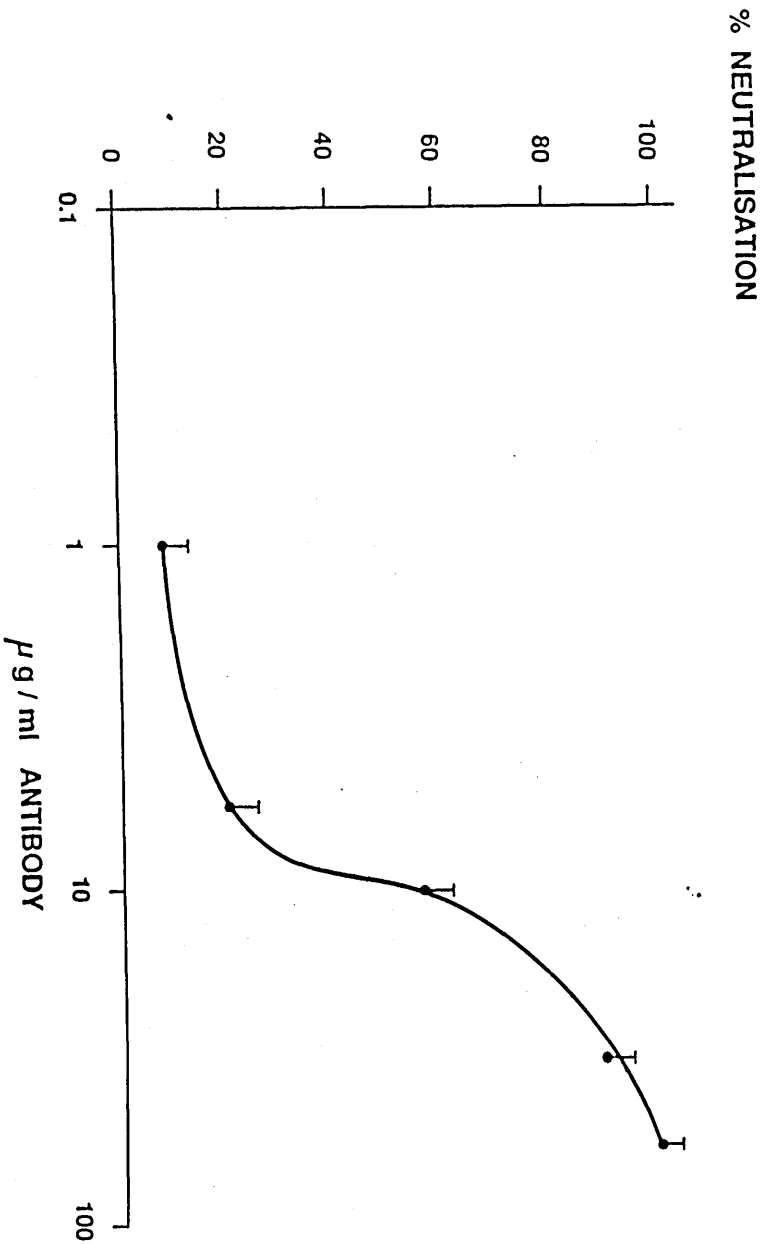


FIGURE 21

Dose response curve: the degree of neutralisation of the effects of 1ng of TGF-β in the mink lung assay by increasing amounts of anti-TGF-β polyclonal antibody. Each point is the mean of 4 estimations and the vertical bars denote 1 standard deviation.

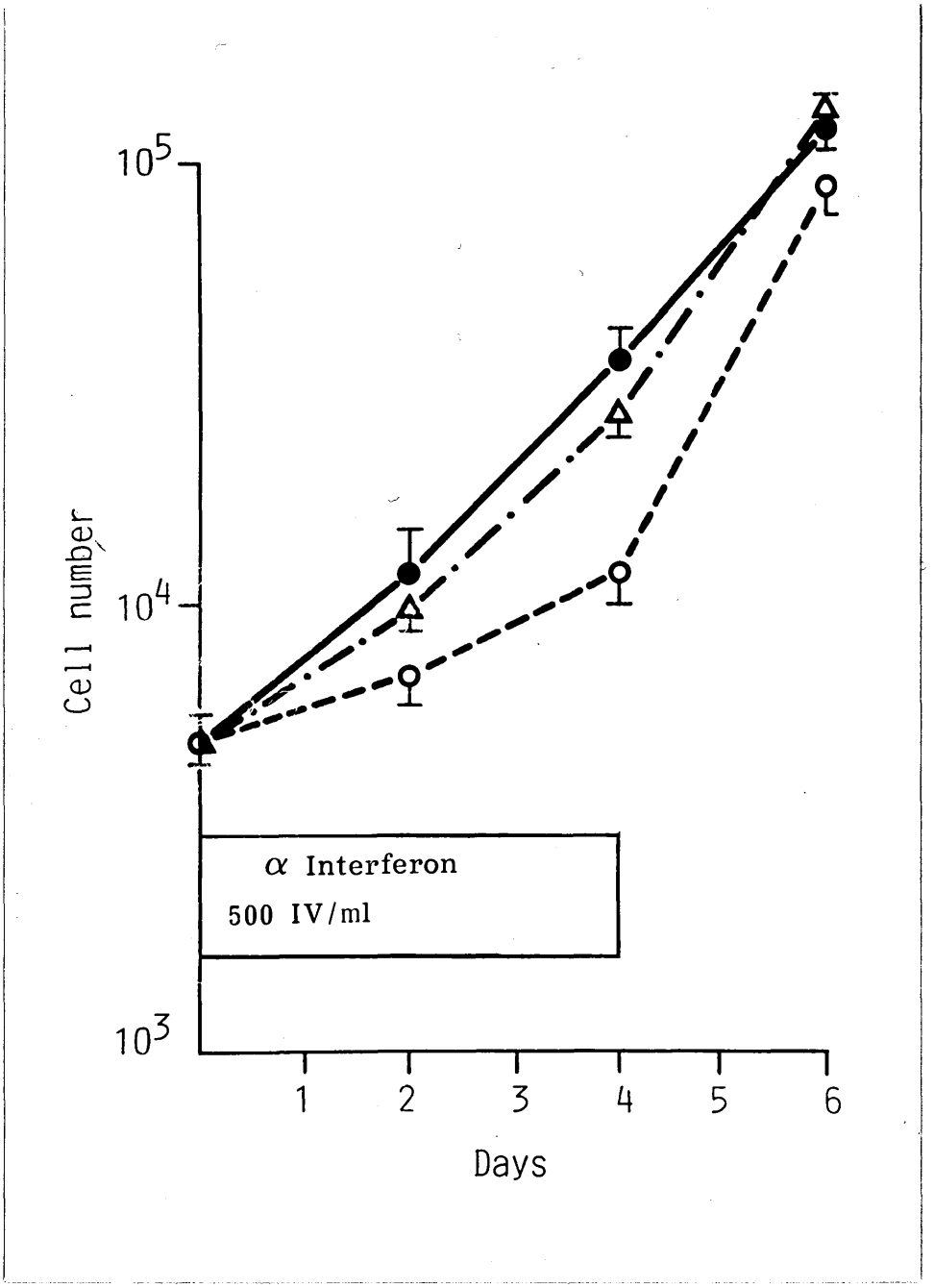


FIGURE 22

The effect of anti-TGF-β antibody on the cytostatic efficacy of α-IF (500iu/ml; 4 day exposure) on ZR-75 cells. Cells were untreated (●), treated with α-IF alone (o) or α-IF plus anti-TGF-β antibody △ (50μg/ml; 4 day exposure). The antibody alone had no antiproliferative effects. Each point is the mean of 6 estimations and the vertical bars denote 1 standard deviation.

SECTION 3.7.1 THE EFFECT OF ONCOGENE TRANSFECTION ON THE SENSITIVITY OF EPITHELIAL CELLS TO TGF-β AND EGF

Two or three derivative cell lines transfected with c-*myc* (cell line = MLMC), H-*ras* proto-oncogene (cell line = HO6N1) and activated H-*ras* oncogene (cell line = HO6T1) were expanded from single colonies.

Southern blot analysis of the c-*myc* transfected cell line (MLMC) hybridised with ^{32}P-labelled human c-*myc* probe isolated by PVU II/Bgl II cleavage of PMC 41 and spanning exon 2. The 8Kb human specific Hind III/EcoRI fragment encompassing the entire human c-*myc* gene was observed in cell line MLMC and was absent from the parental line line CCl-64.

Southern blot analyses of H-*ras* transfected cell lines hybridised with ^{32}P labelled H-*ras*1 probe isolated by Sma I/SST I cleavage of PT24C3 and spanning exons 1-4. The 6.6Kb human specific Bam HI fragment encompassing the H-*ras* gene was observed in cell line HO6T1. An H-*ras* specific fragment of higher molecular weight was observed in cell line H06T1 resulting from a probable integration of H-*ras* sequences with in the 6.6Kb fragment with endogenous mink lung sequences. The parental line CCl-64 shows no hybridisation to the human H-*ras* probe (Dr J C Lang, personal communication).

Immunocytochemical staining of fixed cells with monoclonal antibodies directed against human c-*myc* and H-*ras* proteins showed that human *ras* protein was present in both the *ras* transfected lines and that human *myc* was detectable in the MLMC line (Z Khan, personal communication).

The growth characteristics and tumourigenicity of the derivative cell lines were evaluated by Dr M Z Khan, CRC Department of Medical Oncology.

Although the parental line is epithelioid, the activated H-*ras*-transfected line lost its epithelial morphology and showed a bipolar or fibroblastoid shape with evidence of piling up. Chromosomal analysis gave a diploid number of 30 and there was no indication of a major ploidy change, or gross chromosomal abnormalities in the transfected cell lines.

All the lines grew rapidly with doubling times under of 20 hours and a short lag period of around 5 hours (table 4). The population doubling times decreased from 19.5 hours in the parental line to 14.1 hours in the mutant and normal H-*ras*-transfected lines with the c-*myc*-transfected line intermediate at 18.2 hours. The saturation densities increase from 5.7×10^5 cells/cm^2 in the CCl-64 and MLMC cells to 1.1 and 1.2×10^6 cells/cm^2 in the HO6N1 and HO6T1 cells respectively.

When 1 x 10^6 cells of each line were implanted in the flanks of nunu mice, 20% formed tumour from the CCl-64 cells, while 100% formed tumours in all the other groups (table 5). The lag period for the cell tumour was 60 days while that of the MLMC tumours was 43 days, the HO6N1 35 days, and the H06T1 7 days (Table 5). A similar relationship was seen in the doubling times of the tumours with the CCl-64 cell tumour growing the slowest, and the HO6T1 the fastest.

SECTION 3.7.2 THE EFFECT OF ONCOGENE TRANSFECTION ON EPITHELIAL CELL RESPONSE TO EXOGENOUS GROWTH FACTORS, TGF-β AND EGF.

The cellular response of the derivative lines to TGF-β is summarised in figure 23. The CCl-64 and HO6N1 lines respond in an identical fashion to TGF-B in a dose dependent manner. The activated H-*ras* transfected line does not respond, even at high TGF-B concentrations and the MLMC line has an intermediate response.

EGF stimulates incorporation of ^{3}H-thymidine by CCl-64 and HO6N1 cells to a similar extent whereas the c-*myc* transfected line has an exaggerated response and the HO6T1 cells do not respond to EGF (figure 24).

The effect of combined treatment with EGF and TGF-β on ^{3}H-thymidine incorporation by CCl-64 cells is summarised in figures 25a and 25b. TGF-β reduces the stimulatory effect of EGF whereas EGF reduces the inhibitory effect of TGF-β.

TABLE 2

THE EXTENT OF CHROMATOGRAPHIC PURIFICATION OF TRANSFORMING GROWTH FACTOR-β ACTIVITY BY STAGE

TGF-β Preparation	Protein Concentration (eliciting 1 unit of activity)	Fold Concentration (relative to crude extract)
Crude extract	100ng/ml	-
Biogel fractions	5ng/ml	20
Mono-Q fractions	1ng/ml	100
HPLC fractions	0.1ng/ml	1000

TABLE 3

BIOLOGICAL EFFECTS OF PURIFIED PLATELET-DERIVED TGF-β

Sample	Number of Colonies ($\geq 3000 \mu m^2$)
TGF-β + EGF	1968 ± 185
Reduced TGF-β + EGF	268 ± 48
EGF alone	372 ± 56
TGF-β alone	88 ± 32
Foetal Calf Serum (10%)	50 ± 18
^{125}I-TGF-β + EGF	1705 ± 362

TGF-β concentration = 1ng/ml

EGF concentration = 2.5ng/ml

TABLE 4

SUMMARY TABLE OF DOUBLING TIME AND SATURATION DENSITY FOR PARENT AND TRANSFECTED CELL LINES

All experiments performed in 10% FCS, F10:DMEM, and glutamine (10mM). (Mean $\pm$ standard deviation, n=6)

Cell Line	Transfection	Population Doubling Time (PDT) (hours)	Saturation Density(SD) Cells/cm^2
CCl-64	none	19.5 $\pm$ 0.5	5.7×10^5
MLMC	myc	18.2 $\pm$ 0.4	5.7×10^5
HO6N1	H-ras Proto-oncogene	14.1 $\pm$ 0.9	1.1×10^6
HO6T1	H-ras-1 Activated	14.2 $\pm$ 0.5	1.2×10^6

TABLE 5

GROWTH CHARACTERISTICS OF PARENT AND TRANSFECTED CELL LINES ON IMPLANTATION INTO nu nu MICE

(Mean ± 1 standard deviation)

Sublines	Latent Period (vol>33mm^2) (Days) (4x4mm)	Take(%)	Doubling Time (Days) (400-800mm^3)
CCl-64	60.0 ± 5.2 n = 3	21.4 n = 14	9.0 ± 0.8 n = 4
MLMC	43.4 ± 3.9 n = 9	100 n = 12	6.4 ± 0.6 n = 9
HO6N1	35.0 ± 1.3 n = 9	100 n = 12	7.2 ± 1.0 n = 9
HO6T1	7.1 ± 0.8 n = 9	100 n = 12	4.5 ± 0.2 n = 9

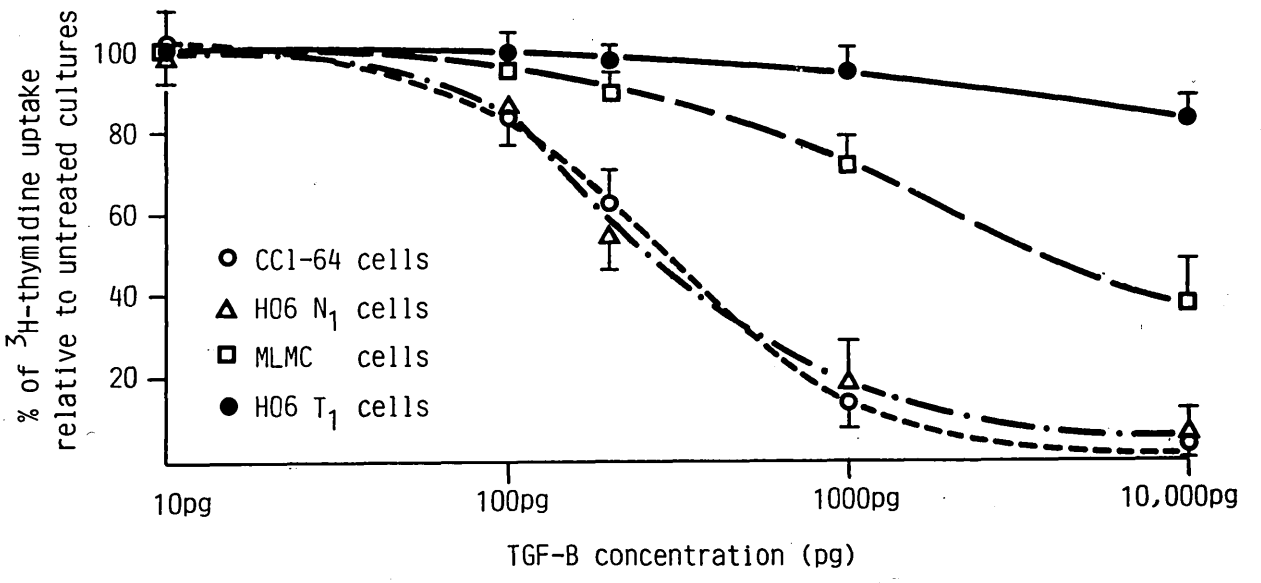


FIGURE 23

Dose response curve: the effect of TGF-β on ^{3}H-thymidine incorporation by parent and transfected cell lines. Each point is the mean of 6 estimations and the vertical line denotes 1 standard deviation.

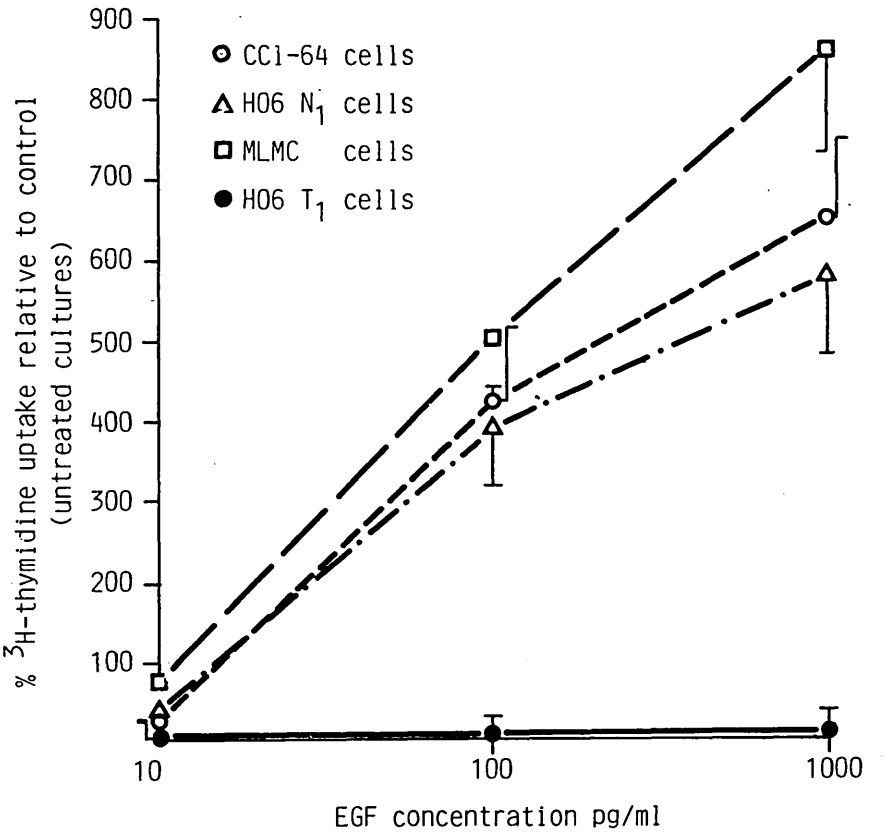


FIGURE 24

Dose response curve: the effect of EGF on ^{3}H-thymidine incorporation by parent and transfected cell lines. Each point represents the mean of 6 estimations and the vertical line denotes 1 standard deviation.

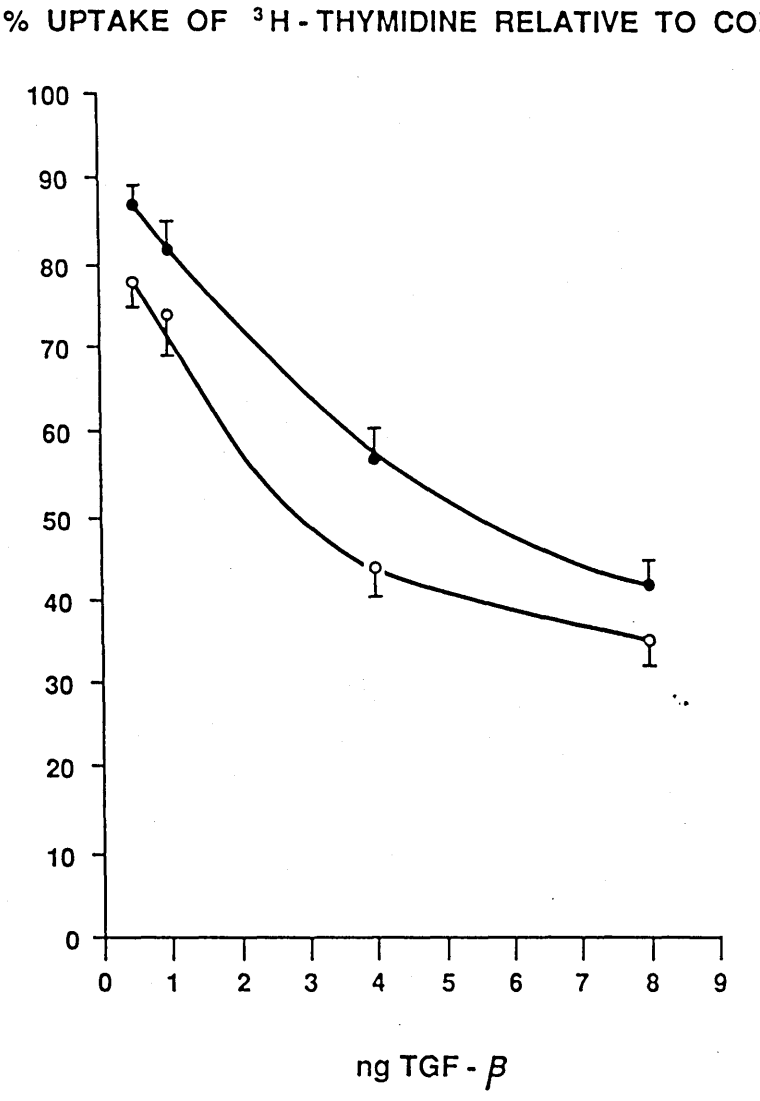


FIGURE 25

A - The effect of a fixed concentration of EGF (1ng) on the dose-response curve to TGF-β in the mink lung epithelial assay. Each point represents the mean of 6 estimations and the vertical line denotes 1 standard deviation. (●) EGF + TGF-β, (O) TGF-β alone.

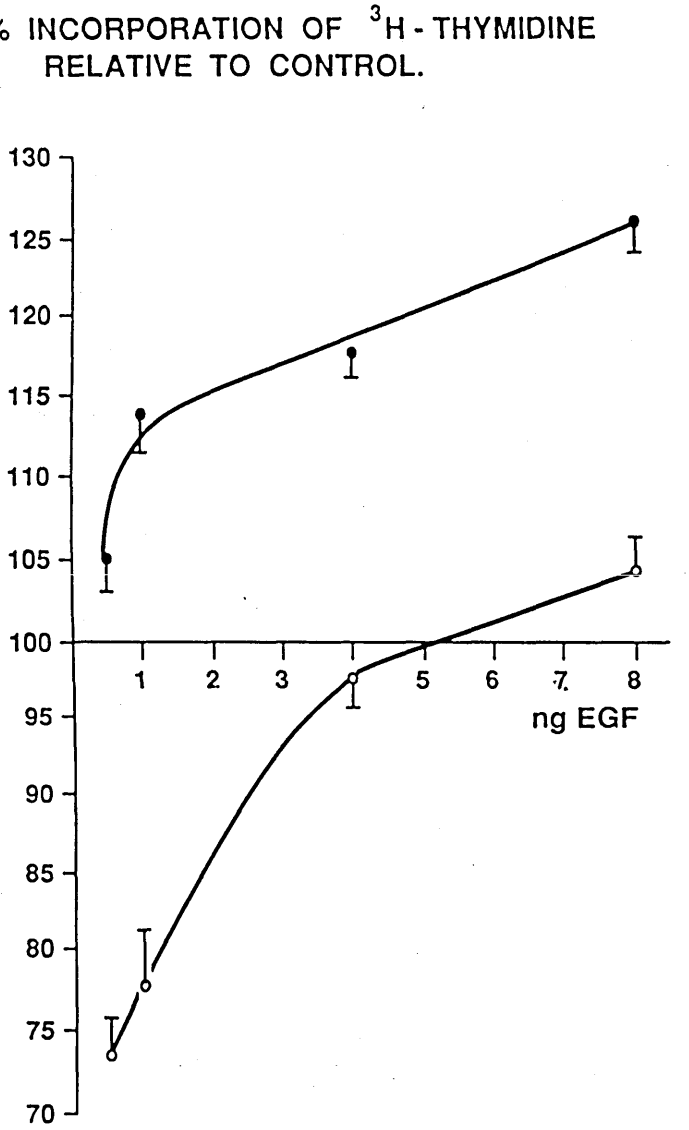


FIGURE 25

B - The effect of a fixed concentration of TGF-β (1ng) on the dose-response curve to EGF in the mink lung epithelial assay. Each point represents the mean of 6 estimations and the vertical line denotes 1 standard deviation. (O) EGF + TGF-β, (●) EGF alone.

CHAPTER 4

DISCUSSION

DISCUSSION

Data are presented in this thesis which demonstrate the importance of growth factors in determining proliferation of breast cancer cells and how growth factor activity can be modulated by exogenous factors such as α-IF and by the genotypic milieu following oncogene transfection.

The initial phase of this thesis described purification to homogeneity of TGF-β (95% on silver staining of SDS-polyacrylamide gels) from expired platelets. The chromatographic method devised was based on the published method of Assoian et al (51) and made use of the acid stability of TGF-β.

The innovation introduced was the use of anion exchange mono Q columns following initial gel filtration chromatography and subsequent HPLC clean up using stepped acetonitrile gradients. The relative yield of TGF-β and its biological activity was very similar to that reported by Assoian (51) but the run-time on anion exchange chromatography is significantly shorter than gel filtration in high concentration urea.

The first series of experiments compared the antiproliferative effects of purified TGF-β with α-IF. α-IF, at a concentration which is pharmacologically achievable in plasma with conventional clinical dosage regimens. α-IF induced cytostasis in ZR-75 cells which persisted for the duration of exposure. The cells started to regrow at a rate roughly parallel to control following removal of α-IF from the medium, after a lag phase of about 24 hours. Interestingly, α-IF did not have an antiproliferative effect on the ER negative cell line, MDA-41. TGF-β at a concentration of 50pM has a more profound antiproliferative effect on ZR-75 cells than α-IF, but with a similar kinetic profile (figure 12a). Growth of MDA-41 cells is inhibited by TGF-β, in a similar manner to the ZR-75 cells (figure 12b).

TGF-β has been shown previously to be a negative regulator of proliferation of both ER positive (79) and ER negative (80) cell lines. However, there is an obvious disparity between the response of the different cell lines to α-IF.

The potential reasons for the lack of response of the ER negative cell line to α-IF include:

a) insufficient interferon receptors

b) absence of ER

c) uncoupling of the mechanism by which α-IF increases the rate of transcription of the TGF-β gene. The MDA-41 cells respond to TGF-β and therefore by imputation, they have sufficient receptors coupled to post receptor signal transduction pathways to react with any TGF-β released by the effect of α-IF.

Subsequent experiments were performed with the ER positive ZR-75 cell line in order to determine if there was a link between the antiproliferative effects of α-IF and TGF-β.

In subsequent experiments it was shown that α-IF has a significant inductive effect on steady state mRNA levels for TGF-β, with an increase to some 8 fold relative to control cultures.

Again, the kinetics of response is such that there is a lag phase, an elevated plateau phase, followed by a decline to roughly control values. The period during which α-IF exerted its maximal, sustained antiproliferative effect corresponded with elevated TGF-β mRNA levels, the TGF-β mRNA levels declined within 24 hours of α-IF withdrawal, at which time the cells began to regrow.

Nuclear run-on assays showed that the site of action of α-IF was to increase the rate of transcription of the TGF-β gene. The increase in the rate of transcription of TGF-β mRNA occurs faidly rapidly after treatment with α-IF, is maintained at a higher rate for the duration of exposure to α-IF and declines to normal on withdrawal of α-IF from the medium. The kinetics of this effect and the extent of induction of mRNA transcription parallel the rise in steady state TGF-β mRNA concentration. It is likely therefore that α-IF exerts its modulating effect at a transcriptional level.

An essential component of any autocrine or paracrine growth control loop is the cell surface receptor for the polypeptide. It was possible to radio-iodinate TGF-β to a high specific activity, yet retain biological activity for receptor binding studies.

Scatchard analysis of this data revealed that ZR-75 cells have 5800 high affinity binding sites (Kd = 70pM) per cell. These results are similar to those described for other TGF-β responsive cell lines such as NRK cells (58) and ER negative breast cancer cell lines (80). The binding dissociation constant of 70pM is similar to the concentration of TGF-β (50pM) which has been shown to have a significant growth inhibiting effect on ZR-75 and MDA-41 cells.

In developing the hypothesis that the cytostatic effects of α-IF could be mediated by TGF-β, it seemed important to link α-IF treatment to activation and secretion of TGF-β protein, as the mRNA data offer indirect support for the hypothesis.

Radioreceptor competition assays have been used (58) to measure TGF-β concentration in conditioned medium. An attempt was made to set up a similar assay in our laboratory but this did not prove sufficiently sensitive. There was unacceptably wide inter and intra-assay variation in the standard curves (>25%). In order to determine whether α-IF increased the synthesis and secretion of active TGF-β into the cell culture medium, we coincubated the cells with both α-IF and commercially available anti-TGF-β polyclonal antibody. In separate experiments it was proven that the antibody was biologically neutralising in the mink lung epithelial assay. Coincubation of α-IF with the antibody (the quantity of antibody capable of abolishing the effect of 1ng purified TGF-β in the mink lung epithelial assay) reduced the cytostatic effects of α-IF by approximately 60%. Further increase in the concentration of antibody did not cause any further reduction in α-IF's antiproliferative effect.

Relatively little is known about the synthesis, packaging, secretion and binding of TGF-β to its cellular receptors, however the fact that the TGF-β antibody reduces the cytostatic effect of α-IF by only 60% has 3 possible explanations:

a) The amount of antibody present is not adequate to bind to and neutralise the quantity of TGF-β produced by the breast cancer cells. We extended the dose response curve by increasing the concentration of antibody present in the culture medium but found no additional inhibitory effect. 50μg of antibody was enough to inhibit 1ng of exogenous TGF-β in the mink lung epithelial assay, therefore it would seem likely that sufficient, even excess antibody, was added to neutralise TGF-β when coincubated with α-IF.

b) TGF-β may be synthesised, secreted and activated by the cells but binds rapidly to its own receptors, therefore reducing the chance of interaction with the anti-TGF-B antibody in the culture medium. Examples of rapid, sequestered binding of ligand to receptor have been reported for other polypeptide growth factors (77, 78).

c) Given the diverse effects that α-IF has on cellular biochemistry, it is possible that other mechanisms contribute to the cytostatic effect of α-IF.

As previously mentioned, ER play a central role in mediating the trophic effects of oestrogen on ER positive cell lines, such as ZR-75. It has been demonstrated that α-IF (500iu/ml) reduces phenotypic expression of ER protein with a decrease in ER concentration to approximately 50% of control values by 24 hours of treatment. ER concentration is maintained at this depressed level for the duration of exposure to α-IF and then rises to approximately normal values within 24 hours of withdrawal of α-IF. The ER mRNA concentrations paralleled the response of ER protein to α-IF, although the fall in ER mRNA occurred 12 hours before the decrease in ER protein. It is interesting to note that TGF-β (50pM) reduced ER expression to 60% of control values and it is possible that TGF-β released in response to α-IF treatment could contribute to the reduction in ER induced by α-IF.

These results are at variance with those of Van den Berg et al (81) who found that exposure of ZR-75 cells to continuous low levels of α-IF (10iu/ml) increased phenotypic expression of ER. The extent of the increase in ER levels ranged from 1.2 to 7.2 fold. This effect was inversely proportional to dose over the range 10-1000iu/ml.

In a small clinical study (71), sequential biopsy of accessible breast cancer metastases during therapy with α-IF and measurement of tissue ER concentration, indicated that ER levels tended to rise following α-IF treatment.

The possible differences for the discrepancy between the work reported here and that of Van den Berg include:

1. Different doses of α-IF were used. The sorts of plasma concentrations of α-IF that are found with clinical dose schedules for α-IF are in the range of 500iu/ml (69), which is why this concentration was chosen for the present study. It is known that the interferons have bell shaped dose response curves in vitro for a range of their immunological effects (80) and it is possible that this could extend to their anti-proliferative properties.

2. The cells were treated with α-IF at different phases of growth . Van den Berg et al (81) noted a marked effect of cell density and in their hands, only cells seeded at low density exhibited induction of ER in response to α-IF. The ZR-75 cells were treated at a relatively high density in the present report to facilitate the extraction of adequate quantities of RNA. Also it was considered closer to the cell kinetic situation found in vivo in the majority of breast carcinomas.

3. There is considerable interlaboratory, phenotypic variation in human ER positive breast carcinoma cell lines and although derived initially from a common source, they have been available for so long and used so widely throughout the world that variation in a range of their biochemical properties has arisen (83).
Taken in concert, the results shown in this thesis imply that a significant proportion of the direct cytostatic effects of α-IF are mediated by TGF-β in this human ER positive breast cancer cell line.

As previously mentioned, the interferons effect a range of immunological indices. However, the mechanisms underlying its direct, anti-proliferative action in vitro are unknown. The anti-proliferative activity of interferon was first reported by Paucker et al (68). There are no general rules to predict which cells will be inhibited and which will not, although interferon elicits its action by binding to cell surface receptors. Interferon treatment of murine and human cells results in the induction of several proteins in addition to those thought to be concerned with its antiviral activity (the 2,5-A_n-dependent endoribonuclease and P1/eIF-2 protein kinase-phosphoprotein phophatase system). A rise in the cellular concentration of a number of proteins ranging in apparent molecular weight from about 15,000 to 120,000 has been reported for a variety of different cell types in response to interferon treatment (82-85).

In certain instances the cDNAs corresponding to the interferon inducible mRNAs have been isolated, a number of which have been identified and shown to encode the following proteins: 2,5-A_n synthetase (88); metallothionein-II (89); thymosin B_4 (89); major histocompatibility antigens (90); protein M_x (91); and 2 proteins of unknown identity, one of 56Kd, (92) and the other of 15Kd (93).

The kinetics of response of the inducible mRNA species is rapid and can be detected within 5 minutes to 2 hours after interferon treatment (89). The mechanism underlying the rise in steady state mRNA levels can be due to increased rates of transcription of genes that are not normally transcribed or are transcribed at very low rates in the absence of interferon (94). It could also be related to post transcriptional mechanisms which alter mRNA stability (94). In terms of its antiproliferative effect, α-IF has been shown to decrease expression of certain oncogenes, which could be important in maintenance of the transformed phenotype. For example, treatment of Daudi cells with α-IF causes a significant decrease in the level of c-*myc* mRNA (95). Interferon treatment has also been shown to reduce the amount of H-*ras* mRNA and protein in mouse and human cells (96) and c-*fos* mRNA in Balb/c 3T3 cells (97).

If oncogene transcription/translation is important in maintenance of the transformed phenotype then the above biochemical effects could contribute to the anti-proliferative action of α-IF. However, there are no significant correlations between the ability of α-IF to alter oncogene mRNA levels and induce cytostasis. The situation is complex as interferon expression can be induced by certain growth factors in an inhibitory autocrine loop; for example, platelet derived growth factor stimulates the rapid expression of a cell division competence gene family including c-*myc* and c-*fos* and the slower induction of expression of interferon-β. It is possible that interferon-β may function as a feedback inhibitor of growth as the expression of the genes regulated by PDGF, including c-*myc* and c-*fos*, are inhibited by interferon treatment (97).

As yet, relatively little is known of the regulatory sequences to which interferon interacts with, in a trans sense, in terms of its ability to regulate gene expression. A consensus sequence has been derived from homologous sequences identified 5' to the putative transcriptional start sites of interferon inducible genes such as HLA and metallothionein but it is not clear how widely applicable this sequence is to other interferon inducible genes (98).

Clearly this area would be of interest as it might be possible to identify consensus sequences flanking the ER or TGF-β genes through which α-IF acts to regulate gene expression. As is always the case, it is possible that the effects of α-IF on ER and TGF-B gene expression is indirect and depends on its induction of an intermediary protein. As mentioned, α-IF can have very rapid effects on gene expression (within 5 minutes) and it is possible that early induction of protein could lead to a secondary cascade effect on expression of other genes mediated by the early induced protein, rather than α-IF itself.

Lippman's group (79) have shown that the cytostatic effects of tamoxifen on an ER positive human breast cancer cell line (MCF-7) were mediated by an increase in synthesis and release of active TGF-β. These authors concluded that in MCF-7 cells, TGF-β is a hormonally regulated growth inhibitor with possible autocrine and paracrine functions in breast cancer cells. It has been shown in this thesis that α-IF has a dual effect on ER positive breast carcinoma cells that could lead to a synergistic antiproliferative interaction with tamoxifen.

a) Tamoxifen is an ER antagonist and therefore seeks to abrogate the ability of oestrogenic hormones to act as trophic factors by competitively blocking their interaction with ER. It has been shown in this study that α-IF decreases phenotypic expression of the ER which would seem a different means of accomplishing the same end-point i.e. depletion of available ER for interaction with oestrogen.

b) Evidence has been provided here that at least some of the cytostatic effect of α-IF is mediated by TGF-β in the ZR-75 cell line. There is induction of TGF-β mRNA by α-IF which was not described in tamoxifen treated cells by Krabbe et al (79).

If the end result is to increase active TGF-β available for auto-inhibition of cell division, then the fact that α-IF (mRNA effect) and tamoxifen (increased synthesis and activation of TGF-β) accomplish this by different means again implies the potential for a synergistic interaction. There is some evidence to suggest that tamoxifen and α-IF could be usefully combined as Van den Berg et al (81) showed that there was a synergistic antiproliferative interaction between tamoxifen and α-IF in vitro.
The combination of α-IF and tamoxifen could be taken into animal models to see if the synergistic interaction is also found in vivo.

Of course, if there were positive results, this would provide compelling evidence to initiate a prospective, randomised clinical trial (possibly in the adjuvant setting) comparing tamoxifen versus tamoxifen plus α-IF in patients with ER positive breast cancer.

In collaboration with Dr A Thompson, MRC Population Genetics Unit, Western General Hospital, Edinburgh, I have performed a series of experiments determining TGF-B mRNA expression in oestrogen and tamoxifen treated nude mice bearing ER positive (MCF-7 human breast carcinoma cell line) human breast carcinoma xenografts. Other than for discussions planning the experiments, my specific contribution was prepare Northern blots from RNA prepared from tumour tissue probed with ^{32}P-cDNA for TGF-β and TGF-α, as previously described. Dr Thompson established xenograft tumours from the oestrogen-dependent MCF-7 breast cancer cell line in thymectomised, irradiated female CBA strain mice. Following infusion of oestrogen via osmotic mini pumps, there was an increase in tumoural DNA synthesis and mitosis was stimulated with a consequent increase in tumour growth.

Laser densitometry was used to quantify mRNA on Northern blots and showed that with oestrogen stimulation, c-*myc* gene expression was increased whereas TGF-β expression was suppressed.

However during the phase of oestrogen withdrawal, the tumours regressed and there was induction of TGF-β RNA. This xenograft system demonstrates changes in gene transcription in response to oestrogen. It thus provides an in vivo model for molecular and biochemical studies of hormone-sensitive human breast cancer (99).

In a further collaborative study with Dr Thompson, the expression of TGF-β mRNA has been quantified in mRNA extracted from breast tumour tissue from 45 untreated and 11 tamoxifen treated patients using densitometry of Northern blots. In the 56 tumours, TGF-β mRNA expression was significantly higher in premenopausal women ($p=0.05$) but was independent of oestrogen receptor status and other clinical and pathological parameters.

Within tamoxifen treated patients, high expression was demonstrated in all 6 patients with progressive tumour growth whereas low levels were present in 4 of 5 with static tumour. These data suggest that failure of tamoxifen treatment in breast tumours is associated with high, rather than low, levels of TGF-β contrary to expectations from in vitro studies. If the asssumption is made that TGF-β is relevant to the mechanism of action of tamoxifen, then tamoxifen resistant tumours could arise due to a breakdown in the potential autoinhibitory loops described in figure 25.

The fact that there are relatively elevated levels of TGF-β mRNA in tamoxifen resistant tumours could imply that the resistance to TGF-β occurs at a post-transcriptional level eg deletion of TGF-β receptors or loss of the ability to cleave the TGF-β precursor.

Although a potential autocrine inhibitory loop involving TGF-β has been described following treatment of ZR-75 cells with α-IF, one would expect that the final outcome of this treatment would depend on other factors which could interact with and alter the cellular response to TGF-β. For this reason, a series of experiments were performed to measure the response to exogenous growth factors of mink lung epithelial cells and their oncogene transfectants.

It was shown in this study that transfection of mink lung epithelial cells with c-*myc* and activated H-*ras* oncogenes altered the cellular reponse to exogenous growth factors. TGF-β inhibits incorporation of ^{3}H-thymidine by mink lung epithelial cells in a dose dependent manner with an ID_{50} of 120pg/ml. EGF has the opposite effect with stimulation of ^{3}H-thymidine incorporation. In addition, we have demonstrated that thymidine incorporation depends on the relative concentrations of the growth factors when the cells are exposed to them simultaneously.

TGF-β and EGF antagonise each other in the mink lung assay. Presumably this reflects the situation in vivo in which stimulatory and inhibitory factors operate simultaneously in the microenvironment of the cells to increase the fidelity of control of proliferation.

The nature of the interaction between EGF and TGF-β is unknown. Assoian et al (100) noted a transient decrease, followed by an increase, in EGF receptors on NRK cells treated with TGF-β. Massague (101) has also investigated the interaction between exogenous growth factors and cell surface receptor number/affinity and has shown that TGF-β treatment decreases phenotypic expression of EGF receptors in exponentially growing NRK cells, but does not alter EGF binding by non-transformed mink lung cells. Coffey et al (102) demonstrated that TGF-β reversibly inhibited the growth of mouse keratinocytes by competitively antagonising the effect of EGF or TGF-α. These authors concluded that the inhibitory effects of TGF-β were mediated by events distal to TGF-β ligand- receptor interactions.

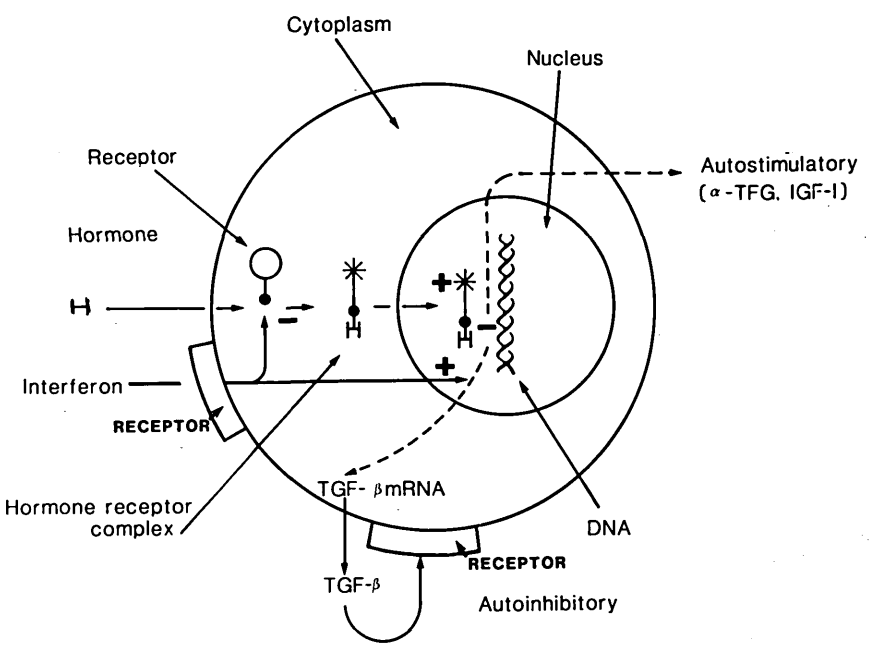
A different pattern of growth factor responsiveness is apparent in the oncogene transfected cells. C-*myc* transfected cells have a a reduced response to TGF-β and an exaggerated response to EGF. Transfection of the mink lung epithelial cells with the normal *ras* protooncogene did not materially alter their response to TGF-β and EGF.

However, activated H-*ras* transfection virtually abolished the effect of both TGF-β and EGF.

Kelekar and Cole (103) immortalised early passage rat kidney cells with c-*myc* and H-*ras* and showed that the c-*myc* transfected cells responded to serum better than EGF and insulin alone and gave a maximal proliferative response to EGF and insulin. The results of Stern et al (104) are more similar to our own. They transfected Fischer rat 3T3 cells with *myc* and *ras* oncogenes and treated them with EGF, PDGF and TGF-β. *Ras* transfected cells were insensitive to all three growth factors and secreted EGF-like activity and TGF-β into cell culture medium. *Myc*-transfected cells did not produce elevated levels of growth factors but did form colonies in soft agar in the presence of EGF, but not TGF-β and PDGF.

Balk et al (105) transfected chicken heart mesenchymal cells with v-*myc* and v-H-*ras* oncogenes and showed that cells transformed with v-*myc* were hypersensitive to EGF whereas those transformed with v-H-*ras* were refactory to EGF and brain fibroblast growth factor. Houck et al (106) showed that resistance to the growth inhibitory effect of TGF-β was induced by transfection of an activated H-*ras* oncogene into rat liver epithelial cells.

In summary, this thesis presents data supportive of the hypothesis that a proportion of the cytostatic effect of α-IF on the human breast carcinoma cell line, ZR-75, is mediated by TGF-β, acting, in a way, like a second messenger. The bioactivity of TGF-β can be modulated by the presence of other exogenous growth factors (e.g. antagonised by EGF) and by oncogene transfection and activation. Therefore, if one supposes that in certain instances, α-IF will increase the rate of transcription of the TGF-β gene leading to an increase in activated TGF-β , an antiproliferative effect will only be seen if the cellular microenvironment and genotypic milieu are cooperative.



SCHEME SHOWING POSSIBLE AUTOCRINE INHIBITION OF BREAST CANCER CELL PROLIFERATION IN RESPONSE TO α-INTERFERON

FIGURE 26

Diagram summarising the biochemical interaction of α-IF with ZR-75 cells.

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