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Development and characterization of an *in vitro* model for liver homeostasis.

by

Caroline Jane Brabyn

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Department of Biochemistry

Faculty of Medicine

University of Ottawa

Ottawa, Canada

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UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

Abstract

The major objective of this work was the development of an *in vitro* model for liver homeostasis which would allow the future study of early events in cell proliferation and cell death.

The model which was set up involves growing T51B rat liver epithelial cells with a single dose of 1nM epidermal growth factor (EGF). This results in a period of hyperplasia where the cells reach double the control cell numbers two days after EGF addition. This is then followed by a decrease in cell numbers and the cell density returns to around the confluent control level five days after EGF addition.

The model was investigated to ascertain whether the decrease in cell numbers three to five days after EGF addition was due to an increase in apoptosis. The results from light and electron microscopy studies, from the electrophoresis of T51B cell DNA and from the quantification of nuclear fragmentation indicated that the cells do die via an increase in apoptosis. The electron microscopy studies also show that healthy T51B cells can phagocytose apoptotic bodies. This suggests that the model is more physiological than other *in vitro* models of apoptosis.

Cell growth studies and EGF binding studies were carried out in order to try to determine which events, if any, are EGF specific. The results from these studies suggest that occupancy of the low affinity binding site of the EGF receptor is responsible for the hyperproliferation seen when the T51B cells are grown with high doses of

EGF. These studies also suggest that the apoptosis could be triggered by the down-regulation of the receptor, in a manner analogous to the removal of a trophic hormone in other systems.

Thus this work describes the development and characterization an *in vitro* model of liver homeostasis which closely parallels *in vivo* systems where animals are given mitogenic stimuli, and it also provides a good system for studying the role of EGF in cell proliferation and apoptosis.

Dedication

This work is dedicated to my husband Graham for his unfailing support, encouragement and understanding.

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Abbreviations

BME.....Basal media eagle

BSA.....Bovine serum albumin

cpm.....Counts per minute

ECM.....Extracellular matrix

EDTA.....Ethylenediamine tetraacetic acid

EGF.....Epidermal growth factor

FN.....Fragmented nucleus

gjc.....Gap-junctional communication

H33258.....Hoechst fluorochrom 33258

IGF-1.....Insulin-like growth factor-1

IL-3.....Interleukin-3

MPF.....Maturation promoting factor

PBS.....Phosphate buffered saline

PKC.....Protein kinase C

PS.....Phosphatidylserine

PTK.....Protein tyrosine kinase

REL.....Rat liver epithelial cell

SH2/3.....Src homology 2/3

SP-40,40.....Serum protein 40,40

TGF α.....Transforming growth factor α

Tdr.....Deoxythymidine

Introduction

There is cell turnover in most tissues of higher animals and cellular homeostasis is maintained by a balance between cell proliferation and cell death. In some tissues such as the epithelium of the skin or the mucosa of the bowel, mitotic activity is almost entirely confined to a layer of stem cells. When these divide, one half must remain in the stem cell layer while the other half must begin a process of differentiation and gradually move towards the organ surface where they are eventually sloughed off (Baserga, 1965). These tissues have what is termed renewing cell populations and the cells proliferate relatively quickly (Zajicek, Oren and Weinreb, 1985). In other tissues, in which surface attrition does not occur, the situation is not as clear. These tissues, for example liver and kidney, have expanding populations of cells which proliferate very slowly. Hepatocytes in the liver have a mean cell cycle time of 37 days and a life expectation of 201 days (Zajicek et al., 1985). However, following partial resection such as hepatectomy, the tissue proliferates rapidly. Whether new cells are generated exclusively from stem cells or differentiated cells is not certain. If stem cells are involved at all then the cell divisions must result, at least statistically, in one half of the daughter cells becoming new stem cells and the other half beginning a program of differentiation that ultimately leads to cell death (Prehn, 1992).

Cell proliferation

When a cell proliferates its DNA is replicated and two daughter cells are produced. The cell cycle can be defined as the interval between the completion of mitosis in the parent cell and the completion of the next mitosis in one or both daughter cells. The cycle itself can then be broken down into various phases; G_1, S, G_2 and M. S and M describe specific events within the cycle; the synthesis of DNA and mitosis. The other phases represent a convenient scientific notation (Baserga, 1985). G_1 and G_2 are the post-mitotic gap and the pre-mitotic gap respectively; intervals of time when cells are not undergoing either DNA synthesis or mitosis. There is also another phase, G_0, that is used to describe cells that are no longer cycling and are outside the cell cycle. These quiescent cells are capable of re-entering the cell cycle given the appropriate stimuli. For example, the long-lived hepatocytes in liver are thought to exist most of the time in G_0 but are capable of re-entering the cell cycle following partial hepatectomy (Zajicek et al., 1985).

The control of cell proliferation involves both regulatory events initiated at the plasma membrane that control re-entry into the cell cycle and intracellular biochemical changes that direct the process of cell division itself. The former events are initiated in higher eukaryotes by growth factors, such as epidermal growth factor (EGF), binding to a plasma membrane receptor and thereby activating the receptor's inherent protein tyrosine kinase activity. The receptor can

then phosphorylate other proteins on tyrosine residues and signal transduction throughout the cell is initiated. Just what these signal transduction events are, particularly in relation to EGF, will be discussed later.

A great deal of progress has been made in recent years in elucidating the controls which operate within the cell cycle of a dividing cell. The frog oocyte and egg have been widely used to study these biochemical reactions underlying the regulation of the cell cycle. Masui and Markert, 1971 showed that the unfertilized egg contained a cytoplasmic activity that could induce immature oocytes to undergo meiotic maturation. They named this activity maturation promoting factor (MPF). MPF has now been purified from frog and starfish eggs (Lohka, Hayes and Maller, 1988; Labbe, Lee, Nurse, Picadr and Doree, 1988). It is a protein kinase complex which comprises a type B cyclin and p34. Cyclins were discovered as major products of maternal mRNA in marine invertebrate eggs. Their sudden destruction by proteolysis just before anaphase in the cell cycle provided their names and suggested their importance in cell cycle regulation (Evans, Rosenthal, Youngblom, Distel and Hunt, 1983). p34 is a serine/threonine protein kinase and the human cDNA was isolated by Lee and Nurse, 1987. The cDNA was shown to have 62% identity to the fission yeast (*Schizosaccharomyces pombe*) and the budding yeast (*Saccharomyces cerevisiae*) genes. The yeast genes, *cdc2* and CDC28, are needed at two points in the cell cycle; in late G_1 and again in G_2 (Nurse and Thuriaux, 1980; Beach, Durkacz, and Nurse, 1982). In all eukaryotic cells studied to date initiation of

mitosis is triggered by activation of an MPF complex (Nurse, 1990). The current oversimplified model for the action of MPF is as follows. Phosphorylated p34 and a type B cyclin come together to form the MPF complex during G_2. Active MPF is then able to phosphorylate various substrates which lead to mitosis. The MPF is then inactivated by dephosphorylation of p34 which leads to degradation of the cyclin; the cell then enters G_1, p34 is phosphorylated again and the cycle continues (Nurse, 1990).

The G_2-M transition is not the only control point in the cell cycle; in most mammalian cell types regulation of cell proliferation occurs in G_1 phase. Various G_1 cyclins have recently been identified in budding yeast (reviewed in Lew and Reed, 1992). They interact with p34 and control cell cycle commitment at "START" in G_1. It also appears that there are yeast cyclins that control the initiation or progression through S phase. However, no structural homologue of a G_1 or S phase cyclin has been found in mammalian cells as yet. It is now becoming evident that in multicellular organisms there are a family of p34-related proteins that regulate different parts of the cell cycle (Pines, 1993). Thus as ever the picture is becoming more complex.

Cell death

There are at least three categories of cell death in higher organisms; necrosis, which occurs as a result of massive tissue damage; terminal differentiation of specialized tissues such as the skin,

intestine and red blood cells; and apoptosis, which is a process of active cellular destruction that requires the expression of a number of genes (Bursch, Kleine and Tenniswood, 1990a). Necrosis represents the traditional view of a passive death, whereas apoptosis, in contrast, is an active process involving gene transcription and protein synthesis.

It is now becoming apparent that apoptosis occurs in most animal tissues at some stage of their development. It is evident in the tadpole tail during metamorphosis (Kerr, Harmon, and Searle, 1974); in the regions between developing digits in amniotes (Hammar and Mottet, 1971) and in self-reactive lymphocytes (Goldstein, Ojcius and Young, 1991) to name but a few examples. When this project was started apoptosis was less well known and was viewed as a rather specialized process which occurred during development and in a few instances in the immune system. It is now recognized that apoptosis is as fundamental a process as cell proliferation and differentiation and that it plays a major role in the day to day functioning of the organism, for example, in tissue homeostasis. When various organs, such as rat liver, kidney and pancreas (Bursch, Lauer, Timmermann-Trosiener, Barthel, Schuppler and Schulte-Hermann, 1984; Ledda-Columbano, Columbano, Coni, Faa and Pani, 1989; Oates, Morgan and Light, 1986), are exposed to mitogenic stimuli there is an initial rapid burst of hyperplasia and an increase in organ weight. Once the stimulus is removed there is an increase in apoptosis and over time the organ is restored to its original size. It appears that this increase in apoptosis is part of a regulatory mechanism triggered to counteract the effects of the hyperplasia.

In morphological terms apoptosis is characterized by nuclear compaction and cytoplasmic condensation followed by the breakdown of the nucleus into discrete fragments. Cells break down into membrane-bound apoptotic bodies which are then either taken up and degraded by adjacent cells or, in some instances, by macrophages (Kerr, Searle, Harmon and Bishop, 1987). Chromatin condensation requires the activation of an endogenous Ca^{2+}, Mg^{2+}-dependent endonuclease that degrades the nuclear DNA to produce the hyperchromatic nucleus typical of apoptotic bodies (Wyllie, 1980). During cytoplasmic condensation the desmosomal contacts between cells and the cytoskeleton are disrupted. This results in the condensation of the intermediate filaments around the nucleus and the formation of blebs on the cell surface (Kinn and Allen, 1981). In the next stage of apoptosis the cell itself is fragmented. Apoptotic bodies contain portions of the fragmented nucleus and an array of intact organelles such as mitochondria and lysosomes. This stage is critical to the whole process because it ensures that the cytoplasm and organelles of the apoptotic cell are repackaged without leakage of any of the cellular components into the extracellular space or blood stream. Thus an inflammatory response is not initiated. This is very different from necrosis which usually follows a gross perturbation to the cellular environment. There is loss of plasma membrane integrity which leads to ions and water flowing down their chemical and osmotic gradients. The result is rapid cell and organelle swelling leading to rupture of the cell and spillage of its contents into the extracellular environment. A cell undergoing necrosis can thus cause further injury and even death to neighbouring cells and can provoke an inflammatory response.

While the morphological events accompanying apoptosis are well documented the precise biochemical events remain to be determined. Since apoptosis is an active process requiring gene transcription and protein synthesis it should be possible to identify the new macromolecules specifically made when a cell starts the apoptotic cycle. Likewise it should also be possible to determine the signal transduction events which initiate and control the process. Unfortunately this area of apoptosis remains a black box. It has been difficult, even using subtractive techniques, to determine unequivocally whether any new mRNA or protein is the product of a dying cell or a surviving cell because apoptotic systems involve populations of cells. Added to this difficulty is the fact that the dying cell is rapidly degraded which means that new mRNA and proteins probably disappear quickly. It can be appreciated that the search is not going to be easy. Moreover, as the search has widened the area has become more complex and various early assumptions have been turned aside. Later, in the discussion, I will consider *de novo* gene expression during apoptosis; the relevance of the activation of an endogenous endonuclease as a definitive marker for apoptosis; and the term "apoptosis" itself.

Over the past few years there has been controversy as to the need for mRNA and protein synthesis during apoptosis. Traditionally the addition of RNA and/or protein synthesis inhibitors to an apoptotic system was seen to inhibit the apoptosis (reviewed in Martin, 1993). However, there are now increasing numbers of reports which show that apoptosis is actually induced, not inhibited, when cells are incubated

with RNA or protein synthesis inhibitors, for example, HL-60 cells and macrophages (Martin, Lennon, Bonham and Cotter, 1990; Waring, 1990). Similar concentrations of inhibitors are used in the various systems, so the solution is not straight forward. Seamus Martin in his recent review (Martin, 1993) suggests that the signal transduction pathway might be such that apoptosis can be triggered at multiple points along the pathway depending upon the initiating stimulus. He postulates that the early events require protein synthesis whereas later events do not. In the early part of this putative pathway the cell must transduce the signal via synthesis of various proteins whereas later-acting stimuli can bypass this stage and also bypass the requirement for protein synthesis. Built into this model are inhibitors of apoptosis which also need to be synthesized. Thus if the pathway is accessed at some other point the inhibition of protein synthesis would remove the inhibitor(s) and the cell would die via apoptosis. Judging by the varied reports in the literature on the role of protein synthesis in apoptosis this model fits very well even though the full details of the apoptotic pathway have not yet been described.

Apoptosis, like cell proliferation, depends on the ability of a cell to respond to external stimuli. Apoptosis can be seen as a normal physiological response to environmental influences, for example the regression of the lactating breast after weaning and other normal involutional processes triggered by hormonal effects (Kerr and Harmon, 1991). It is reasonable, therefore, to speculate that the components of signal transduction pathways regulating cell proliferation and cell

cycle progression might also be involved in the anti-proliferative aspects of apoptosis. Calcium is thought to play a regulatory function in apoptosis because early studies indicated that sustained elevation of cytosolic calcium could induce apoptosis in thymocytes (Durant, Homo and Duval, 1980; Cohen and Duke, 1984). The exact role that calcium plays in apoptosis is unclear although one obvious role could be the activation of the Ca^{2+}, Mg^{2+}-dependent endonuclease (Wyllie, 1980). However, an increase in intracellular calcium may not be a universal requirement for initiation of apoptosis. For example, in human leukemia HL-60 cells induced to undergo apoptosis by treatment with calcium ionophores, DNA fragmentation correlated with intracellular acidification rather than increased calcium concentration (Barry and Eastman, 1992). An increase in cytosolic calcium does occur at a later stage of apoptosis in these cells (Lennon, Kilfeather, Hallett, Campbell and Cotter, 1992), which suggests that calcium influx might play different roles during apoptosis in different cell types. It is also possible that calcium's role in apoptosis could be through the activation of a calcium-binding protein such as calmodulin. Calmodulin antagonists have been shown to inhibit DNA fragmentation in thymocytes treated with glucocorticoids (McConkey, Nicotera, Hartzell, Bellomo, Wyllie and Orrenius, 1989). This implies that activation of calcium/calmodulin-dependent enzymes could be part of the apoptotic pathway.

Protein phosphorylation appears to be the major intracellular mechanism by which cells respond to extracellular stimuli such as

hormones and growth factors (Hardie, 1989). This means that protein kinases are bound to play a role in the signal transduction and control of the apoptotic process. One of the major signal transduction pathways involves the enzyme protein kinase C (Parker, Coussens, Totty, Rhee, Young, Chen, Stabel, Waterfield and Ullrich, 1986) and this kinase has been widely implicated in serving both a positive and a negative role in apoptosis. Activation of protein kinase C (PKC) by phorbol esters inhibits apoptosis in a number of systems; differentiating leukemic cells grown without interleukin-3 (IL-3) (Lotem, Cragoe and Sachs, 1991); CL8 mouse fibroblasts exposed to ionizing radiation (Tomei, Kanter and Wenner, 1988); vascular endothelial cells after fibroblast growth factor removal (Araki, Simada, Kaji and Hayashi, 1990) and mature lymphocytes grown without fetal bovine serum (Lucas, Solano and Sanz, 1991). On the other hand, activation of PKC also appears to induce apoptosis in some systems. T cell hybridoma cells undergo apoptosis following PKC-α activation by anti-CD3 antibodies (Jin, Inaba and Saitoh, 1992) and this effect is blocked by inhibitors of protein phosphorylation. Similar findings have been reported in HL-60 cells (Cotter, Lennon, Glynn and Green, 1992b) and thymocytes (Ojeda, Guarda, Maldonado and Folch, 1990). In general PKC appears to inhibit rather than promote apoptosis, but again the image of an apoptotic pathway with multiple access points is brought to mind. At some points PKC activation will inhibit apoptosis whereas at other points, maybe in different cell types, PKC activation leads to apoptosis.

Because apoptosis is viewed as requiring the induction of an apoptosis-specific genetic program proteins which regulate transcription are also thought to play a role in this form of cell death. Recently it has been found that overexpression of the proto-oncogene *c-myc* leads to apoptosis in an IL-3-dependent murine myeloid cell line following IL-3 withdrawal (Askew, Ashmun, Simmons and Cleveland, 1991), and in rat fibroblasts under conditions of serum growth factor deprivation (Evan, Wyllie, Gilbert, Littlewood, Land, Brooks, Waters, Penn and Hancock, 1992). It has also been shown that the expression of *c-fos* and *c-jun* (but not *c-myc*) is rapidly induced in IL-2 and IL-6-dependent mouse myeloma cell lines after induction of apoptosis by growth factor removal (Colotta, Polentarutti, Sironi and Mantovani, 1992). The addition of *c-fos* and *c-jun* antisense oligonucleotides to the system also protected the cells from apoptosis, suggesting an active role for *fos* and *jun* in the onset of cell death.

Over the past few years interest has been growing in the *bcl-2* gene which encodes an inner mitochondrial membrane protein that can block programmed cell death. Hockenbery, Zutter, Hickey, Nahm and Korsmeyer, 1991 reported that the protein is associated with tissues characterized by apoptotic cell death such as surviving T cells in the thymic medulla; glandular epithelium in which hormones regulate hyperplasia and involution; complex differentiating epithelium, such as skin and intestine, characterized by long-lived stem cells; and long-lived postmitotic cells such as neurons. They suggested that the Bcl-2 protein functions as an antidote to apoptosis, conferring longevity to

progenitor and effector cells in these tissues. The findings that the gene also has an oncogenic form and that expression of the human gene in the nematode *Caenorhabditis elegans* reduces the number of programmed cell deaths in this worm (Vaux, Weissman and Kim, 1992) suggest that this protein plays a very important role in the control of apoptosis in a wide range of organisms. However, the precise function(s) of the protein are unclear. It was thought that the mitochondrial localization of the protein might provide a clue to its function, for example, it could be involved in oxidative phosphorylation. However, it is now known that Bcl-2 can block apoptosis in cells lacking mitochondrial DNA suggesting that its localization in other areas of the cell, such as the nucleus and endoplasmic reticulum, could be important in preventing cell death (Jacobson, Burne, King, Miyashita, Reed and Raff, 1993). More recently it has been found that Bcl-2 heterodimerizes *in vivo* with a protein termed Bax (Oltvai, Milliman and Korsmeyer, 1993). Interestingly Bax is a conserved homologue of Bcl-2 which accelerates apoptosis. It has been suggested that the ratio of Bax to Bcl-2 in a cell, and thus whether there is a predominance of either Bax:Bax or Bcl-2:Bcl-2 homodimers, determines whether a cell receiving a "death order" accepts it or ignores it (reviewed in Barinaga, 1994). It seems certain that once the biochemical functions of the Bcl-2 family of proteins have been determined a clearer picture of the apoptotic pathway will start to emerge.

In summary, apoptosis is a fundamental process as important to the life of a cell as proliferation and differentiation. It is easy to

imagine how deregulation of apoptosis could lead to a disease state, for example, neuron degeneration in Alzheimer's disease. It is also possible that in some diseases cells lose their ability to undergo apoptosis, for example, in autoimmune diseases where self-reactive T-cells are produced. While the morphological aspects of apoptosis are well documented the biochemical details still need to be fully described in order to provide further insight into the general role of apoptosis in animal development and normal and malignant cell growth.

Epidermal Growth Factor

Epidermal growth factor was first reported over thirty years ago as a purified protein that could induce precocious eyelid opening and incisor eruption in newborn mice (Cohen, 1962). It was the second growth factor to be identified, following shortly after the discovery of nerve growth factor. Since its discovery, EGF has served as a model system, particularly for the identification of molecular pathways that describe the intracellular activity of growth factors. One reason for this is that the EGF system, which consists of EGF-like ligands and receptors, is ubiquitously distributed, perhaps with the exception of mature hematopoietic cell types, in all mammals examined (Carpenter and Wahl, 1990). EGF-like growth-regulating molecules have also been reported in a variety of non-mammalian organisms such as birds, reptiles, fish, flies and worms (Carpenter, 1993). However, in spite of the huge body of scientific literature surrounding EGF its true biological role(s) still remains unclear (Browne, 1991). EGF appears to

play a role in organismic development, affecting both growth and differentiation and it exerts its biological activity not only in epithelial cells, but in mesothelial and endothelial cells as well, both *in vivo* and *in vitro* (Marti, Burwen and Jones, 1989).

EGF is a small, heat-stable polypeptide of between 48 and 53 amino acids and has a molecular weight of about 6,000 (Browne, 1991). It has three disulphide bonds, all of which are required for full biological activity (Taylor, Mitchell and Cohen, 1972), and it has a highly conserved structure (Browne, 1991). It is now becoming apparent that there is a family of EGF-like molecules, all of which are encoded by distinct genes. The other members of this family are transforming growth factor α (TGF-α) (Marquardt, Hunkapiller, Hood and Todaro, 1984) and amphiregulin (Shoyab, Plowman, McDonald, Bradley and Todaro, 1989), both of which are now established as small, soluble growth factors capable of being produced by human tissues. These EGF-like molecules are defined by two main characteristics: high affinity binding to the EGF receptor and production of mitogenic responses in EGF-sensitive cells (Carpenter and Cohen, 1990). There are a series of viral growth factors which display considerable homology to EGF and it is also clear that the EGF structural motif occurs in a wide range of proteins, such as blood clotting factors, which have not been formally classified as growth factors (Browne, 1991). This suggests that the EGF-like sequences have a stable and useful structural element, and may also represent a universally recognizable signal structure.

The structure of the EGF precursor is quite surprising. The cDNA for the prepro-EGF predicts a protein of 1217 amino acids with a molecular weight of 128,000 (Gray, Dull and Ullrich, 1983). The first 25 amino acids from the N terminus are thought to represent the "pre"-signal sequence, while it is only residues 976-1029 of the precursor which represent the mature EGF. The precursor contains glycosylation sites and seven other cysteine-rich regions with a high degree of sequence homology to EGF (Scott, Urdea, Quiroga, Sanchez-Pescador, Fing, Selby, Rutter and Bell, 1983). The extreme C terminus of the precursor has a 20 amino acid hydrophobic region (residues 1039-1058). It has been suggested that this motif could act as a transmembrane region which serves to present the bulk of the EGF precursor as a cell membrane-bound glycoprotein with all the carbohydrate sites and EGF-like sites on the extracellular surface of the plasma membrane (Scott, Patterson, Rall, Bell, Crawford, Penschow, Niall and Coughlan, 1985). The portions of prepro-EGF which contain the EGF-like sequences are found in two clusters separated by 400 amino acids. This arrangement is remarkably homologous to the low-density lipoprotein receptor (Sudhof, Russell, Goldstein, Brown, Sanchez-Pescador and Bell, 1985), which has one extracellular domain containing two well-separated clusters of EGF-like sequences. Therefore we have the interesting possibilities that EGF can act as a "classical" soluble growth factor, released from its precursor when it is required; that the precursor can act as a receptor for some other factor, whose action involves the release of EGF and that the precursor represents a membrane-bound factor, perhaps involved in cell-

cell or cell-extracellular matrix interactions, since EGF-like sequences have been found in blood clotting proteins.

The sources of EGF in the human body are not clear; it is secreted from the submaxillary gland, and Brunner's glands, located in the small intestine, are known to synthesize and secrete significant amounts of EGF (Marti et al., 1989). However, these are not likely to be the only sites of synthesis and secretion because all body fluids are enriched in EGF relative to plasma (Marti et al., 1989). This suggests either an active transport mechanism from plasma into secretions or production of EGF by fluid-secreting cells.

The roles of EGF in liver are uncertain and there is some controversy over its effects on liver regeneration after partial hepatectomy. It has been found that low doses of EGF stimulate liver regeneration (Rasmussen, Jorgensen, Almdal, Kirkgaard and Skov Olsen, 1992) and that an antibody to EGF, administered at the same time as partial hepatectomy in rats, is associated with a reduced regenerative response (Skov Olsen, Boesby, Kirkegaard, Therkelsen, Almdal, Poulsen and Nexo, 1988). However, in a re-examination of this last observation, it was found that injection of an anti-EGF antibody did not significantly alter the DNA synthetic response to partial hepatectomy 24 hours after resection (Vesey, Selden, Woodman and Hodgson, 1992b). But the authors did find that the antibody could completely block the EGF-induced proliferative response of hepatocytes in culture.

The major requirement for a cell's responsiveness to EGF is of course possession of an EGF receptor. The receptor is a single-chain glycoprotein of 1186 amino acids and has a molecular weight of 170,000 (Browne, 1991). It comprises an extracellular EGF-binding domain, a transmembrane domain, an intracellular protein tyrosine kinase domain and an extreme C-terminal domain which contains autophosphorylation sites (Hunter, Ling and Cooper, 1984; Ullrich, Coussens, Hayflick, Dull, Gray, Tam, Lee, Yarden, Livermann, Schlessinger, Downward, Mayes, White, Waterfield and Seeburg, 1984). One of the earliest events following EGF binding to its receptor is an increase in the phosphorylation of a number of key intracellular regulatory proteins (Cohen, Ushiro, Stoscheck and Chinkers, 1982), including several phospholipases (Margolis, Rhee, Felder, Mervic, Lyall, Levitzki, Ullrich, Zilberstein and Schlessinger, 1989), and protein kinases and phosphatases (Yang, Chou, Huang, Song and Chen, 1989). The EGF receptor has been demonstrated to be a potent protein kinase which can be autophosphorylated at tyrosine residues (Ushiro and Cohen, 1980; Hunter and Cooper, 1981) as well as being phosphorylated at serine and threonine residues by PKC (Hunter et al., 1984).

One very interesting finding about the EGF receptor is the large degree of homology that it shows with the *erb*-B viral oncogene product (Downward, Yarden, Mayes, Scrace, Totty, Stockwell, Ullrich, Schlessinger and Waterfield, 1984). The *erb*-B protein resembles the EGF receptor without the EGF-binding domain and thus its oncogenic activity

is thought to be expressed as a constitutive, permanently activated, unregulatable receptor.

A number of recent advances have been made in our understanding of how signals generated at the plasma membrane are transduced to the nucleus, and they have a direct bearing on the action of EGF. It has been found that most proteins that associate with the activated EGF receptor (for example, phospholipase C-γ1 and $p21^{ras}$ GTPase-activating protein) contain conserved noncatalytic domains termed Src homology (SH) regions 2 and 3 (Koch, Anderson, Moran, Ellis and Pawson, 1991). Genetic and biochemical data have implicated the SH2 domain in regulating general protein-protein interactions whereas SH3 domains are found in proteins that comprise or associate with the cytoskeleton and membrane (Koch et al., 1991). More recently a series of papers has been published which describe how an SH2 and SH3 containing protein, Grb2, causes Ras activation (reviewed in McCormick, 1993). This leads to a complete description of a signal transduction pathway from the plasma membrane to the nucleus. An outline of the pathway is as follows. Grb2 binds to activated (autophosphorylated) receptors, such as the EGF receptor, by the SH2 domain and to another protein, Sos, by the SH3 domain. Receptor-associated Sos provokes guanine-nucleotide exchange on Ras. Inactive GDP-bound Ras is converted to the active GTP-bound form, triggering an activation cascade involving the Raf-1 and MAP serine-threonine kinases. This results in the phosphorylation of transcription factors, such as the components of AP-1, and ultimately to changes in gene expression.

The binding of EGF to its receptor causes the rapid formation of clusters of EGF-receptor complexes on the cell surface (Carpenter and Cohen, 1976). These complexes become "captured" by clathrin-coated pits and are then rapidly internalized. This event involves EGF degradation within lysosomal vesicles and a form of receptor down-regulation by removal of receptor sites from the cell surface. However, the receptor-mediated endocytosis and degradation of EGF is not necessary for biological activity (Savion, Vlodavsky and Gospodarowicz, 1980). It has also been suggested that a prolonged (8 hour) occupancy of the receptor is needed for the full expression of mitogenic activity and thus that the rapid EGF internalization, degradation and receptor down-regulation may be irrelevant to the effects of the growth factor (Shechter, Hernaez and Cuatrecasas, 1978). It also seems that receptor recycling occurs and that, on average, each receptor enters a degradation pathway after a second or third internalization event (Carpenter, 1987). The presence of EGF might also increase the expression of the receptor by exerting control at transcriptional, post-transcriptional and post-translational levels (Das, Kasavan and Khire, 1990) but at present the literature appears confused on this issue.

It is generally accepted that the EGF receptor has two binding affinities for EGF but there is some debate over their physiological relevance. Two classes of models have been proposed to explain how the binding of EGF to the extracellular binding domain of the receptor activates the cytoplasmic kinase domain. In one class of models,

activation occurs through an intramolecular mechanism (Koland and Cerione, 1988). Binding of EGF is conjectured to induce a conformational change in the receptor which is transmitted through the transmembrane domain and activates intrinsic receptor protein tyrosine kinase (PTK) activity (Bertics and Gill, 1985). In the other class of models, activation is controlled by receptor aggregation (Yarden and Schlessinger, 1987). In these models, the binding of EGF to monomeric receptors enhances receptor aggregation. The aggregated receptors have higher affinity for EGF than the non-aggregated receptors and possess elevated PTK activity. This has led to the latter model being favoured as physiologically relevant (Schlessinger, 1988). The low affinity binding site has been taken to be the monomeric (inactive) receptor and the high affinity site the aggregated (active) receptor. However, a recent paper by Wofsy, Goldstein, Lund and Wiley, 1992 throws doubt on this conclusion by re-examining EGF receptor Scatchard plots. They suggest that this interpretation of the two types of binding site is incorrect because it predicts that a Scatchard plot will have curvature characteristic of positive cooperativity, the opposite of what is seen experimentally. They postulate that the most likely model involves EGF binding with higher affinity to a receptor in a dimer with both sites free than to the second receptor in a dimer with one site already occupied. With this model there is negative cooperativity of the distinct receptors in the dimer (or higher oligomer) and the Scatchard plot would be negatively curved. This suggests that the occupancy of both high and low affinity binding sites could affect the signal transduction of the EGF receptor.

Objectives

The major objective of this project was to set up an *in vitro* model of tissue homeostasis that would facilitate future study of early events, such as signal transduction and gene expression, occurring in cell proliferation and cell death.

I used the T51B rat liver epithelial cell line to set up the model because it was already known that liver could be induced to enter hyperplasia/apoptosis cycles *in vivo* (Bursch et al., 1984). This cell line was established from the liver of a normal adult Fisher rat (Swierenga, Whitfield and Karasaki, 1978) and the cells seem to be derived from the bile duct epithelium (Marceau, Germain, Goyette, Nöel and Gourdeau, 1986). While these cells are not hepatocytes it has been found that during liver regeneration, and presumably regression, all cell types in the liver are affected (Callea, Brisigotti, Fabbretti, Sciot, Van Eyken and Favret, 1991). One other advantage is that numerous studies have been carried out on T51B cell proliferation under various conditions (Boynton and Whitfield, 1980; Franks, Kleine and Aasheim, 1990).

Two early models were set up using drugs to induce apoptosis in the cells; the calcium ionophore A23187 (Brabyn and Kleine, 1990) and DNA topoisomerase II inhibitors (Walker, Sikorska, Brabyn, Kleine, Franks, Welsh, Brown and Roy, 1991). However, once set up these systems were not pursued further because it was felt that they did not provide good models for cell proliferation and there was the concern that the drugs

might bypass many of the early signaling events in apoptosis. In other words I might be missing the early events in cell proliferation and cell death that the model was being designed to study. The final model was based on a report of the effects of growing primary cultures of neonatal rat liver cells with repeated exposures to EGF (Armato, Romano, Andreis, Paccagnella and Marchesini, 1986). The authors observed that the hepatocytes and stromal cells went through the hyperplasia/cell death cycle seen in the *in vivo* systems. It therefore seemed probable that the T51B cell line could also be induced to undergo this growth pattern with high concentrations of EGF. The advantage of this model over the drug models is that the signaling events originate outside the cell when the growth factor binds to its receptor.

Once the main objective had been achieved the further objectives were as follows: 1) To show that the cell death in the system was occurring through apoptosis; 2) To investigate the system further to determine why the cell proliferation and cell death were occurring and, following on from this, 3) To determine which events, if any, were EGF-specific.

Materials and Methods

Materials

Bovine calf serum was purchased from Flow Laboratories Inc. (McLean, VA). Human recombinant epidermal growth factor and insulin-like growth factor-1 were obtained from Upstate Biotechnology Industries Inc. (Lake Placid, NY). Proteinase K, DNA ladder standard and basal media eagle were ordered from Gibco BRL (Burlington, Ont). RNase A was purchased from Boehringer Manheim Canada Ltd. (Laval, PQ). The [^{3}H methyl] thymidine was obtained from NEN (Boston, MA). The NTB-2 nuclear track emulsion was obtained from Eastman Kodak (Rochester, NY). Hoechst dye (fluorochrom 33258), bovine serum albumin fraction V (BSA) and gentamycin sulphate were purchased from Sigma Chemicals Co. (St Louis, MO). The (3-[^{125}I]iodotyrosyl) human recombinant epidermal growth factor was obtained from Amersham Canada Ltd, (Oakville, Ont). All other chemicals of reagent grade were purchased from BDH Chemicals Ltd. or Fisher Scientific Ltd. (Ottawa, Ont).

Cell culture

T51B rat liver epithelial cells were cultured in a medium consisting of 90% (v/v) BME (basal media eagle) and 10% (v/v) bovine calf serum containing 0.1g/litre gentamycin sulphate and maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Prior to

experiments cells were detached by brief exposure to 0.05% (w/v) trypsin, 1mM EDTA in phosphate buffered saline (PBS). For the majority of the experiments the cells were then plated at low density (approximately 7,000 cells/cm^2) and grown for 3 days before the addition of epidermal growth factor dissolved in PBS, to a final concentration of 1nM. Control cultures were left untreated. All assays were performed in duplicate.

Cell number counts

The cells were grown as described above and at various time intervals the cells were detached from the culture plates by exposure to 0.05% (w/v) trypsin, 1mM EDTA in PBS. A single cell suspension was obtained by passing the liquid through a 21 gauge needle two times. The number of cells per dish was determined using a Coulter Counter. Each sample was counted twice.

Electron microscopy

The cells were grown as described above and at various time intervals after EGF addition were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in cacodylate buffer (pH 7.4) for 1 hour. Samples were washed with 0.1M cacodylate buffer and post fixed in 1% osmium tetroxide for 1 hour. After post fixation the cells were stained with 2% uranyl acetate and dehydrated through ethanol series and embedded. The embedding was done by inverting the epon-filled capsules over the cell

culture dishes. Electron microscopic blocks were "popped off" and thin sections were made by *en-face* cutting. The sections were mounted on copper grids coated with formvar and carbon, stained with lead citrate and viewed using a Zeiss 109 electron microscope.

DNA Fragmentation

Gel analysis of DNA from T51B cells was carried out as described by Smith, Williams, Kingston, Jenkinson and Owen, 1989. Cells were harvested as two pools; floating cells were pelleted from the culture media and attached cells were removed from the culture dish by trypsinization and then pelleted. The cell pellets were washed with PBS before being resuspended in 150μl lysis buffer (10 mM EDTA, 50 mM Tris-HCl (pH 8.0) containing 0.5% (w/v) sodium lauryl sarkosinate). The lysed cells were then either stored at -80°C or treated immediately with 10μl proteinase K (20mg/ml) and 10μl RNase A (2mg/ml) and incubated at 25°C overnight. The DNA samples were then extracted using phenol/chloroform, precipitated overnight at -20°C and resuspended in 50-100μl 10mM Tris-HCl (pH 8.0), 1mM EDTA. The samples were mixed with 5μl of TAE (30mM Tris-HCl (pH 8.0), 2mM EDTA, 1% (v/v) glacial acetic acid) containing 30% (w/v) sucrose and 0.25% (w/v) Bromophenol blue before being loaded into the dry wells of a 2% (w/v) agarose gel containing 0.1μg/ml ethidium bromide. Electrophoresis was carried out in TAE until the marker dye had migrated half way down the gel.

DNA synthesis and nuclear fragmentation

To determine the proportion of cells undergoing DNA synthesis and nuclear fragmentation cells were grown on glass coverslips and exposed to 0.5μCi/ml [^{3}H methyl] thymidine (specific activity 10-25 Ci/mmol) for 24 hours. The cells were then fixed by the addition of 4% paraformaldehyde (w/v) in PBS and stored at 4°C. The coverslips were mounted on slides and covered with a layer of NTB-2 nuclear track emulsion. Five days later the slides were developed and stained with Hoechst dye, fluorochrom 33258 (H33258), 1μg/ml in PBS as described by Oberhammer, Pavelka, Sharma, Tiefenbacher, Purchio, Bursch and Schulte-Hermann, 1992.

Fluorescent H33258-stained nuclei were scored as follows: (i) normal nucleus, uncondensed chromatin dispersed over the whole nucleus; (ii) fragmented nucleus, groups of isolated pieces of condensed chromatin after nuclear fragmentation. An illustration is shown in Figure 5. In addition, nuclei which had incorporated [^{3}H methyl] thymidine, as evidenced by silver grains above the nuclei, were also counted. Fluorescence was observed using a Zeiss Axioskop microscope. Three fields per dish were counted and the average number of nuclei per field was calculated.

EGF receptor binding assays

The EGF receptor binding assays were carried out as described by Gladhaug and Christoffersen (1987). T51B cells were grown in 24 well plates under various conditions depending on the experiment. The cells were washed twice with ice-cold assay buffer (Krebs Ringer buffer pH 7.4 containing 1% (w/v) BSA) and then incubated in 200μl of buffer at 4°C for 30 minutes. The binding reaction was initiated by the addition of a 50μl mixture of labeled ((3-[^{125}I]iodotyrosyl)EGF) and unlabeled EGF. The cells were then incubated at 4°C for 24 hours to achieve equilibrium binding (Figure 15). The ratio of labeled to unlabeled EGF was varied depending on the final concentration of EGF being used. This was done in order to obtain sufficiently high counts per minute (cpm) in each well. The specific activity of the (3-[^{125}I]iodotyrosyl)EGF used was approximately 1,400 Ci/mmol. The binding reaction was terminated by removing the incubation media and washing the cells three times with ice-cold wash buffer (PBS pH 7.4 containing 0.2% (w/v) BSA). The cells were then dissolved in 1ml NaOH (1N) at room temperature for 2 hours. The dissolved cells were counted in a γ-counter in order to determine the amount of bound ligand in each well.

When binding studies were carried out on cells incubated with EGF the cells were washed twice with an acid buffer (50mM glycine and 0.1M NaCl (pH 4); 2 times 1 minute) prior to the assay buffer washes in order to remove any surface-bound EGF (Vesey, Selden and Hodgson, 1992a). The cells were then treated as described above.

The protein concentration in the wells was determined by the method of Bradford, 1976.

Data for the Scatchard plots were analysed using the computer assisted ENZFITTER program (Elsevier-Biosoft Ltd).

Statistics

Statistical calculations were performed by the computer assisted INSTAT program (GraphPad Software Inc). The level of significance (P-value) was obtained using the paired two-tailed Student's t-test comparing the mean ±SE of treated values to untreated (control) values. P values <0.05 were considered statistically significant.

Results

The first part of this section describes the setting up and characterization of an *in vitro* model system to study cell proliferation and cell death of rat liver cells. I took as a starting point work carried out by Armato et al., 1986 where primary cultures of neonatal rat liver cells were grown with repeated additions of 1nM EGF.

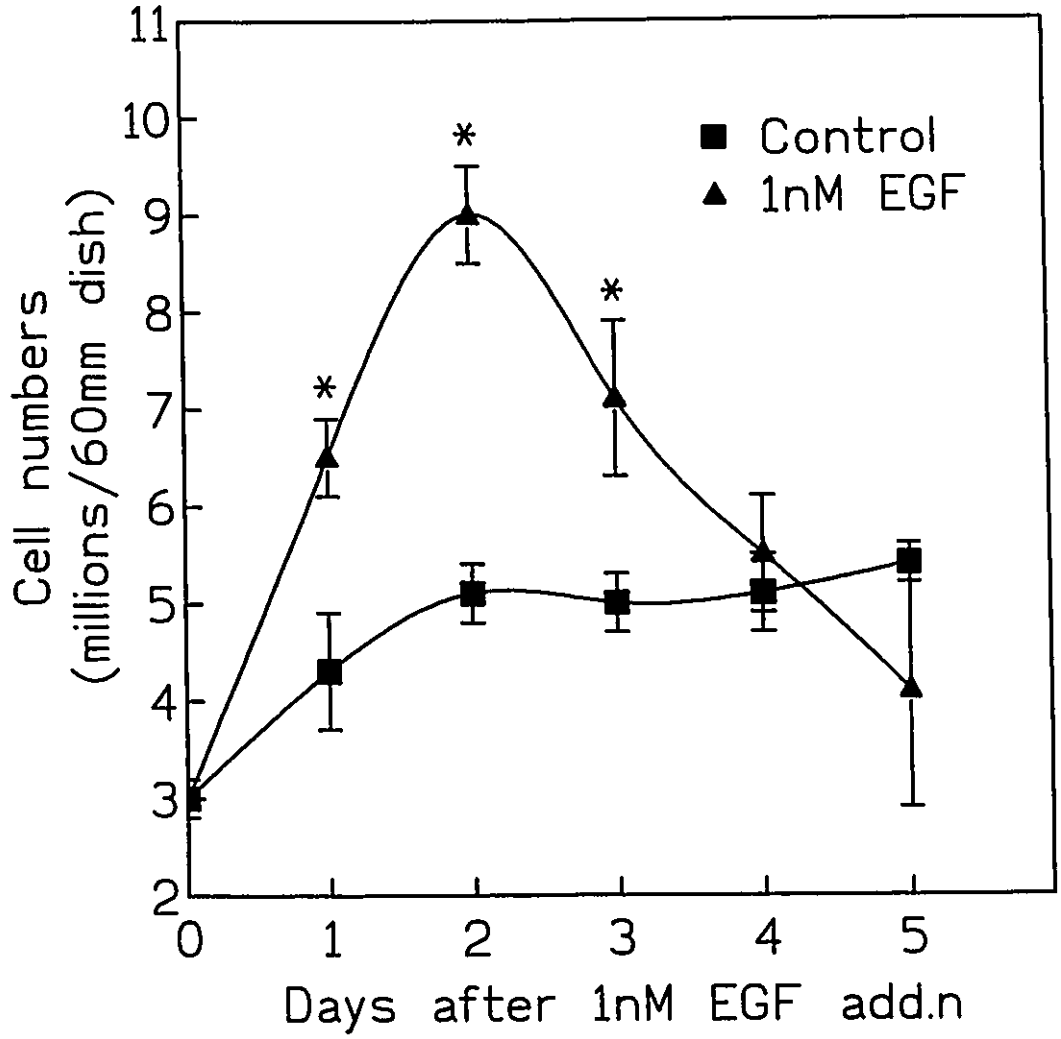
Effect of EGF on cell numbers

The addition of 1nM EGF to proliferating T51B cells results in significant changes in cell numbers (Figure 1). The cell numbers increase rapidly, peaking two days after EGF addition at about twice the control level. This is followed by a decrease in cell counts with the numbers returning to around the control level by day 5. This cell growth curve parallels *in vivo* systems where rats are given various stimuli which give rise to liver hyperplasia (Bursch et al., 1984; Columbano, Ledda-Columbano, Coni, Faa, Liguori, Santa-Cruz and Pani, 1985). After removal of the hyperplastic stimulus livers atrophy through apoptosis back to the original organ weight.

Figure 1: Effect of 1nM EGF on cell numbers.

T51B cells were plated at low density (7,000 cells/cm^2) and grown for 3 days before the addition of EGF. Control cultures were left untreated. Cell numbers were determined daily using a Coulter Counter. Statistical analysis performed was the two-tailed paired Student's t-test, comparing the mean ±SE from three different experiments of control and EGF-treated cultures. A level of significance of $P<0.05$ is indicated as *.

Figure 1


Cell numbers
(millions/60mm dish)
11
10
9
8
7
6
5
4
3
2
0
1
2
3
4
5
Days after 1nM EGF add.n
Control
1nM EGF
*
*
*

Light microscopy of cells treated with EGF

The control cells are confluent by day 3 (6 days after plating) which means that the EGF-treated cells at days 1 to 3 are either more densely packed or smaller than the controls. Using light microscopy it appears that although the EGF-treated cells might be smaller than the control cells they are definitely much more densely packed onto the culture plates (Figures 2.1 and 2.2).

Changes in cell morphology are seen as early as 24 hours after EGF addition (Figure 2.1). At first the cells become elongated, and then, as the cell density increases, the cells appear to grow over one another (Figure 2.2; day 3). A very clear example of this is shown with the Hoechst dye (Figure 5). However, it is not certain whether the cells retain the elongated morphology as the density increases. Even as the cell numbers are returning towards the control levels the EGF-treated cells do not have the regular cobblestone appearance of the control cells (Figure 2.3; day 5). Continuing the experiment for another two days (Figure 2.4) indicates that the EGF-treated cells again exhibit the elongated morphology seen at day 1. This photograph also shows three clear examples of "blebbing" as cells start to go through the apoptotic pathway and it also indicates that by this stage the cells need refeeding since numerous vacuoles can be seen. This is in contrast to the control cultures which still maintain a healthy cobblestone appearance (not shown).

Figures 2.1 to 2.4: Light microscopy of cells treated with EGF.

T51B cells were plated at low density (7,000 cells/cm^2) and grown on coverslips. The cells were grown for 3 days before the addition of EGF to a final concentration of 1nM. Control cultures were left untreated. The cells were then fixed and stained with haematoxylin and eosin at various times after EGF addition and viewed using a Zeiss Axioskop microscope. Magnification: Days 1, 3 and 5 x220; Day 7 x440.

Figure 2.1

Day 1 Control

Day 1 EGF-treated

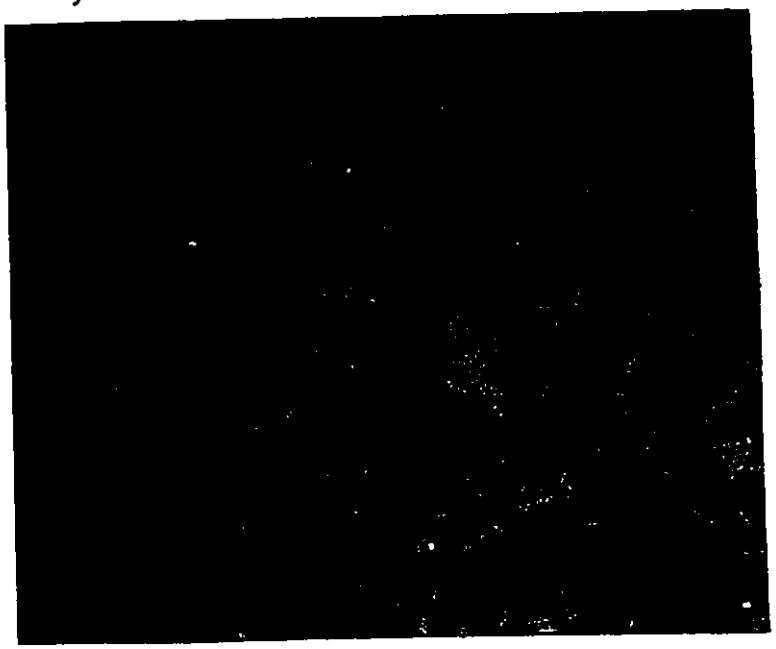


Figure 2.2

Day 3 Control

Day 3 EGF-treated

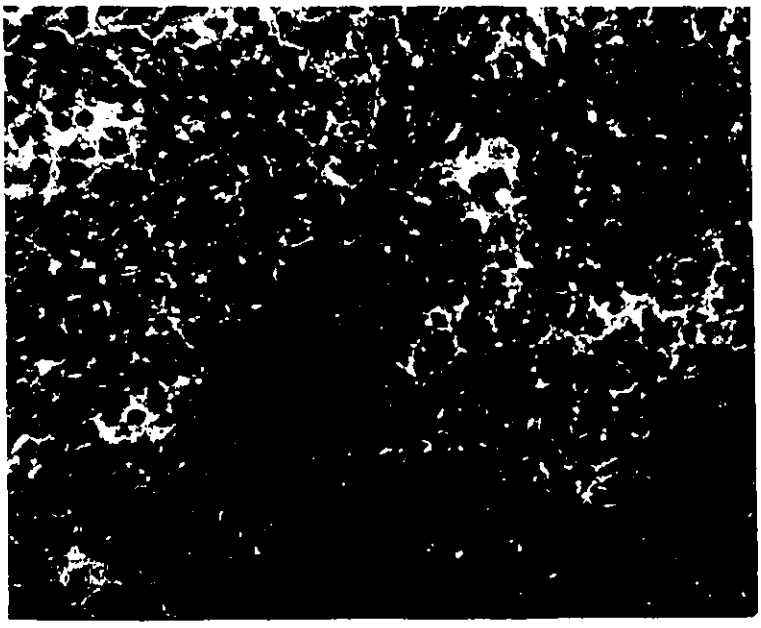
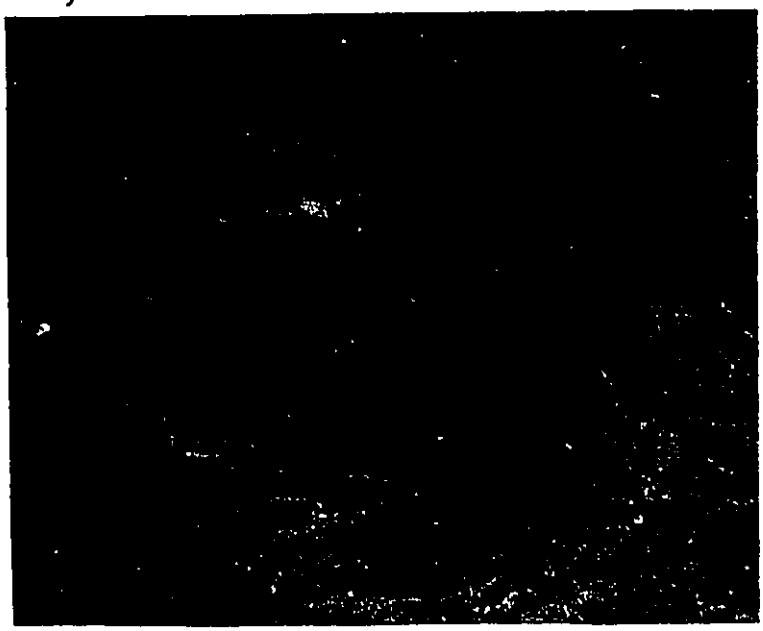


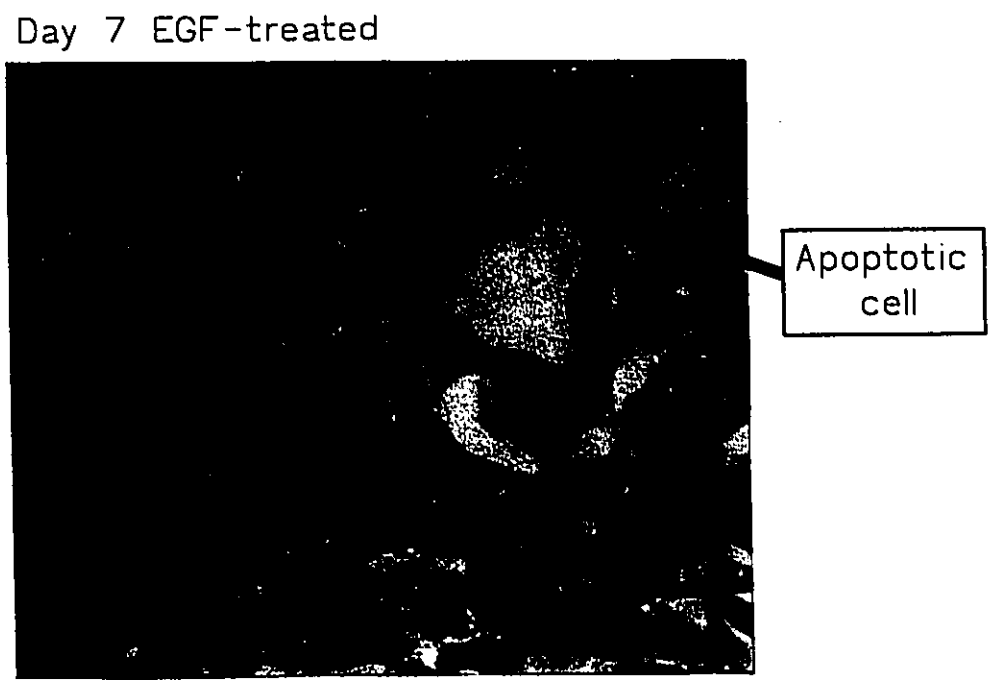
Figure 2.3

Day 5 Control



Day 5 EGF-treated

Figure 2.4



In order to further characterize the effects of the addition of 1nM EGF to proliferating T51B cells I decided to investigate the decrease in cell numbers to determine if the cells were dying through apoptosis.

Electron microscopy of cells treated with EGF

Under the electron microscope many of the treated cells exhibit secondary lysosomes, presumably from the engulfment of apoptotic bodies. Figure 3.2 shows representative examples of this four days after EGF addition. These cells, and the control cells (Figure 3.1), appear to be healthy because their nuclei are intact, they have plentiful mitochondria and endoplasmic reticula and energy-dependent processes are seen to be forming. It is of interest that the healthy cells are capable of phagocytosis and this is consistent with the findings of Ledda-Columbano, Coni, Curto, Giacomini, Faa, Oliverio, Piancentini and Columbano, 1991 in their studies on rat hepatocytes *in vivo* and the work of Armato et al., 1986 on primary cultures of neonatal rat liver cells.

DNA fragmentation

The degradation of DNA into oligonucleosomal-sized fragments is characteristic of apoptosis in various systems (Orrenius et al., 1989; Wyllie, 1980), especially in cells of lymphatic or thymic origin (Wyllie, 1980). Figure 4 shows that T51B cell DNA can also be degraded into this characteristic ladder. The most striking pattern is seen with

Figures 3.1 and 3.2: Electron microscopy of cells treated with EGF.

T51B cells were plated at low density (7,000 cells/cm^2) and grown for 3 days before the addition of EGF to a final concentration of 1nM. Control cultures were left untreated. The cells were then fixed and treated as described in Materials and Methods at various time intervals after EGF addition. Magnification: Control x 6,890; EGF-treated x 5,380. Photographs a and b in Figure 3.2 are two representative examples of EGF-treated cells; 'S' indicates the secondary lysosomes.

Figure 3.1

Day 4 Control

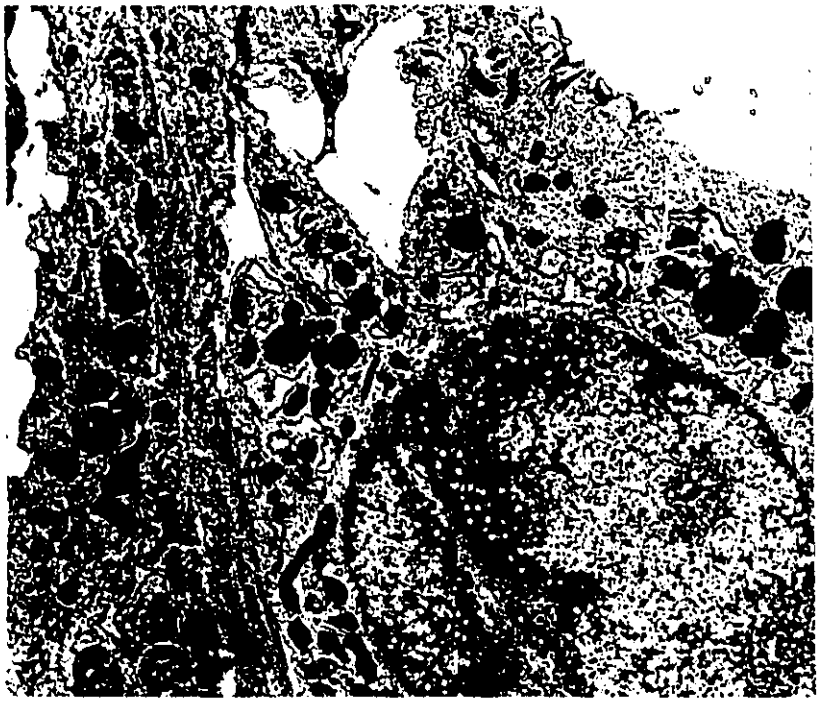
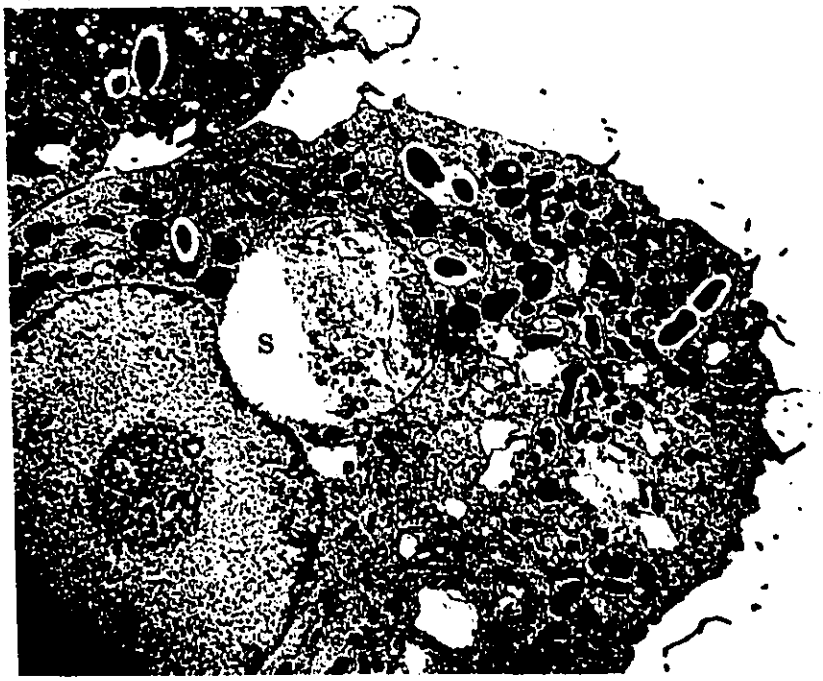


Figure 3.2

Day 4 EGF-treated

a.



b.

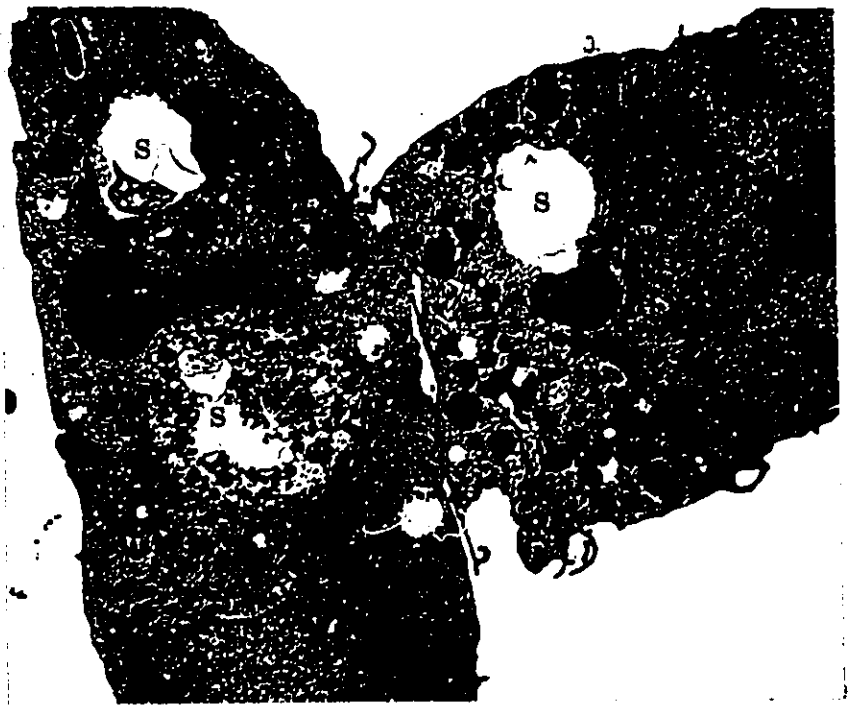
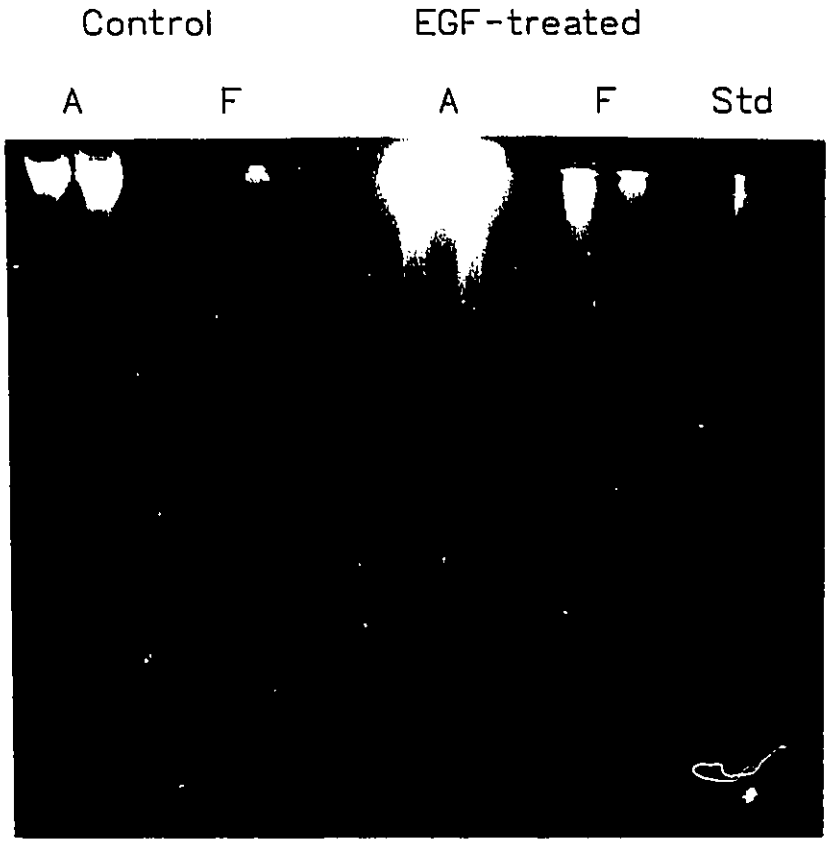


Figure 4: Agarose gel electrophoresis of total cellular DNA from T51B cells.

T51B cells were plated at low density (7,000 cells/cm^2) in 150 mm tissue culture dishes and grown for 3 days before the addition of EGF to a final concentration of 1nM. Control cultures were left untreated. The cells were then harvested 5 days after EGF addition and the DNA was prepared and electrophoresed as described in Materials and Methods. A = Attached cells; samples prepared from one dish of cells, in duplicate. F = Floating cells; samples prepared from the pooled media of three dishes of cells, in duplicate. Std = 123 base pair DNA ladder.

Figure 4



attached cells which have been exposed to EGF for 5 days. However, it must be noted that this procedure was carried out in a purely qualitative fashion. Faint ladders can also be seen with the cells floating in the media and the attached control cultures.

DNA synthesis and nuclear fragmentation

Condensation of chromatin and nuclear fragmentation are known to reflect sequential stages of apoptosis (Kerr et al., 1987). Staining nuclei with the Hoechst dye, fluorochrom 33258 (H33258), provides a very useful method for distinguishing between normal and fragmented nuclei. An illustration is shown in Figure 5. This allows quantification of a process which is often only analyzed in a qualitative fashion. I developed a double-labeling technique with tritiated thymidine incorporation and H33258 staining which enabled me to quantify DNA synthesis and nuclear fragmentation in my system.

The cell number data (Figure 1) suggest that the control cultures are confluent by day 3 and this is borne out by the DNA synthesis data (Figure 6). During days 3 to 5 the control cells are proliferating at a low rate and the cell numbers remain fairly constant as does the percentage of fragmented nuclei (Figure 7). Thus cell proliferation is balanced by cell death. The EGF-treated cells are still proliferating at day 3 but then there is an almost complete cessation of DNA synthesis by day 5 (Figure 6). There is a sharp rise in the number of fragmented

Figure 5: DNA synthesis and nuclear fragmentation in T51B cells after EGF treatment.

T51B cells were plated at low density (7,000 cells/cm^2) into wells containing glass coverslips. The cells were grown for 3 days before the addition of EGF to a final concentration of 1nM. Control cultures were left untreated. The cells were exposed to 0.5μCi/ml [tritiated-methyl] thymidine for 24 hours before being fixed, treated as described in Materials and Methods and then incubated with Hoechst dye at various time intervals after EGF addition. The cells were viewed using a Zeiss Axioskop microscope. Magnification: x440. The figure shows A) nuclei which have not incorporated Tdr (pale, diffuse H33258 staining); B) nuclei which have incorporated Tdr (dark silver grains over H33258 staining) and C) fragmented nuclei (bright H33258 staining).

Figure 5

Day 4 EGF-treated

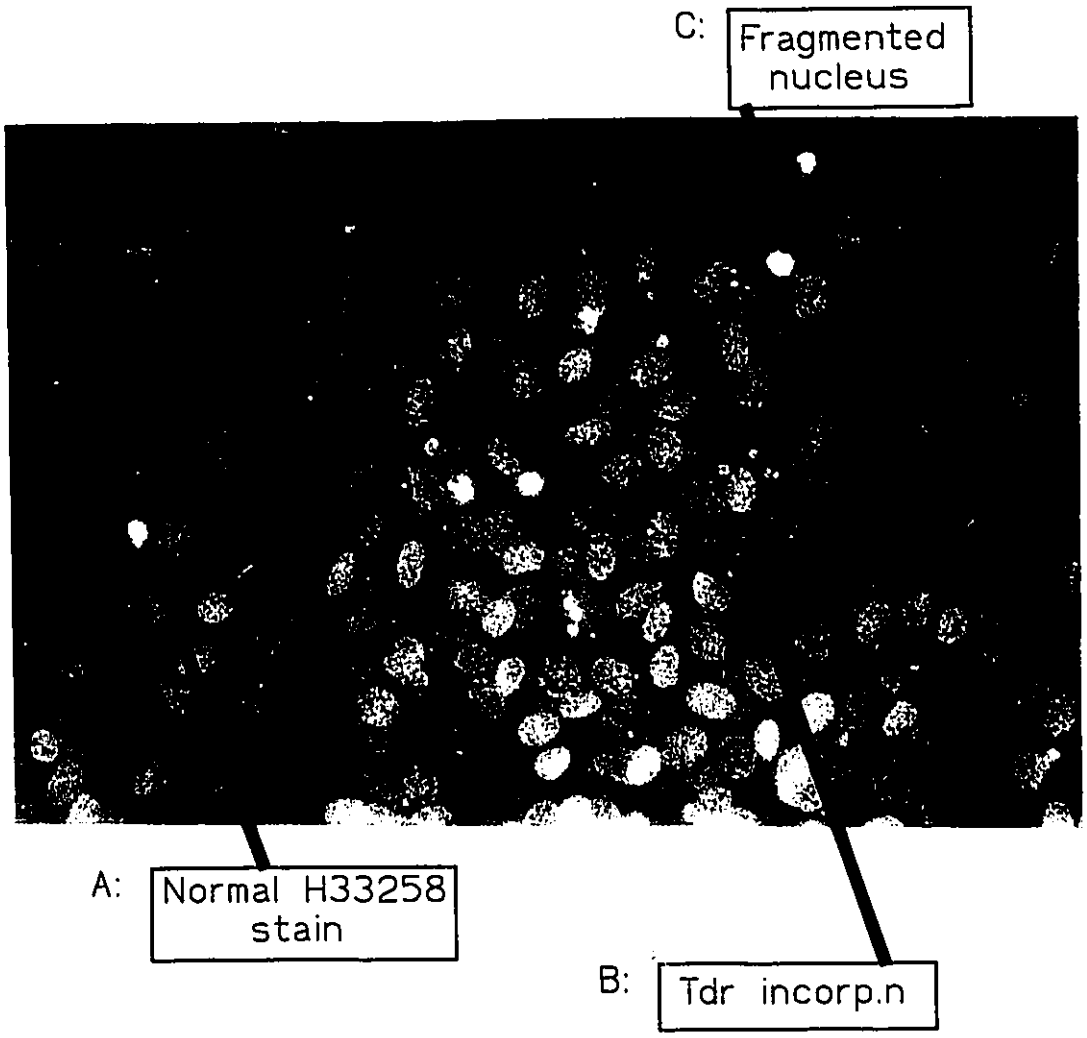


Figure 6: Effect of 1nM EGF on DNA synthesis.

T51B cells were plated at low density (7,000 cells/cm^2) into wells containing glass coverslips. The cells were grown for 3 days before the addition of EGF. Control cultures were left untreated. The cells were exposed to 0.5μCi/ml tritiated thymidine for 24 hours prior to fixation at various time intervals after EGF addition. The number of nuclei per field incorporating tritiated thymidine was determined as described in Materials and Methods. Statistical analysis performed was the two-tailed paired Student's t-test, comparing the mean ±SE from three different experiments of control and EGF-treated cultures. The levels of significance of $P<0.01$ and $P<0.10$ are indicated as * and ** respectively.

Figure 6

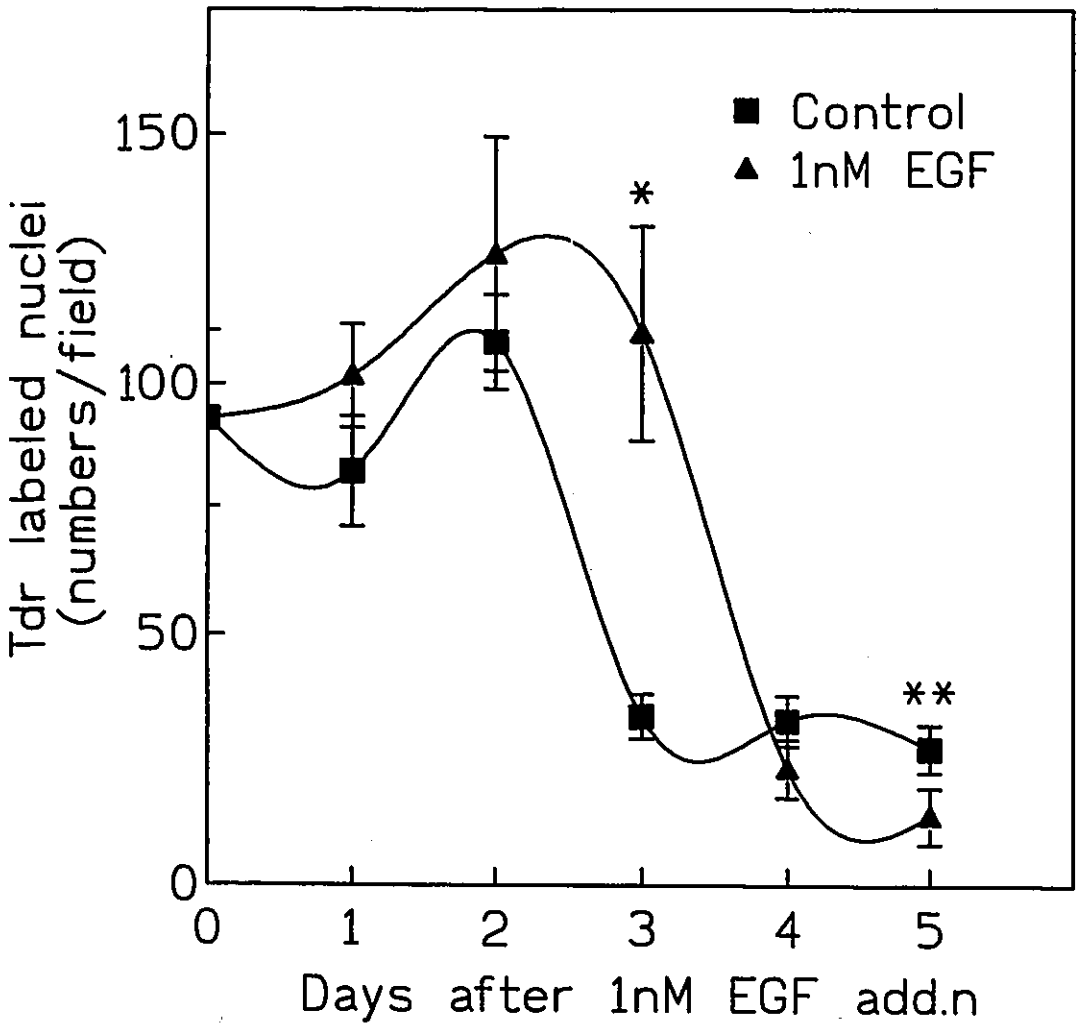
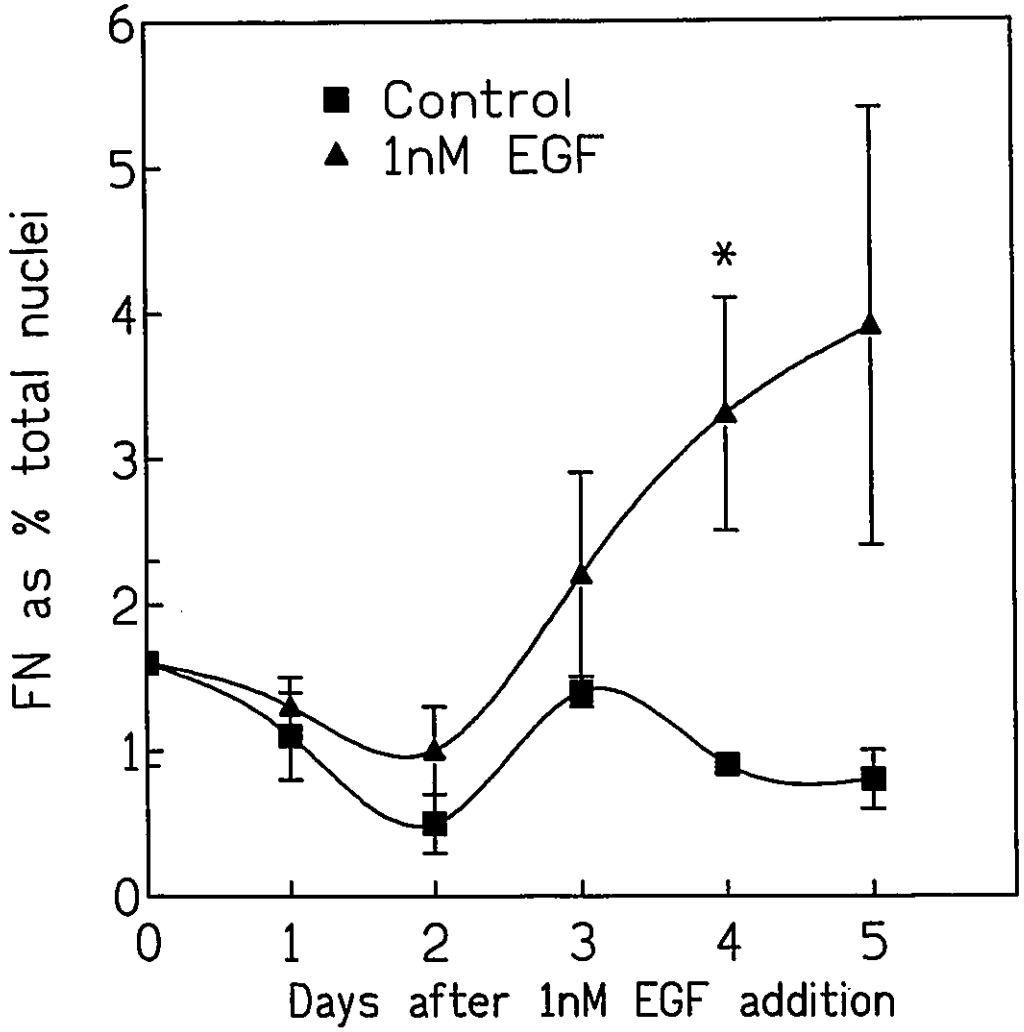

Control
1nM EGF
Tdr labeled nuclei (numbers/field)
150
100
50
0
0
1
2
3
4
5
Days after 1nM EGF add.n
*
**

Figure 7: Effect of 1nM EGF on nuclear fragmentation.

T51B cells were plated at low density (7,000 cells/cm^2) into wells containing glass coverslips. The cells were grown for 3 days before the addition of EGF. Control cultures were left untreated. The cells were fixed daily and later stained with Hoechst dye. The number of fragmented nuclei (FN) as a percentage of total nuclei was determined as described in Materials and Methods. Statistical analysis performed was the two-tailed paired Student's t-test, comparing the mean ±SE from three different experiments of control and EGF-treated cultures. A level of significance of $P<0.05$ is indicated as *.

Figure 7


Control
1nM EGF
*
FN as % total nuclei
Days after 1nM EGF addition
0
1
2
3
4
5
6

nuclei starting around day 3 (Figure 7) which coincides with the decrease in cell numbers seen in the growth curve (Figure 1).

Through the double labeling technique I have noticed that very few (less than 5%) of the fragmented nuclei have incorporated tritiated thymidine (data not shown). This suggests that the cells which die have not recently gone through the S phase of the cell cycle.

The next part of this section describes the results from cell growth experiments carried out to explore the model system further. Having established that the addition of 1nM EGF to proliferating T51B cells results in a period of hyperplasia followed by a rapid decrease in cell numbers as cells die by apoptosis I wanted to alter various conditions in the system and observe how it responded. The aim was to gain insights into why the hyperplasia and apoptosis were occurring and which events, if any, were EGF-specific.

Effect of IGF-1 on cell numbers

Insulin-like Growth Factor-1 (IGF-1) appears to be relevant to liver growth *in vivo* (Quin, 1992; Caro, Poulos, Ittoop, Pories, Flickinger and Sinha, 1988) so I wanted to determine whether this growth factor could also cause the hyperplasia and/or cell death seen with 1nM EGF in T51B cells. Three doses of IGF-1 were used, 0.1, 1.0 and 10nM, in order to provide a wide range of receptor occupancy levels. The Kd for the IGF-1 receptor is approximately 1nM (Tollefsen, Thompson and Peterson, 1987; Werner, Woloschak, Stannard, Shen-Orr, Roberts and

LeRoith, 1991). Figures 8.1 and 8.2 indicate that the doses of IGF-1 used do not cause significant changes in cell growth compared to control cultures.

Effect of daily media changes on cell numbers

Serum contains numerous growth factors at concentrations that are much closer to physiological levels than the large excesses that are used in this model system (Delbert, Salido and Barajas, 1989). Changing the cells' media daily replenishes the supply of hormones and nutrients and possibly removes any growth inhibitors produced as the cells become confluent. I speculated that one way to obtain the hyperplasia seen with 1nM EGF might be to give the cells fresh media and serum every day. However, Figure 9 indicates that this is not the case and that changing the media daily has no effect on cell numbers.

Effect on cell numbers of adding EGF daily versus once

Armato et al., 1986 in their work with primary cultures of neonatal rat liver cells added EGF to the cells daily whereas I found that a

Figures 8.1 and 8.2: Effect of different IGF-1 doses on cell numbers.

T51B cells were plated at low density (7,000 cells/cm^2) and grown for 3 days before the addition of IGF-1 dissolved in PBS. Control cultures were left untreated. Cell numbers were determined daily using a Coulter Counter. Statistical analysis performed was the two-tailed paired Student's t-test, comparing the mean ±SE from three different experiments of control and IGF-1-treated cultures. There was no significant difference between experimental and untreated cultures.

Figure 8.1

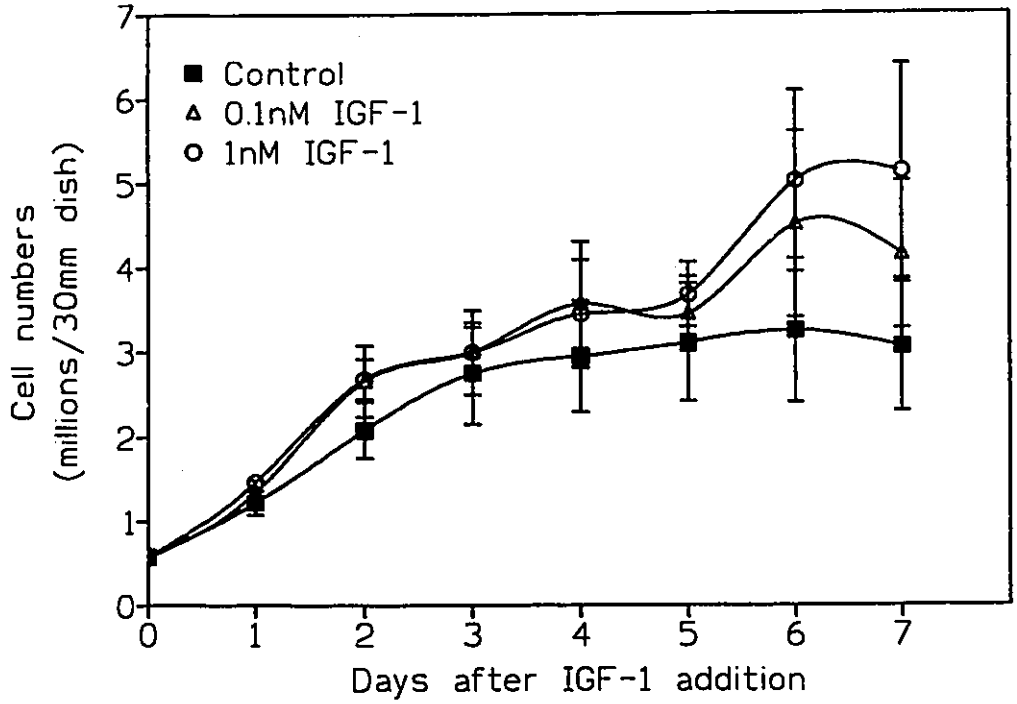


Figure 8.2

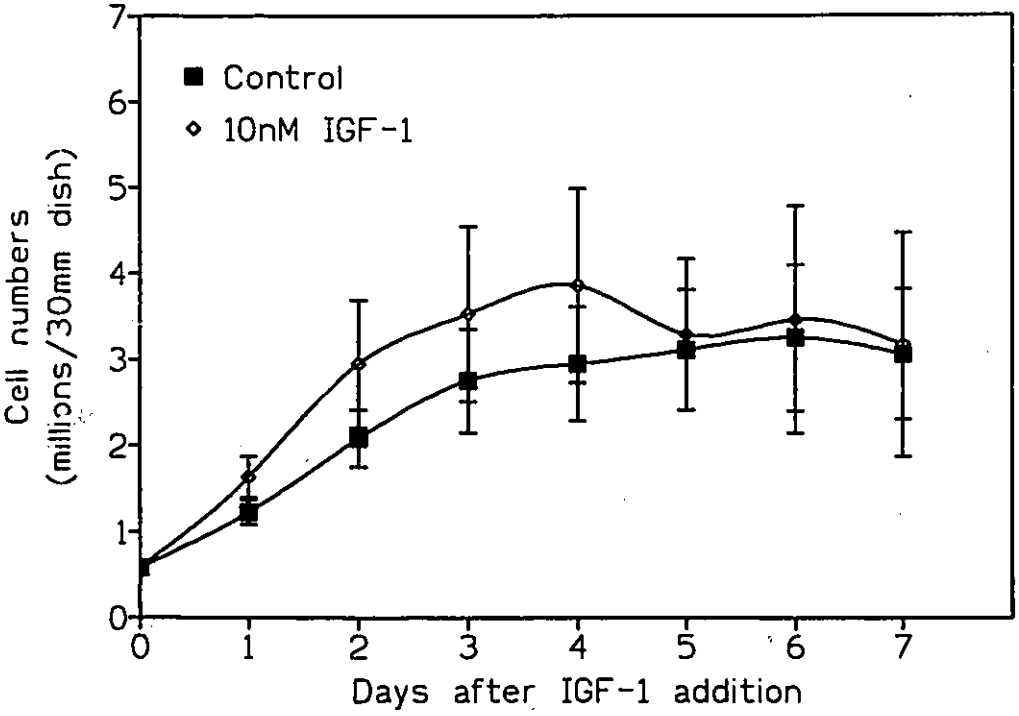
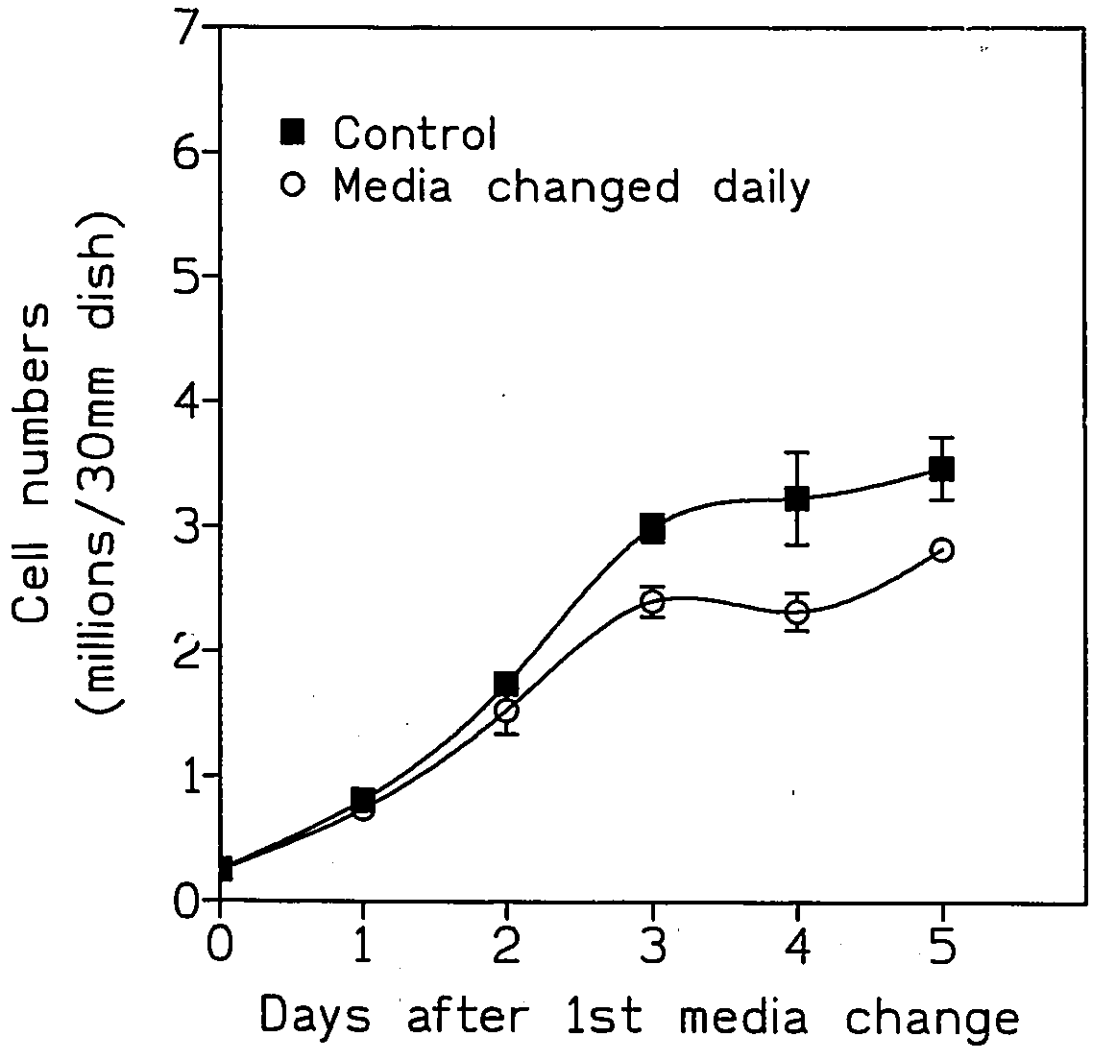


Figure 9: Effect of daily media changes on cell numbers.

T51B cells were plated at low density (7,000 cells/cm^2) and grown for three days before the start of the experiment. Control cultures were left untreated while experimental cultures were subject to a daily media change. Cell numbers were determined daily using a Coulter Counter. Statistical analysis performed was the two-tailed paired Student's t-test, comparing the mean ±SE from three different experiments of control and media-changed cultures. There was no significant difference between experimental and untreated cultures.

Figure 9


Control
Media changed daily
Cell numbers
(millions/30mm dish)
Days after 1st media change
0
1
2
3
4
5
6
7

single dose was sufficient to generate levels of cell proliferation and cell death significantly different from controls. Interestingly when I did add EGF daily the cell growth curve was identical to that produced when the EGF was added once (Figures 10.1 to 10.4). This suggests that if the EGF is rapidly taken up by the cells and degraded it is the initial dose that is important to generate the growth curve seen. On the other hand if the EGF is not degraded rapidly and the daily additions lead to higher and higher concentrations then at 1nM EGF the growth response is at its maximum.

Effect on cell numbers of adding EGF 1 day versus 3 days after plating.

It is not clear what is causing the EGF-treated cells to die once they reach the higher density. It is unlikely that the cells are running out of nutrients because control cultures are able to remain confluent for up to a week without replenishing the media and in the experiments carried out by Armato et al., 1986 fresh media was added daily and the cells still died after a period of hyperplasia. It is possible that the cell death is specifically due to EGF addition or that, for example, some form of growth inhibition is initiated once the cells reach the higher density. In order to investigate this the cells were plated at half the usual density and then EGF was added either one day or three days after plating. The rationale was that if the cell death was EGF-specific then the cells might die at, for example, three days after EGF addition rather than at a certain density. Figures 11.1

Figure 10.1: Effect on cell numbers of adding EGF daily versus once.

T51B cells were plated at low density (7,000 cells/cm^2) and grown for 3 days before a single addition of EGF to a final concentration of 1nM. Control cultures were left untreated. Cell numbers were determined daily using a Coulter Counter. Statistical analysis performed was the two-tailed paired Student's t-test, comparing the mean ±SE from three different experiments of control and EGF-treated cultures. A level of significance of $P<0.05$ is indicated as *.

Figure 10.2: Effect on cell numbers of adding EGF daily versus once.

T51B cells were plated at low density (7,000 cells/cm^2) and grown for 3 days before the addition of EGF. The same dose of EGF was added daily to give a concentration of 1nM, ignoring prior additions. Control cultures were left untreated. Cell numbers were determined daily using a Coulter Counter. Statistical analysis performed was the two-tailed paired Student's t-test, comparing the mean ±SE from three different experiments of control and EGF-treated cultures. A level of significance of $P<0.05$ is indicated as *.

Figure 10.1

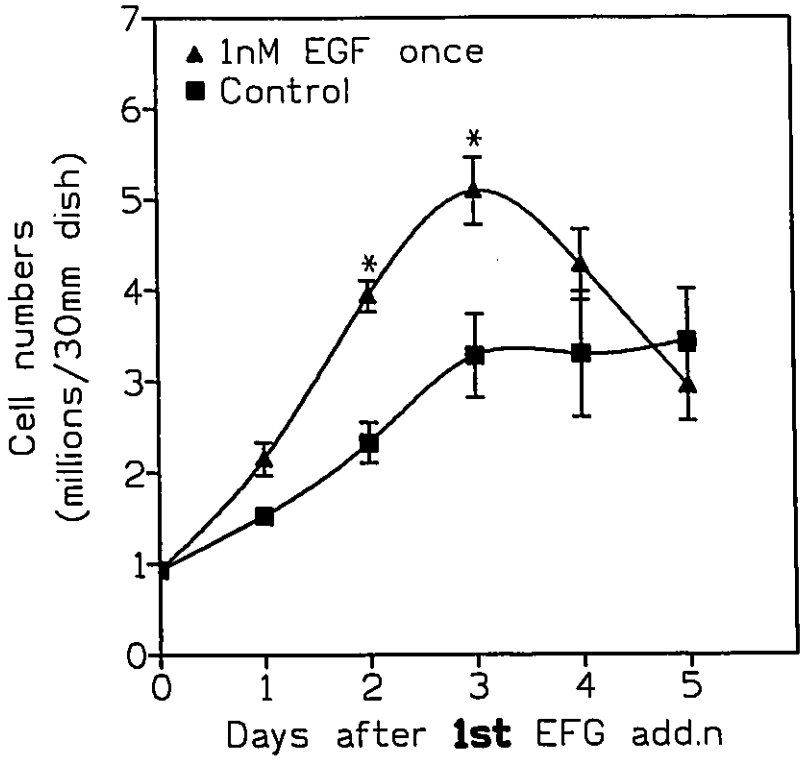


Figure 10.2

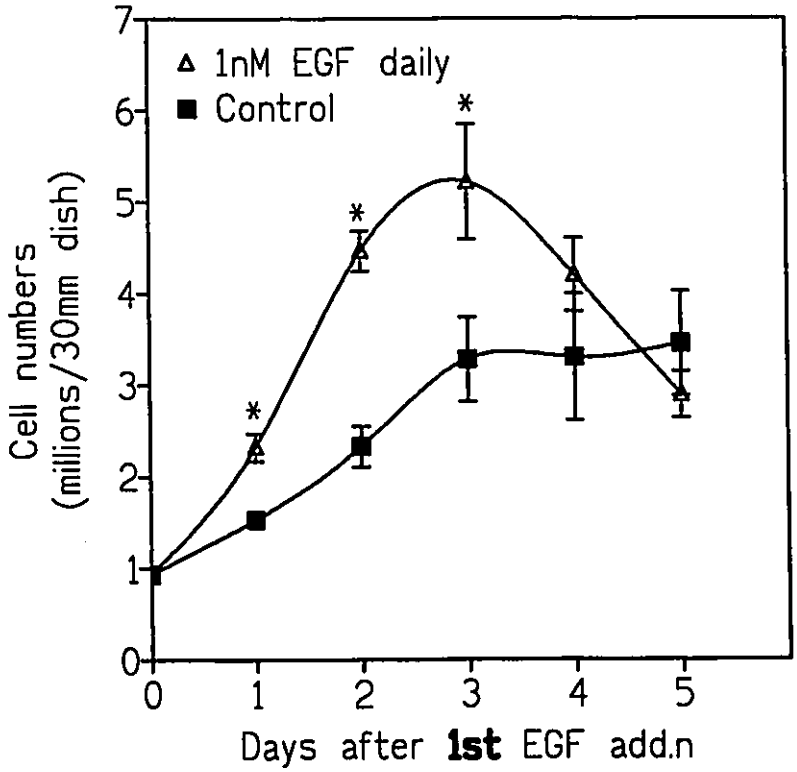


Figure 10.3: Effect on cell numbers of adding EGF daily versus once.

This figure combines the EGF growth curves from Figures 10.1 and 10.2. The experimental conditions are as set out for those figures. Statistical analysis performed was the two-tailed paired Student's t-test, comparing the mean ±SE from three different experiments of "EGF added once" and "EGF added daily". There was no significant difference between the two treatments.

Figure 10.4: Summary of effect on cell numbers of adding EGF daily versus once.

This figure is a summary of figures 10.1 and 10.2 plotting the mean cell number for each EGF treatment and the control. The standard error bars and levels of significance have been omitted for clarity.

Figure 10.3

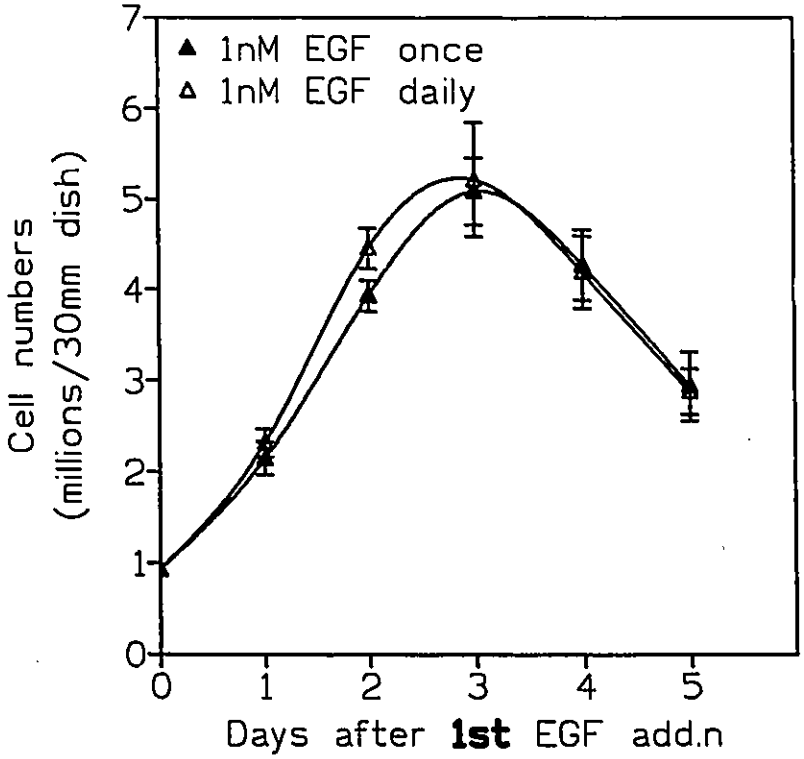


Figure 10.4

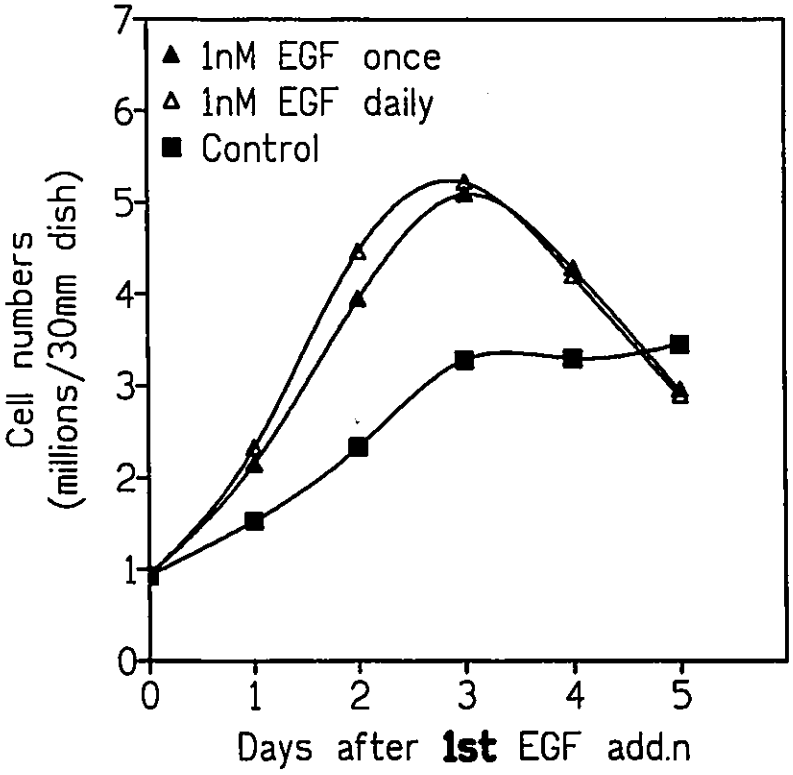


Figure 11.1: Effect on cell numbers of adding EGF one day versus three days after plating.

T51B cells were plated at half the normal density (3,500 cells/cm^2) and grown for 1 day before the addition of EGF to a final concentration of 1nM. Control cultures were left untreated. Cell numbers were determined daily using a Coulter Counter. Statistical analysis performed was the two-tailed paired Student's t-test, comparing the mean ±SE from three different experiments of control and EGF-treated cultures. A level of significance of $P<0.05$ is indicated as *.

Figure 11.2: Effect on cell numbers of adding EGF one day versus three days after plating.

T51B cells were plated at half the normal density (3,500 cells/cm^2) and grown for 3 days before the addition of EGF to a final concentration of 1nM. Control cultures were left untreated. Cell numbers were determined daily using a Coulter Counter. Statistical analysis performed was the two-tailed paired Student's t-test, comparing the mean ±SE from three different experiments of control and EGF-treated cultures. A level of significance of $P<0.05$ is indicated as *.

Figure 11.1

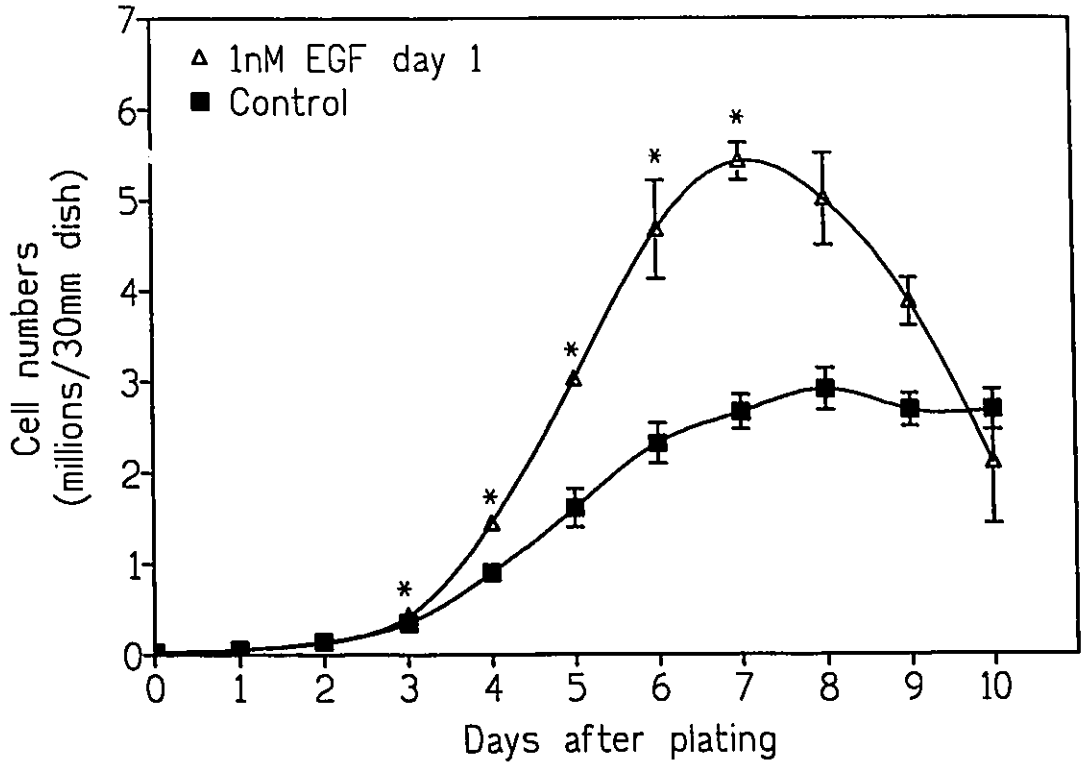


Figure 11.2

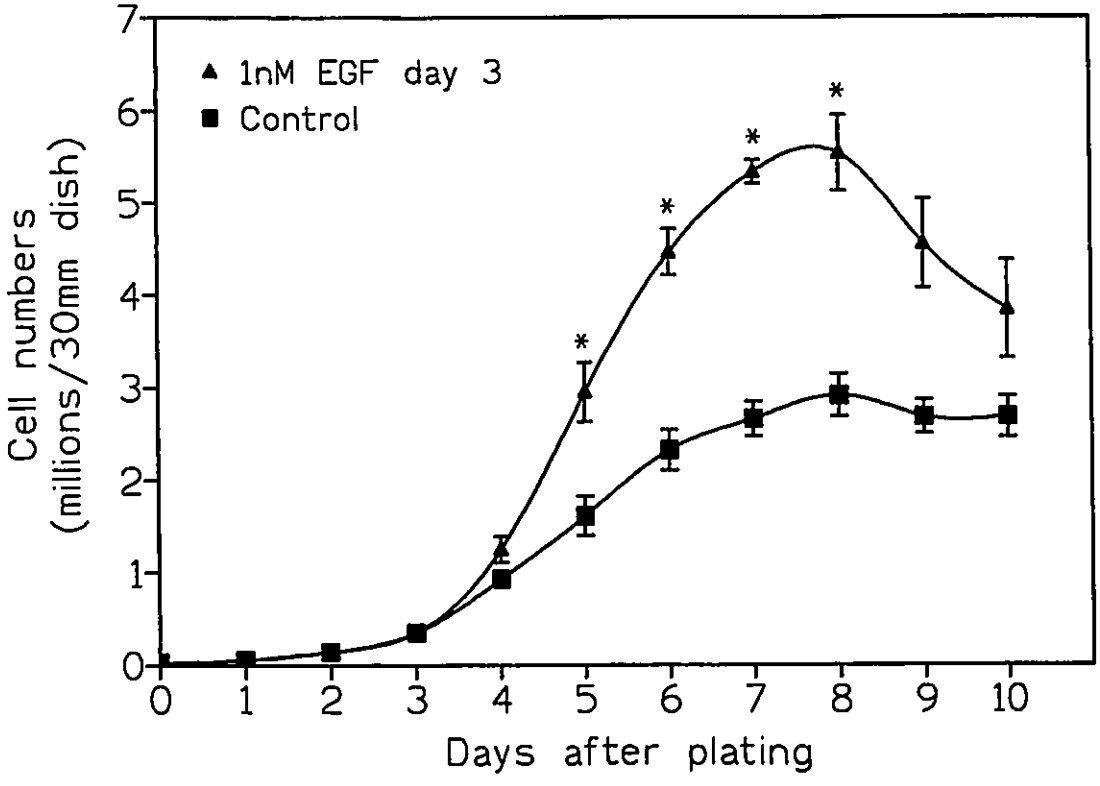


Figure 11.3: Effect on cell numbers of adding EGF one day versus three days after plating.

This figure combines the EGF growth curves from figures 11.1 and 11.2. The experimental conditions are as set out for those figures. Statistical analysis performed was the two-tailed paired Student's t-test, comparing the mean ±SE from three different experiments of "EGF added on day 1" and "EGF added on day 3". A level of significance of $P<0.05$ is indicated as *.

Figure 11.4: Summary of effect on cell numbers of adding EGF one day versus three days after plating.

This figure is a summary of figures 11.1 and 11.2 plotting the mean cell number for each EGF treatment and the control. The standard error bars and levels of significance have been omitted for clarity.

Figure 11.3

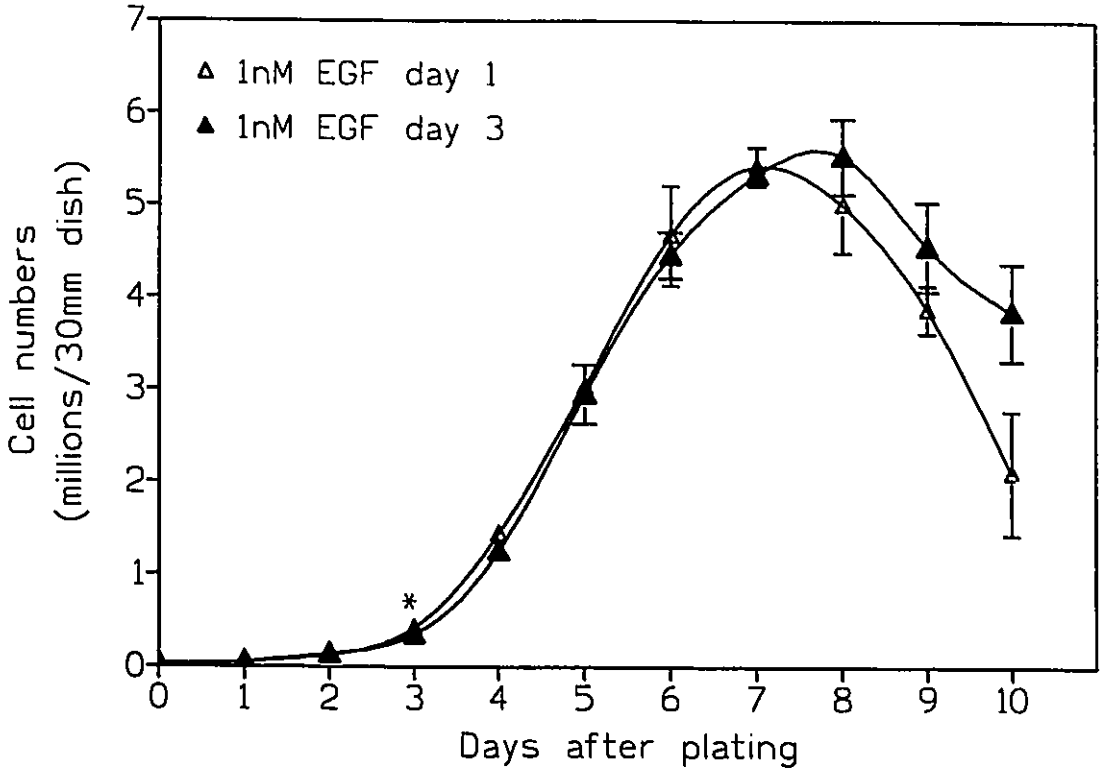
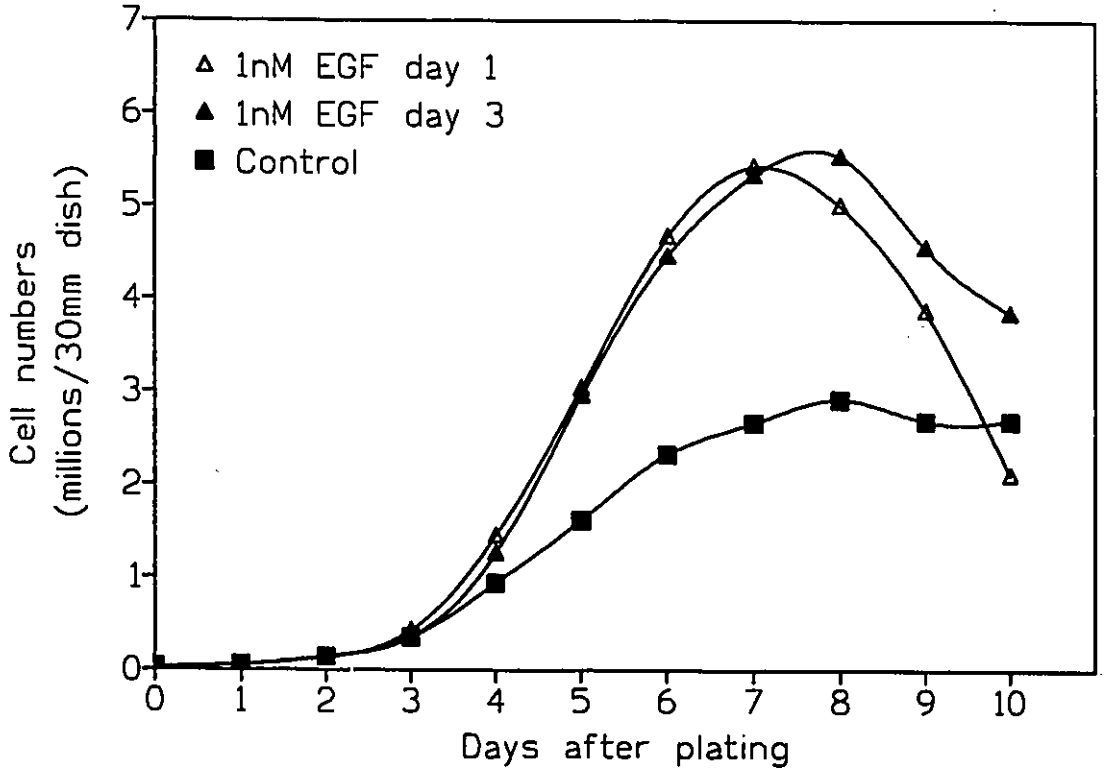


Figure 11.4



to 11.4 show that time of EGF addition does not affect the growth curve significantly: the cells reach a density of $5.5x10^6$ cells per plate 7 or 8 days after plating and then start to die. This would suggest that EGF is not specifically causing the increase in apoptosis.

Effect of replating cells at various points in the EGF growth curve

I wanted to determine whether growing cells with 1nM EGF for a number of days altered their phenotype in any way. For example, are the dying cells programmed to die or are the surviving cells, at the end of the growth curve, resistant to further mitogenic stimuli from EGF? Control and EGF-treated cells were replated at Days 2, 4 and 7 in the growth curve which represents proliferating, dying and surviving populations of EGF-treated cells respectively. Figures 12.2 and 12.3 indicate that the cells replated at Days 2 and 4 proliferate until they reach the usual control cell density of around $5x10^6$ cells per dish. There is no growth difference between the control cultures and those previously grown with EGF. The surviving population of cells (Day 7) was replated at the usual low density, grown for 3 days and then re-exposed to EGF (Figure 12.4). The cells proliferate for three or four days and then start to die, very much like they did during their first exposure to EGF (Figure 12.1). Although it must be noted that this experiment was only carried out twice it does suggest that growing the cells with 1nM EGF does not significantly alter their growth phenotype.

Figure 12.1: Effect on cell numbers of replating cells at various points in EGF growth curve.

T51B cells were plated at low density (7,000 cells/cm^2) and grown for 3 days before the addition of EGF to a final concentration of 1nM. Control cultures were left untreated. Cell numbers were determined daily using a Coulter Counter. Each point represents the mean of duplicate samples from a representative experiment. The experiment was performed two times. This growth curve represents the base curve for the experiment; the cells for replating were taken from it.

Figure 12.2: Effect on cell numbers of replating cells at Day 2 in EGF growth curve.

Control and EGF-treated cells were taken at Day 2 in the growth curve and replated at ¼ of their density. All cells were left untreated and the cell numbers were determined daily using a Coulter Counter. Each point represents the mean of duplicate samples from a representative experiment. The experiment was performed two times.

Figure 12.1

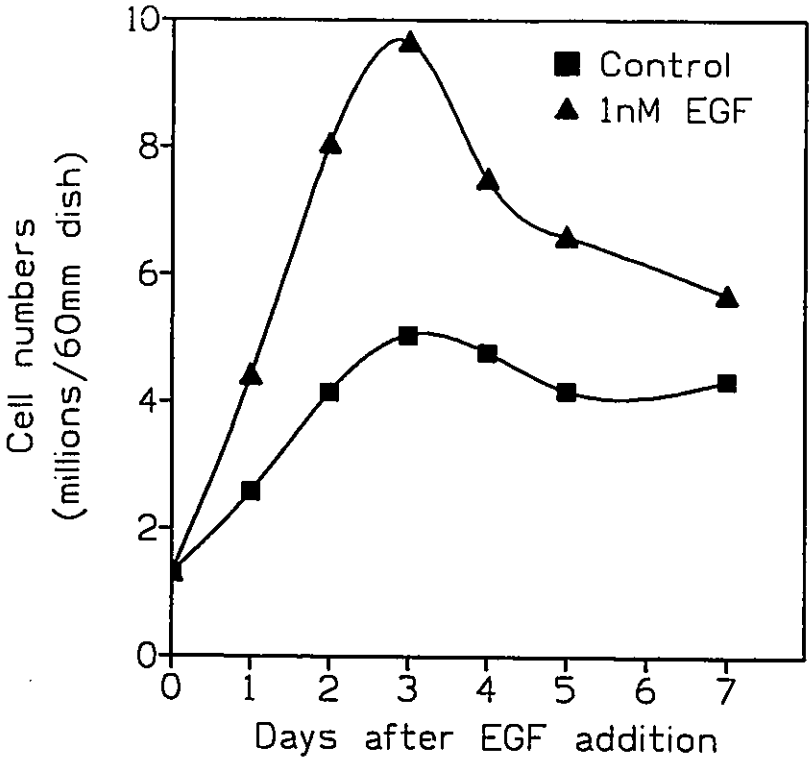


Figure 12.2

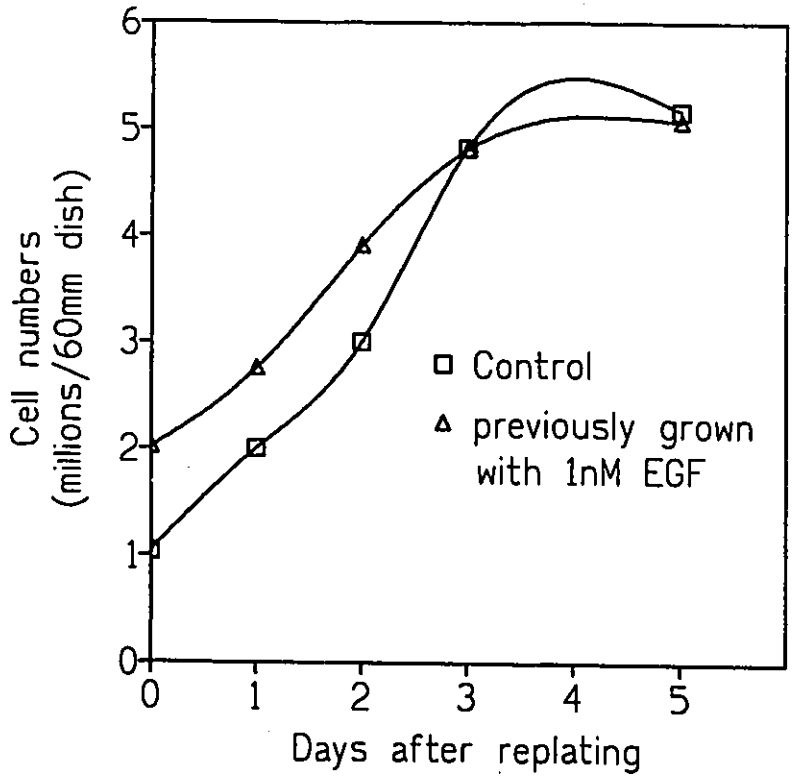


Figure 12.3: Effect on cell numbers of replating cells at Day 4 in EGF growth curve.

Control and EGF-treated cells were taken at Day 4 in the growth curve and replated at ¼ of their density. All cells were left untreated and the cell numbers were determined daily using a Coulter Counter. Each point represents the mean of duplicate samples from a representative experiment. The experiment was performed two times.

Figure 12.4: Effect on cell numbers of replating cells at Day 7 in EGF growth curve and retreating them with EGF.

Control and EGF-treated cells were taken at Day 7 in the growth curve and replated at the normal density (7,000 cells/cm^2). The cells were grown for 3 days before EGF (to a final concentration of 1nM) was again added to the cells previously grown with EGF. Control cultures were left untreated. Cell numbers were determined daily using a Coulter Counter. Each point represents the mean of duplicate samples from a representative experiment. The experiment was performed two times.

Figure 12.3

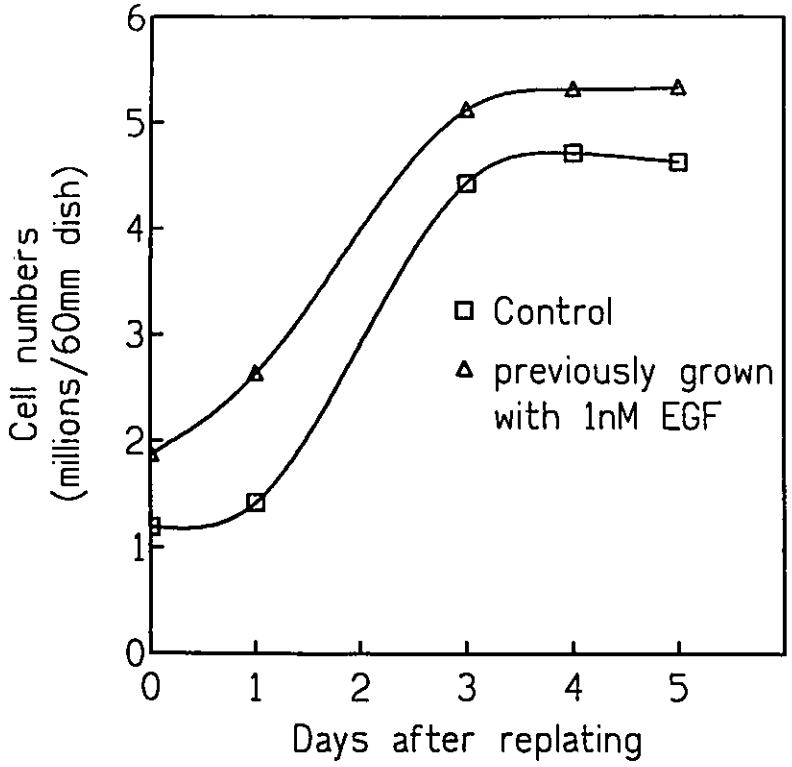
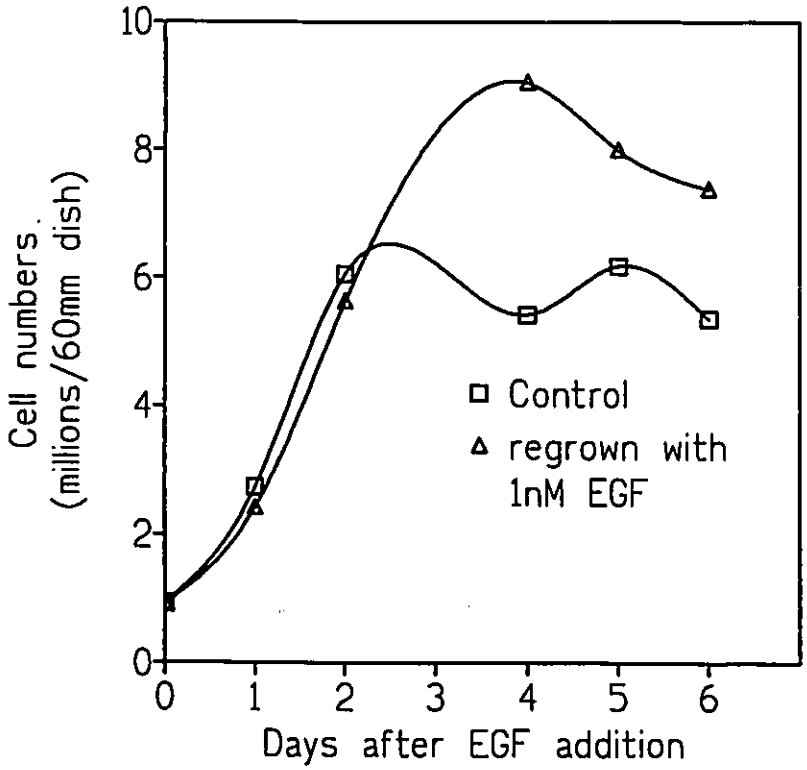


Figure 12.4



Effect of different EGF doses on cell numbers

Growing T51B cells in 10% bovine calf serum does not produce the hyperplasia and cell death that occurs when 1nM EGF is added to the media, which suggests that this growth curve is concentration dependent. Figures 13.1 to 13.6 plot the growth curves of cells cultured in a wide range of EGF concentrations. When cells are grown in 10pM EGF there is no change in cell numbers as compared to the controls (Figure 13.1). However, at 0.1nM EGF some hyperplasia and cell death are seen at days 2 and 3 (Figure 13.2). Interestingly the maximum cell number reached before the cells start to die is $4x10^6$ cells per dish rather than the usual high density of about $5.5x10^6$. Once the EGF concentration reaches 0.5nM EGF the usual growth curve is seen and continues to concentrations as high as 10nM EGF (Figures 13.3 to 13.5). Figure 13.6 represents a summary of the EGF dose response curves where the mean cell numbers have been plotted but the standard error bars have been omitted for clarity. This figure clearly shows the three different growth curve responses that T51B cells exhibit as the EGF concentration increases from 10pM up to 10nM EGF.

Figures 13.1 and 13.2: Effect of different EGF doses on cell numbers.

T51B cells were plated at low density (7,000 cells/cm^2) and grown for 3 days before the addition of EGF. Control cultures were left untreated. Cell numbers were determined daily using a Coulter Counter. Statistical analysis performed was the two-tailed paired Student's t-test, comparing the mean ±SE from three different experiments of control and EGF-treated cultures. A level of significance of $P<0.05$ is indicated as *.

Figure 13.1

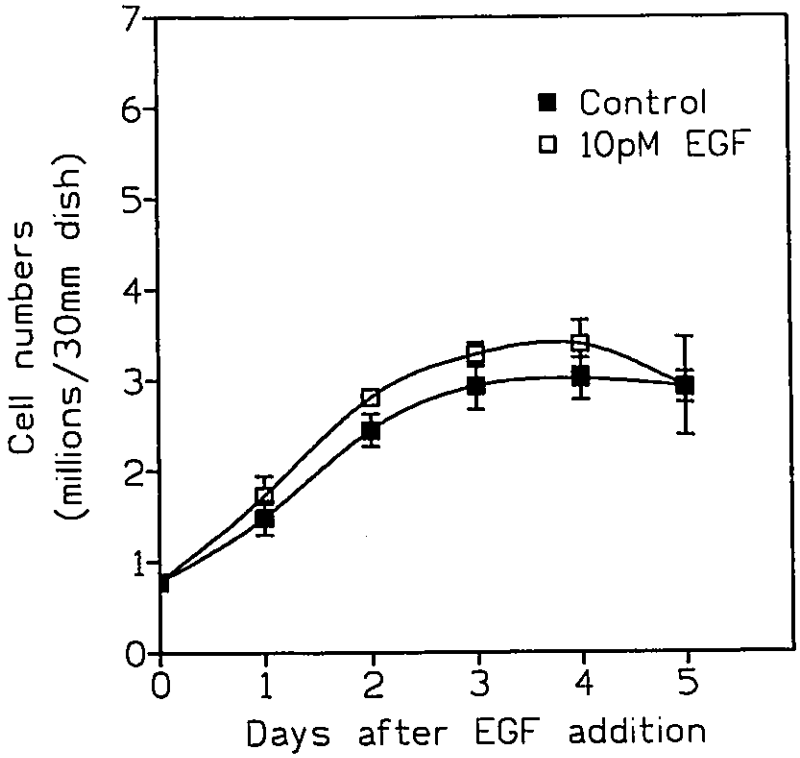
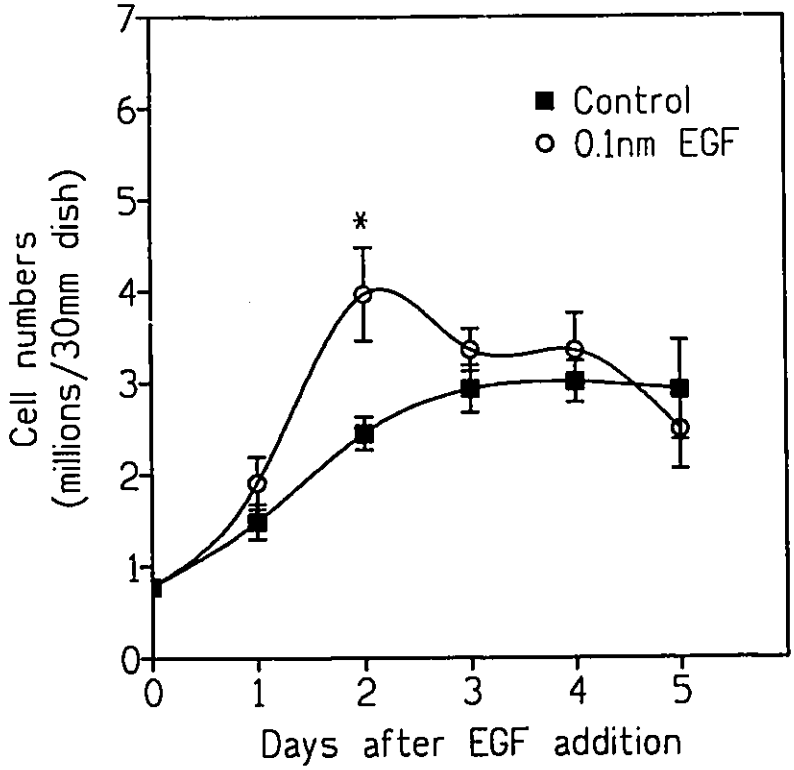


Figure 13.2



Figures 13.3 and 13.4: Effect of different EGF doses on cell numbers.

T51B cells were plated at low density (7,000 cells/cm^2) and grown for 3 days before the addition of EGF. Control cultures were left untreated. Cell numbers were determined daily using a Coulter Counter. Statistical analysis performed was the two-tailed paired Student's t-test, comparing the mean ±SE from three different experiments of control and EGF-treated cultures. A level of significance of $P<0.05$ is indicated as *.

Figure 13.3

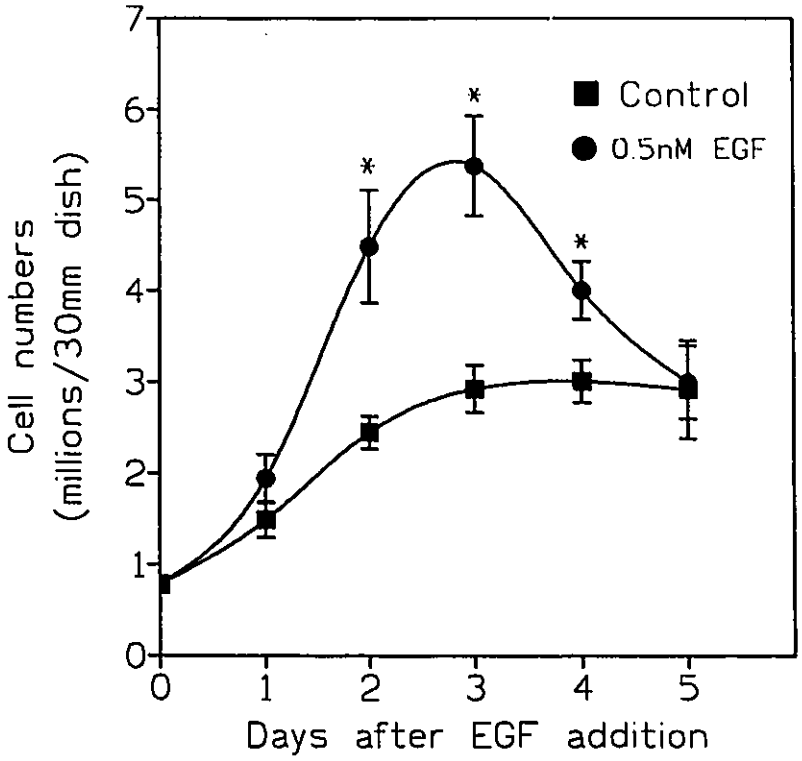


Figure 13.4

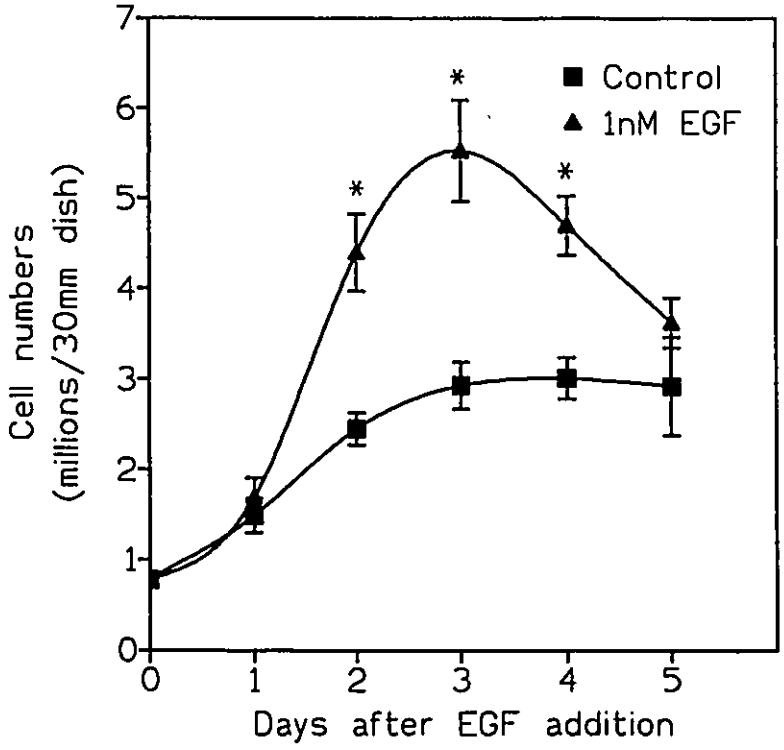


Figure 13.5: Effect of different EGF doses on cell numbers.

T51B cells were plated at low density (7,000 cells/cm^2) and grown for 3 days before the addition of EGF. Control cultures were left untreated. Cell numbers were determined daily using a Coulter Counter. Statistical analysis performed was the two-tailed paired Student's t-test, comparing the mean ±SE from three different experiments of control and EGF-treated cultures. A level of significance of $P<0.05$ is indicated as *.

Figure 13.6: Summary of effect of different doses on cell numbers.

This figure is a summary of figures 13.1 to 13.5 plotting the mean cell number for each EGF dose and the control. The standard error bars and levels of significance have been omitted for clarity.

Figure 13.5

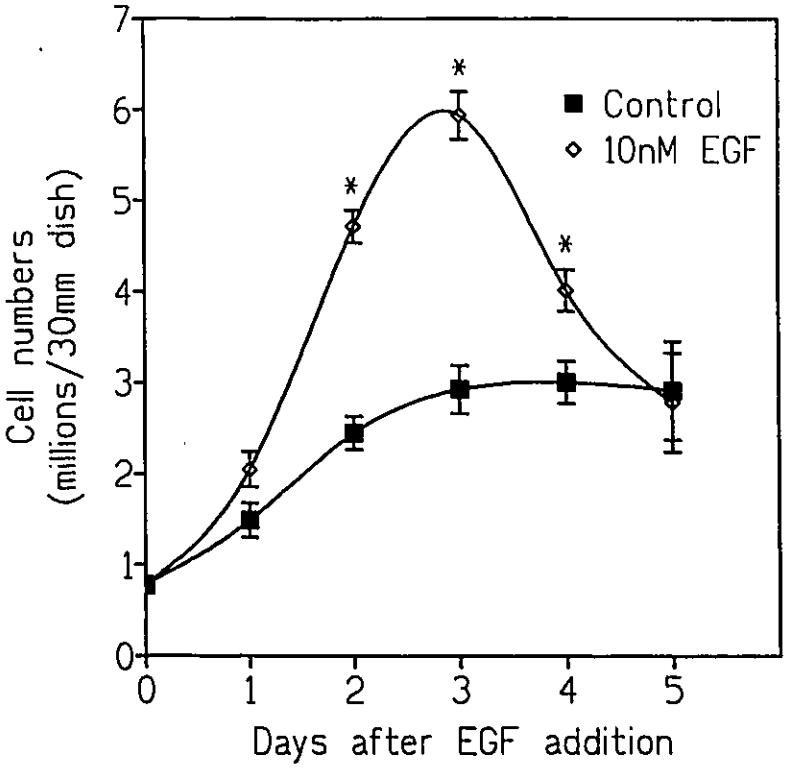
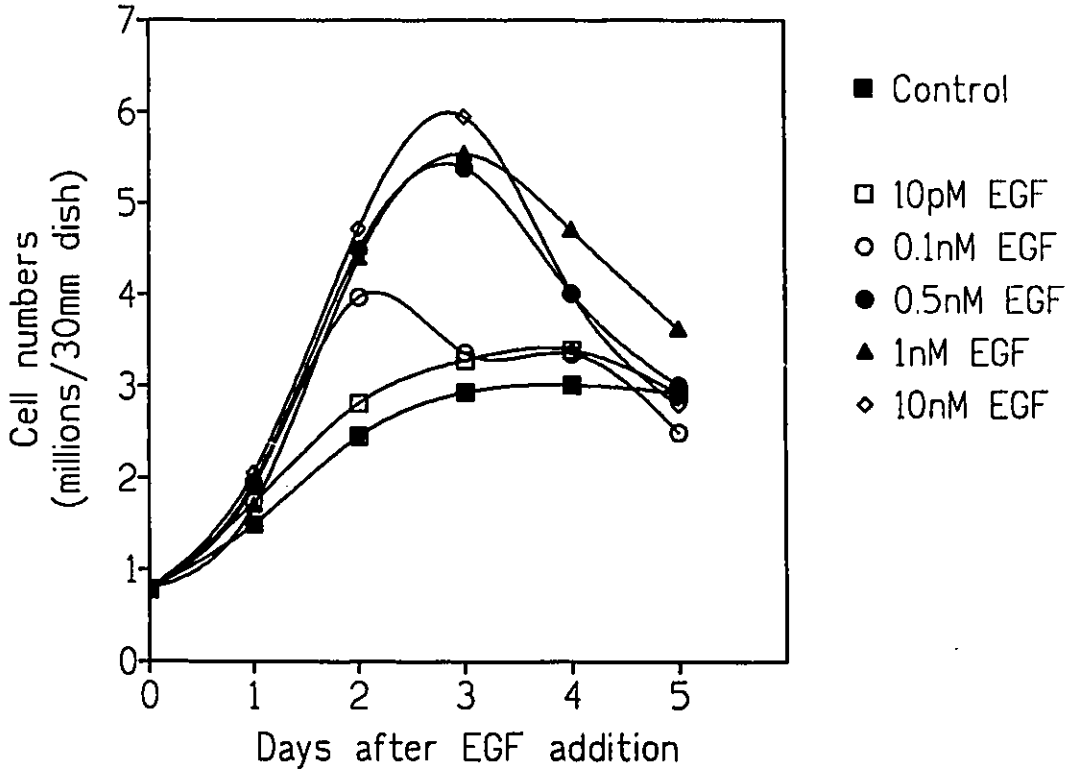


Figure 13.6



The final part of this section explores the interesting EGF dose response further by determining the binding characteristics of the EGF receptor in T51B cells. This was done in order to investigate whether the hyperplasia and/or apoptosis were induced by occupancy of the low affinity binding site. I also examined changes in binding when the cells were grown with two different concentrations of EGF to determine which events in the growth curve are EGF specific.

EGF receptor binding characteristics

The first two figures show the results from initial experiments that were performed to determine the basic parameters for the assay. The level of non-specific binding to the cells was found to be low. Figure 14 shows a typical non-specific binding curve where a fixed concentration of (3-[^{125}I]iodotyrosyl)EGF is diluted with increasing concentrations of unlabeled EGF. The graph shows that cpm bound drop off rapidly and plateau at around 450 cpm which represents 8.2% of maximum binding. The experiment was carried out three times and the non-specific binding found to be 7.7%, 9.0% and 8.2%. The mean (8.3%) was used to determine the level of non-specific binding in further experiments.

Figure 15 shows the results from an association time course carried out with non-saturating concentrations of EGF. The curves indicate that equilibrium binding has been achieved by 8 hours at 4°C and that the

Figure 14: EGF receptor binding: determination of non-specific binding.

T51B cells were plated in 24 well culture plates at high density (56,000 cells/cm^2) and grown for 4 days before the start of the binding assay. The assay was carried out as described in Materials and Methods. The cells were incubated with a fixed concentration of (3-[^{125}I]iodotyrosyl)EGF (0.27nM) and increasing concentrations of unlabeled EGF. The amount of radioactivity bound to the cells was determined using a γ-counter. Each point represents the mean of triplicate measurements from a representative experiment. The experiment was performed three times.

Figure 14

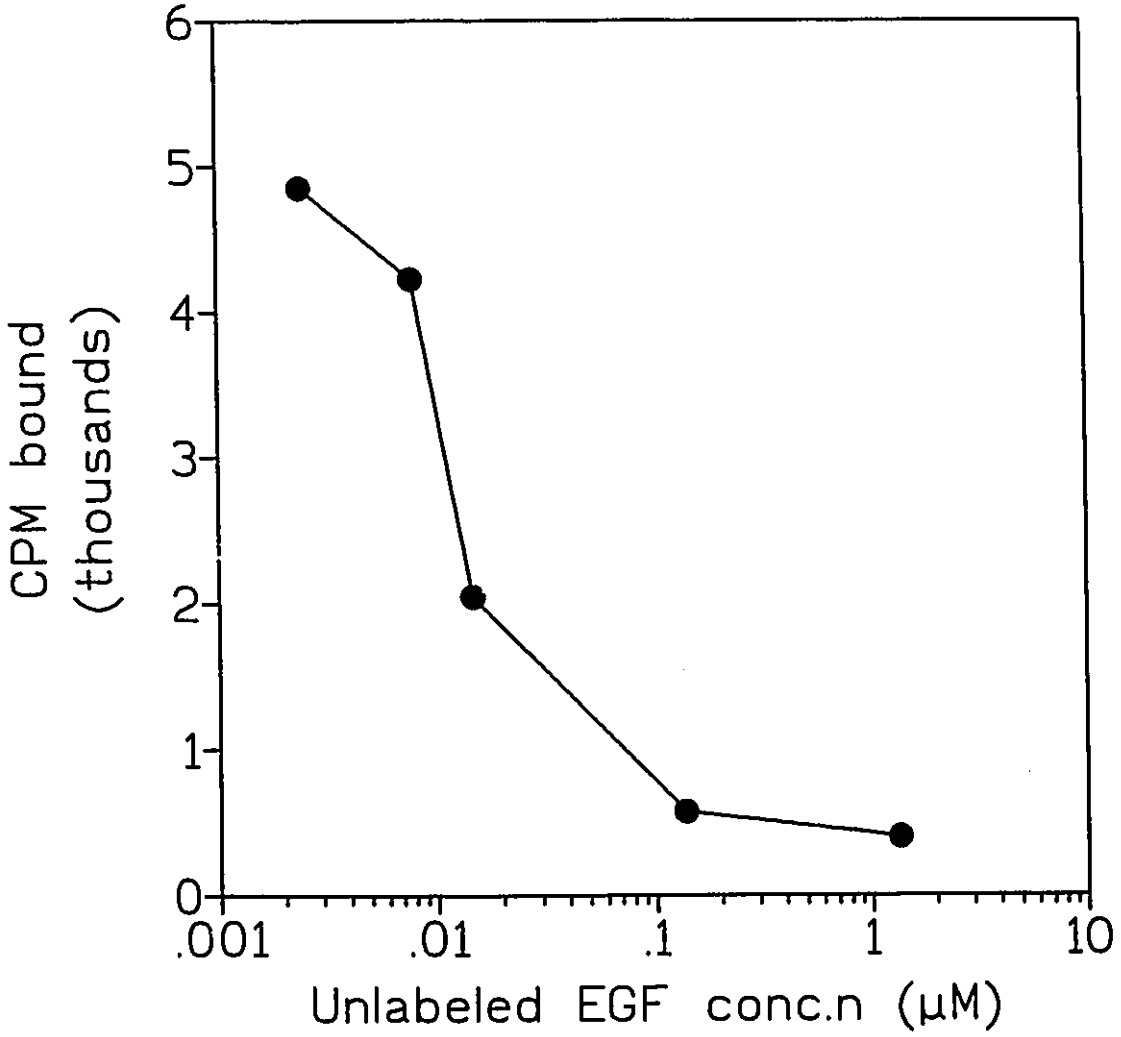
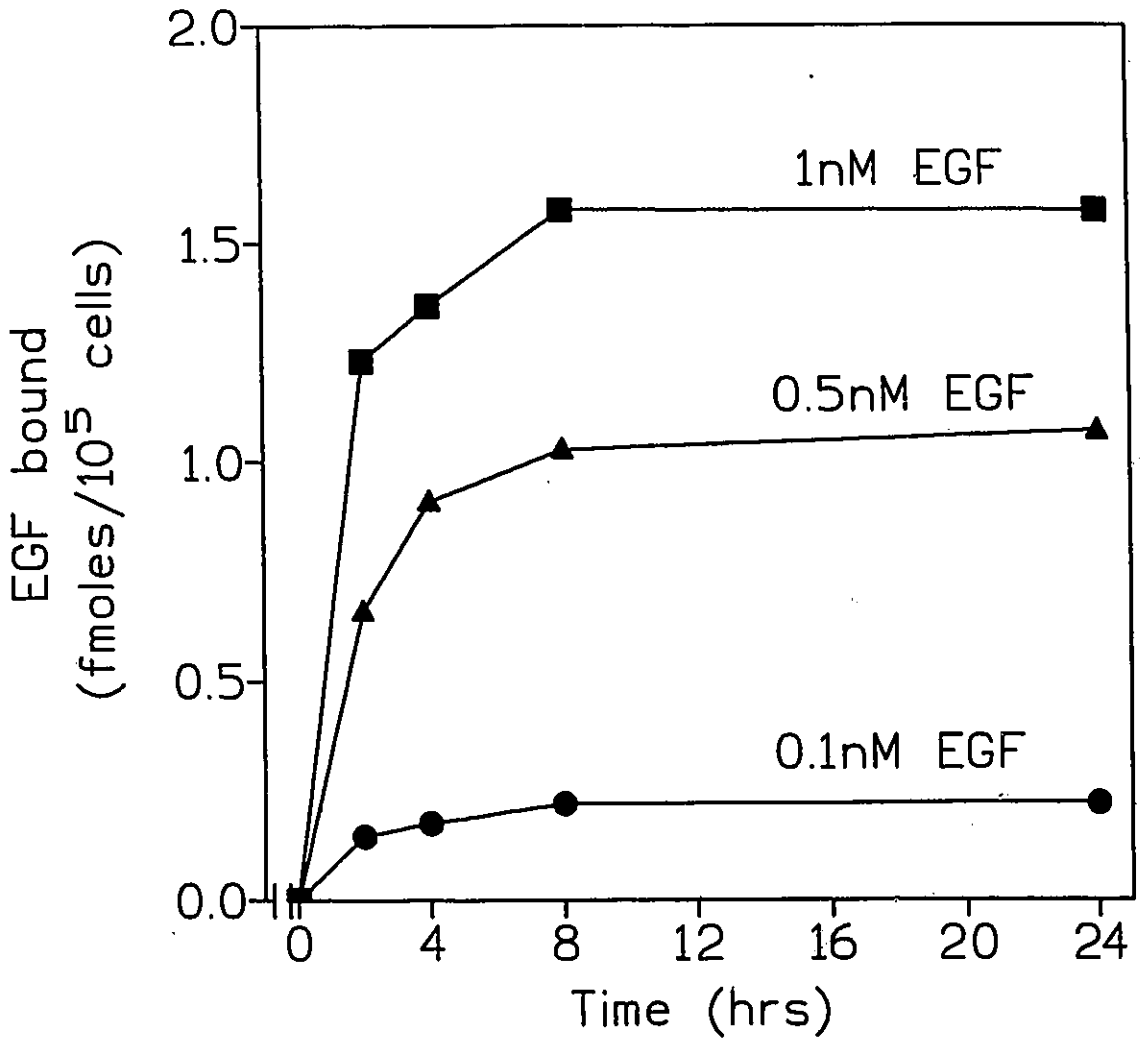

CPM bound
(thousands)
6
5
4
3
2
1
0
.001
.01
.1
1
10
Unlabeled EGF conc.n (μM)

Figure 15: EGF receptor binding association time course.

T51B cells were plated in 24 well culture plates at high density (56,000 cells/cm^2) and grown for 4 days before the start of the binding assay. The assay was carried out as described in Materials and Methods. The cells were incubated with a fixed concentration of (3-[125I]iodotyrosyl)EGF (0.1nM) and varying amounts of unlabeled EGF required to achieve the total EGF concentrations of 0.1, 0.5 and 1.0nM. The amount of radioactivity bound to the cells was determined using a γ-counter. Cell numbers were determined using a Coulter Counter. Each point represents the mean of triplicate measurements from a representative experiment. The experiment was performed three times.

Figure 15


2.0
1.5
1.0
0.5
0.0
EGF bound
(fmoles/10^5 cells)
1nM EGF
0.5nM EGF
0.1nM EGF
0
4
8
12
16
20
24
Time (hrs)

binding then remains steady up to 24 hours. The incubation time of 24 hours was subsequently used for all binding experiments.

Once the assay had been set up it was possible to use it to determine the binding characteristics of the EGF receptor in the T51B cells. Figures 16 and 17 show the results from the binding study carried out over a wide concentration range (6pM to 10nM). The association curve (Figure 16) indicates that the binding has reached saturation at around 5nM EGF. The data were then transformed into a Scatchard plot (Figure 17). The cells displayed a curvilinear binding plot indicating that there is more than one binding site for EGF. Based on the literature it was assumed that two binding sites are present and the data were analysed accordingly. However, it is possible, but unlikely, that more than two binding sites could be present in T51B cells. The data were analysed using the computer assisted ENZFITTER program and fit a two site binding model whose parameters are shown in Table 1. The component lines for the two binding sites have been plotted on the Scatchard graph (Figure 17). The standard errors associated with the high affinity binding site are large and, in the case of the dissociation constant, suggest that the value could be less than zero. This is a common problem with this type of analysis because in the reciprocal plot of Bound/Free ligand small errors are magnified, especially at low ligand concentrations where the ratio (B/F) is highest. The experiment was performed twice and the average values from the second experiment (1,760 sites, Kd = 26pM; 7,965 sites, Kd = 320pM) are close to those of the first, derived from Figure 17.

Figure 16: EGF receptor binding association concentration curve.

T51B cells were plated in 24 well culture plates at high density (56,000 cells/cm^2) and grown for 4 days before the start of the binding assay. The assay was carried out as described in Materials and Methods. The cells were incubated with various concentrations of EGF over the range 6pM to 10nM. The ratio of (3-[125I]iodotyrosyl)EGF to unlabeled EGF used was 1:2. The amount of radioactivity bound to the cells was determined using a γ-counter. Cell numbers were determined using a Coulter Counter. Each point represents the mean of duplicate measurements from a representative experiment. The experiment was performed two times.

Figure 16

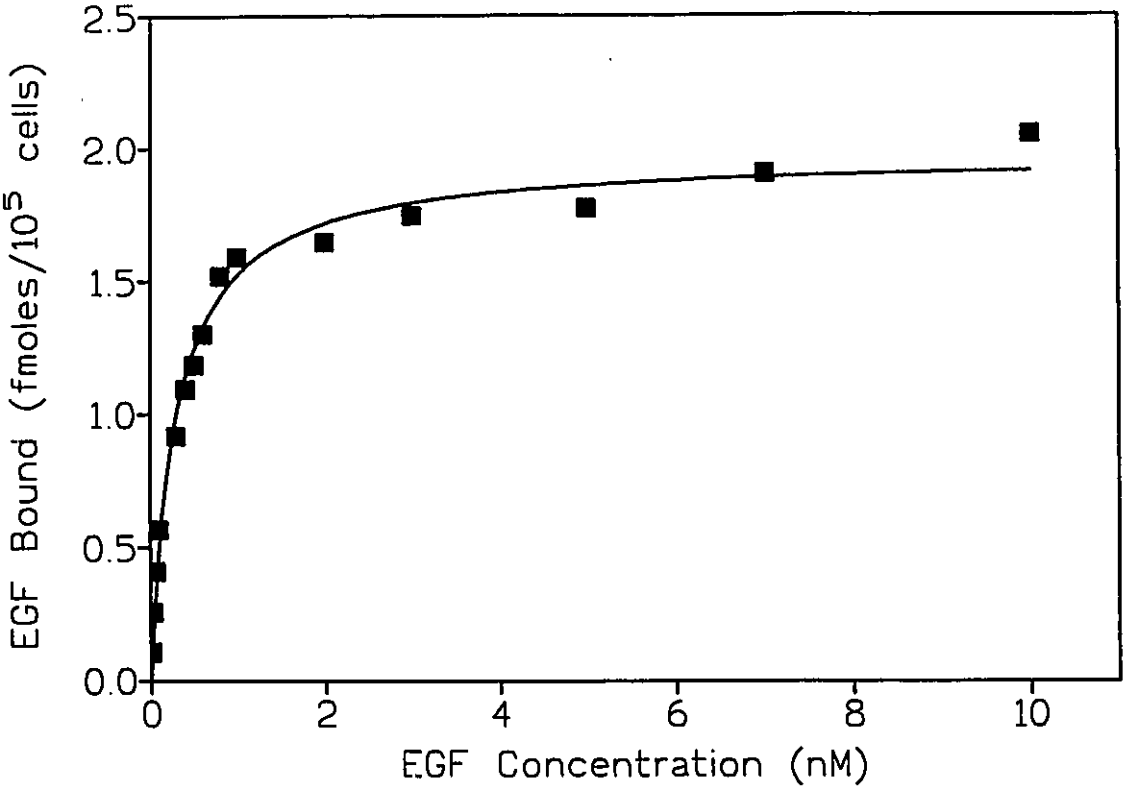

EGF Bound (fmoles/10^5 cells)
2.5
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
10
EGF Concentration (nM)

Figure 17: EGF receptor binding Scatchard curve.

This figure shows the Scatchard transformation of the EGF receptor binding association data plotted in Figure 16. Each square symbol represents the mean of duplicate measurements from a representative experiment. The experiment was performed two times. The curve represents the line of best fit to the data points generated from the computer assisted ENZFITTER program. The two straight line (Y=0.406-0.29X and Y=0.0947-0.0103X) represent the components of this curve due to each individual binding site.

Figure 17

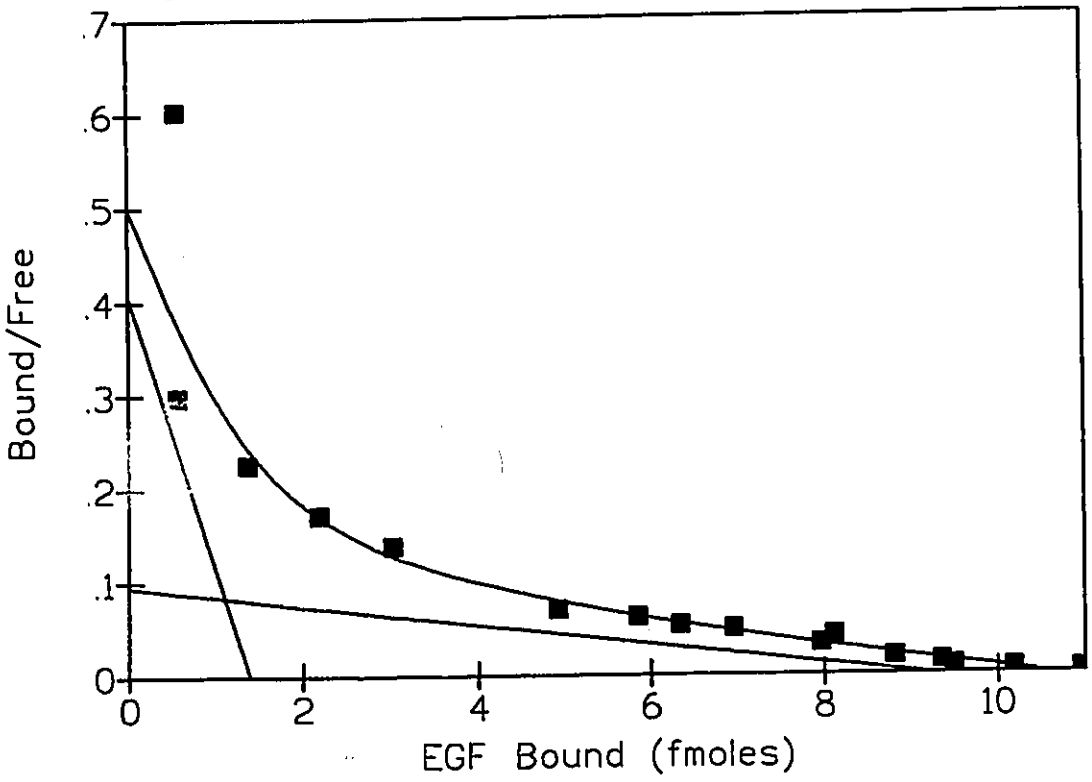


Table 1: Estimation of EGF receptor binding parameters based on Scatchard analysis of equilibrium binding data.

This table presents the binding parameters (±SE) calculated for the EGF receptor from the binding association data plotted in Figure 16 and the Scatchard transformation shown in Figure 17. The binding parameters were calculated by computer analysis using the ENZFITTER program assuming there to be two binding sites.

Table 1: Estimation of EGF receptor binding parameters based on Scatchard analysis of equilibrium binding data.

	High affinity site	Low affinity site
sites/cell	1,577 ± 855	10,318 ± 799
Kd (pM)	13.8 ± 16	387 ± 68

Effect of growing cells with EGF on receptor binding

Once the dissociation constants had been obtained for the EGF receptor in T51B cells it was possible to design binding experiments to assess qualitatively how the numbers of binding sites changed when the cells were grown with EGF. The growth curves were performed as usual and then the cells were incubated with either 20pM or 3nM EGF in the binding assay to represent predominantly high affinity and low affinity binding sites respectively. The percentage of sites occupied (using the Kd values from Table 1) at the two concentrations is as follows:

20pM: 59% of high affinity sites
5% of low affinity sites

3nM: 99.5% of high affinity sites
88.6% of low affinity sites

The cells were grown with either 1nM or 10pM EGF in order to determine the differences in binding between the hyperplasia/apoptosis growth curve and the growth curve obtained with a non-mitogenic dose of EGF (Figures 13.4 and 13.1 respectively). Figures 18.1 to 18.3 represent the growth curve and the levels of EGF binding when the cells were grown with 1nM EGF. The cells were plated at a high density, approximately $56x10^3$ cells/well, in order to obtain sufficient binding to be able to measure the bound (3-[^{125}I]iodotyrosyl)EGF reliably.

Figure 18.1: Effect of growing cells with 1nM EGF on receptor binding: Cell growth curve.

T51B cells were plated at high density (56,000 cells/cm^2) and grown for 3 days before the addition of EGF to a final concentration of 1nM. Control cultures were left untreated. Cell numbers were determined daily using a Coulter Counter. Each point represents the mean of duplicate samples from a representative experiment. The experiment was performed two times.

Figure 18.1

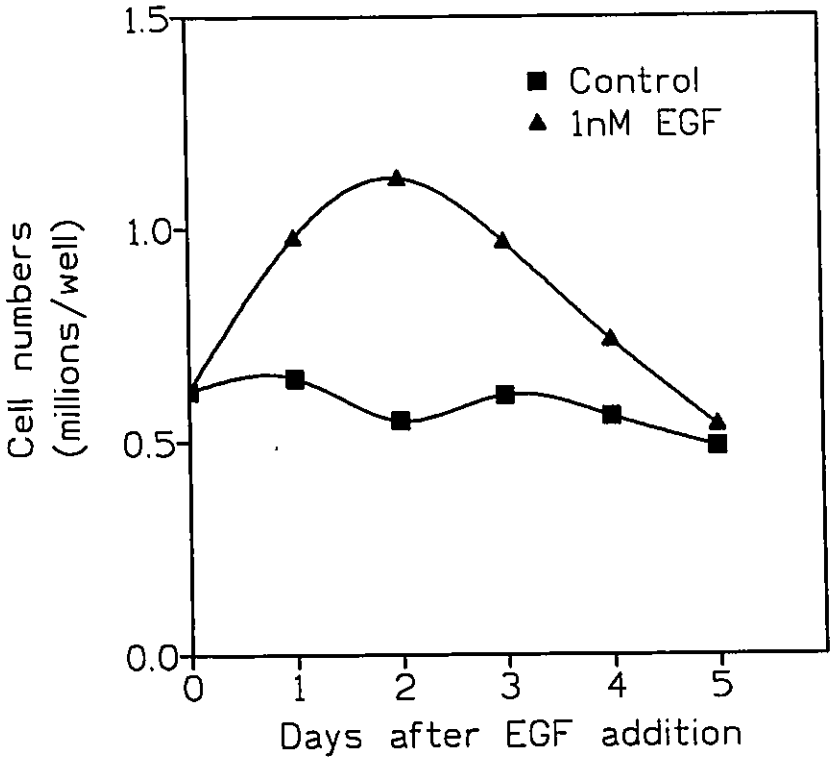

1.5
1.0
0.5
0.0
Cell numbers (millions/well)
Control
1nM EGF
0
1
2
3
4
5
Days after EGF addition

Figure 18.2: Effect of growing cells with 1nM EGF on receptor binding: High affinity binding.

T51B cells were plated in 24 well culture plates at high density (56,000 cells/cm^2) and grown for 3 days before the addition of EGF to a final concentration of 1nM. Control cultures were left untreated. The binding assay was started at various intervals after EGF addition. The assay was carried out as described in Materials and Methods incorporating the acid wash to remove any surface-bound EGF. The cells were incubated with a fixed concentration of (3-[125I]iodotyrosyl)EGF (20pM). Each point represents the mean of duplicate samples from a representative experiment. The experiment was performed two times.

Figure 18.3: Effect of growing cells with 1nM EGF on receptor binding: Low affinity binding.

T51B cells were plated in 24 well culture plates at high density (56,000 cells/cm^2) and grown for 3 days before the addition of EGF to a final concentration of 1nM. Control cultures were left untreated. The binding assay was started at various intervals after EGF addition. The assay was carried out as described in Materials and Methods incorporating the acid wash to remove any surface-bound EGF. The cells were incubated with 3nM EGF at a fixed ratio of 1:9 (3-[125I]iodotyrosyl)EGF to unlabeled EGF. Each point represents the mean of duplicate samples from a representative experiment. The experiment was performed two times.

Figure 18.2

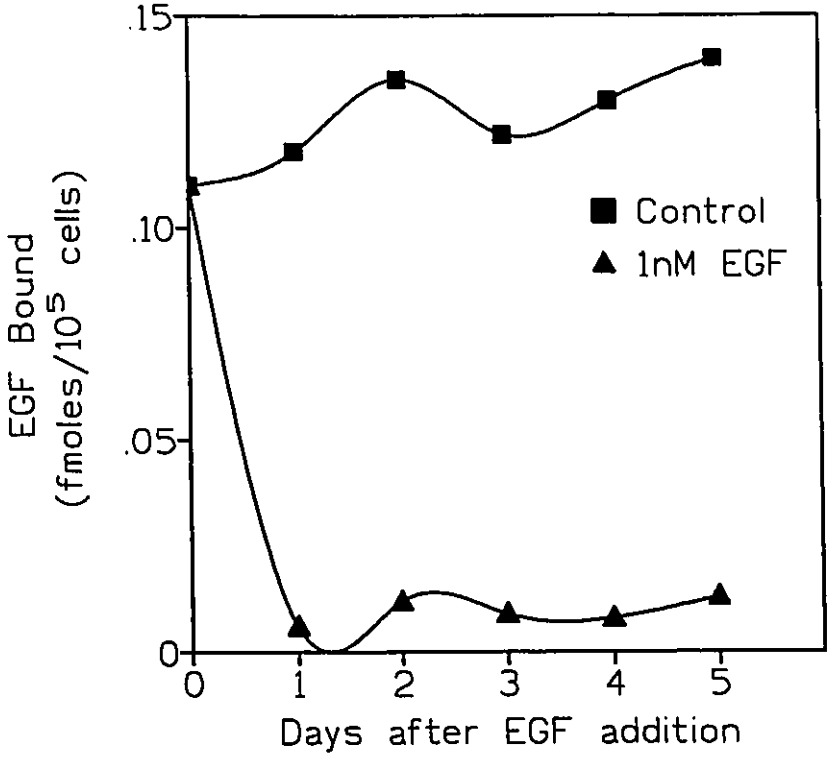


Figure 18.3

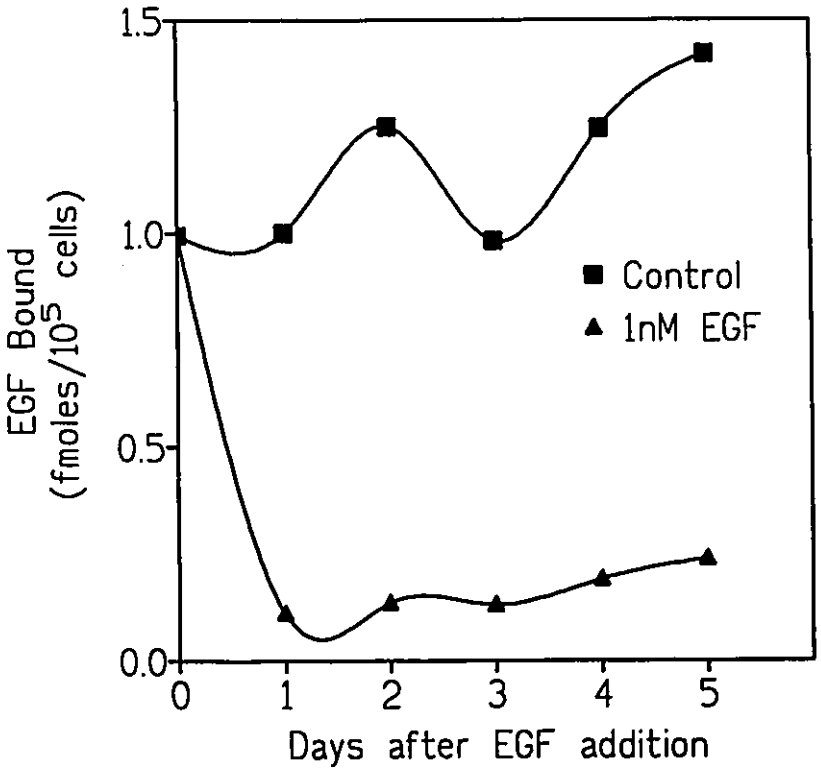


Figure 18.1 shows that the control cells are confluent at the start of the experiment and that exposure to 1nM EGF results in the usual growth curve; the cell numbers double and then decline back to the control level over a period of 5 days.

Figures 18.2 and 18.3 indicate that the amount of EGF bound in the 20pM assay is about 10% of that bound in the 3nM assay. This is consistent with the ratio of high to low binding sites obtained from the Scatchard analysis. The binding to the control cells, although not constant, is taken as a baseline to compare with the EGF-treated cells. The most striking observation is that the level of binding is reduced to around 10% of the control value at day 1 and does not recover significantly during the entire experiment. This suggests that the EGF receptor is down regulated during the initial exposure to 1nM EGF.

Figures 19.1 to 19.3 represent the growth curve and the levels of EGF binding when the cells were grown with 10pM EGF. Figure 19.1 indicates that there is no difference in cell numbers between the controls and the cells grown with 10pM EGF. Figures 19.2 and 19.3 show that the amount of EGF bound also remains the same for the duration of the experiment. The one exception is the increase in binding to the low affinity site in cells grown with 10pM EGF. The experiment was only performed two times and a smaller increase was seen with the second experiment suggesting that the increase seen in Figure 19.3 may be due to experimental error.

Figure 19.1: Effect of growing cells with 10pM EGF on receptor binding: Cell growth curve.

T51B cells were plated at high density (56,000 cells/cm^2) and grown for 3 days before the addition of EGF to a final concentration of 10pM. Control cultures were left untreated. Cell numbers were determined daily using a Coulter Counter. Each point represents the mean of duplicate samples from a representative experiment. The experiment was performed two times.

Figure 19.1

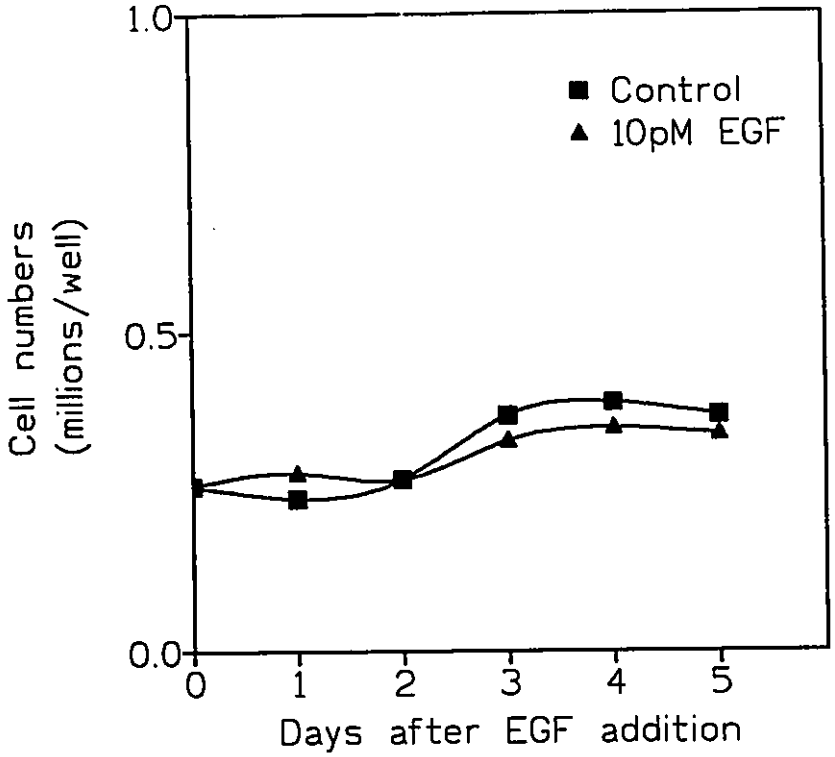

1.0
0.5
0.0
Cell numbers (millions/well)
Control
10pM EGF
0
1
2
3
4
5
Days after EGF addition

Figure 19.2: Effect of growing cells with 10pM EGF on receptor binding: High affinity binding.

T51B cells were plated in 24 well culture plates at high density (56,000 cells/cm^2) and grown for 3 days before the addition of EGF to a final concentration of 10pM. Control cultures were left untreated. The binding assay was started at various intervals after EGF addition. The assay was carried out as described in Materials and Methods incorporating the acid wash to remove any surface-bound EGF. The cells were incubated with a fixed concentration of (3-[125I]iodotyrosyl)EGF (20pM). Each point represents the mean of duplicate samples from a representative experiment. The experiment was performed two times.

Figure 19.3: Effect of growing cells with 10pM EGF on receptor binding: Low affinity binding.

T51B cells were plated in 24 well culture plates at high density (56,000 cells/cm^2) and grown for 3 days before the addition of EGF to a final concentration of 10pM. Control cultures were left untreated. The binding assay was started at various intervals after EGF addition. The assay was carried out as described in Materials and Methods incorporating the acid wash to remove any surface-bound EGF. The cells were incubated with 3nM EGF at a fixed ratio of 1:9 (3-[125I]iodotyrosyl)EGF to unlabeled EGF. Each point represents the mean of duplicate samples from a representative experiment. The experiment was performed two times.

Figure 19.2

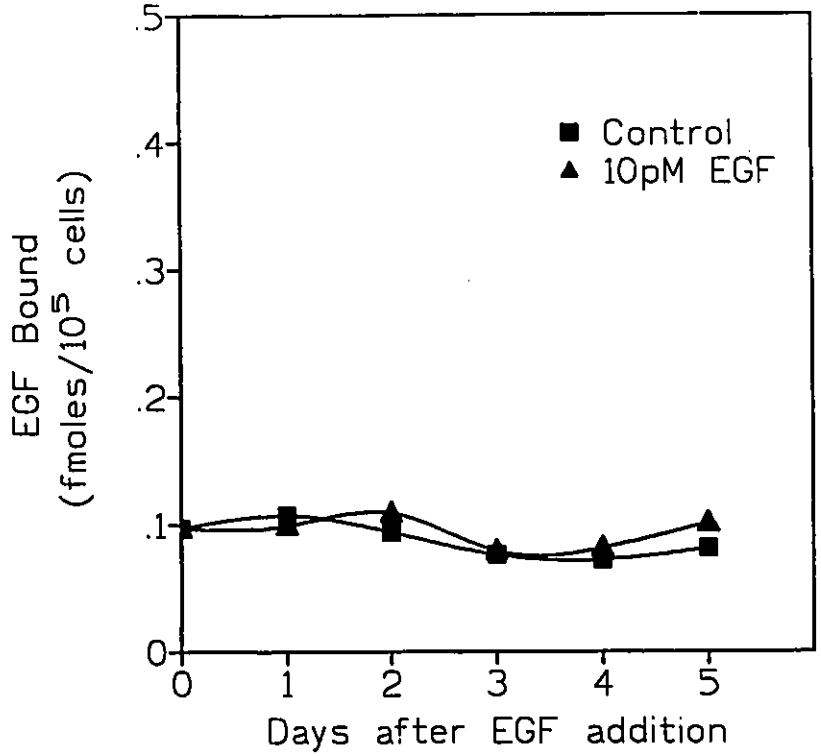


Figure 19.3

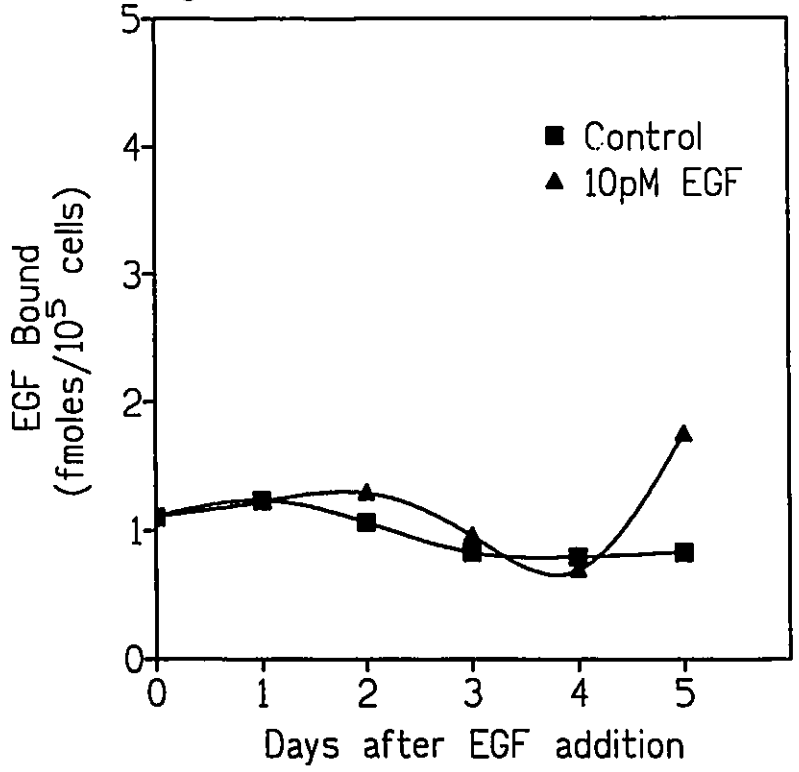


Figure 18.3 indicates that about 90% of the low affinity sites are removed from the cell surface within 24 hours of 1nM EGF addition. This means that it is impossible to determine from Figure 18.2 whether the residual binding is due to occupancy of a reduced number of high and low affinity sites or whether the high affinity sites have disappeared completely and the residual binding is due to occupancy of the low affinity sites alone. To determine what had happened to the two binding sites the cells were grown with 1nM EGF for 24 hours before carrying out an equilibrium binding study. Figure 20 shows the association concentration curves of the control and the EGF-treated cells. The data were then transformed into Scatchard plots (Figure 21). The control cells exhibit a curvilinear binding plot as before but the EGF-treated cells display a linear plot indicating that one binding site has almost completely disappeared. The data were analysed using the computer assisted ENZFITTER program assuming one binding site. The Kd was determined to be 1.4nM ±0.06 with 3,062 ±53 sites/cell. This suggests that it is the high affinity site which has almost completely been down-regulated and that the residual binding in figure 18.2 is due solely to the remaining low affinity sites. This experiment was only carried out 1 Day after EGF addition and not for the duration of the entire growth curve so it is possible that some high affinity sites do return after Day 1.

The Kd value for the remaining low affinity site is about three times higher than that found previously in the control cells (Table 1). It is not certain whether this represents a genuine change in the

Figure 20: Effect of growing cells with 1nM EGF for 24 hours on binding association concentration curve.

T51B cells were plated in 24 well culture plates at high density (56,000 cells/cm^2) and grown for 4 days before the addition of EGF to a final concentration of 1nM. Control cultures were left untreated. The binding assay was started 24 hours after EGF addition. The assay was carried out as described in Materials and Methods incorporating the acid wash to remove any surface-bound EGF. The cells were incubated with various concentrations of EGF over the range 6pM to 5nM. The ratio of (3-[125I]iodotyrosyl)EGF to unlabeled EGF used was 1:2. The amount of radioactivity bound to the cells was determined using a γ-counter. Cell numbers were determined using a Coulter Counter. Each point represents the mean of duplicate measurements from a single experiment.

Figure 20

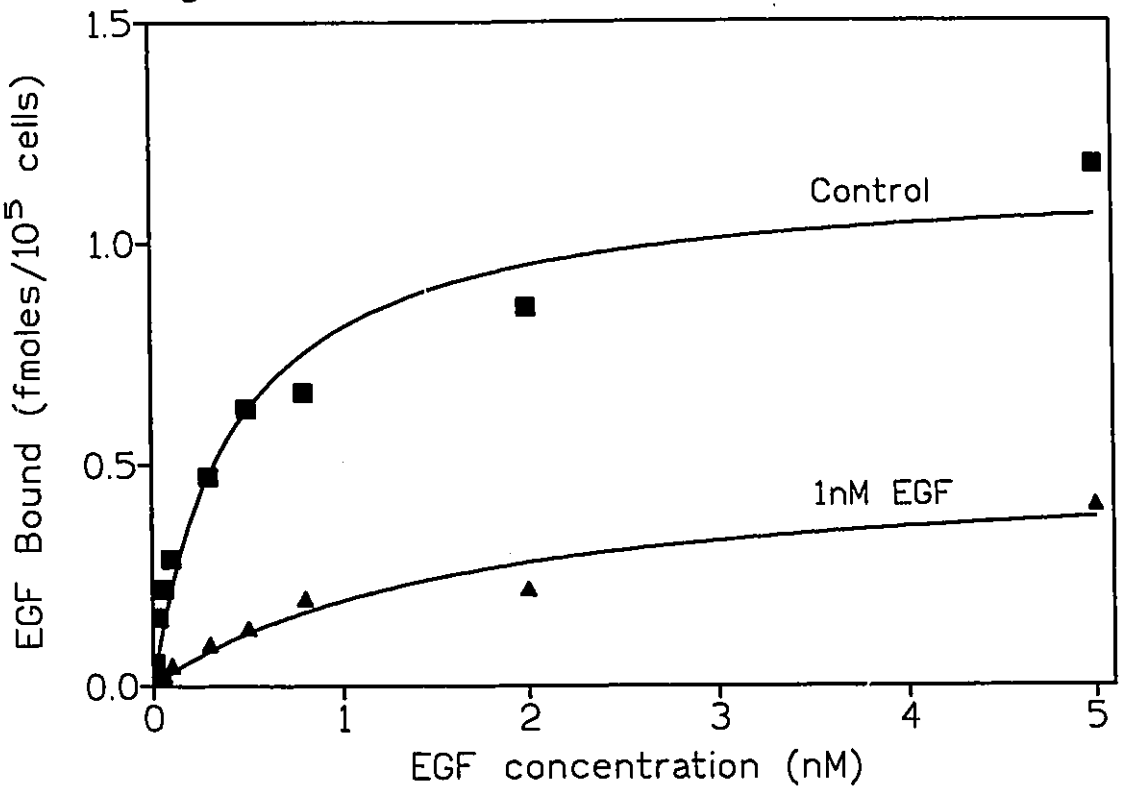
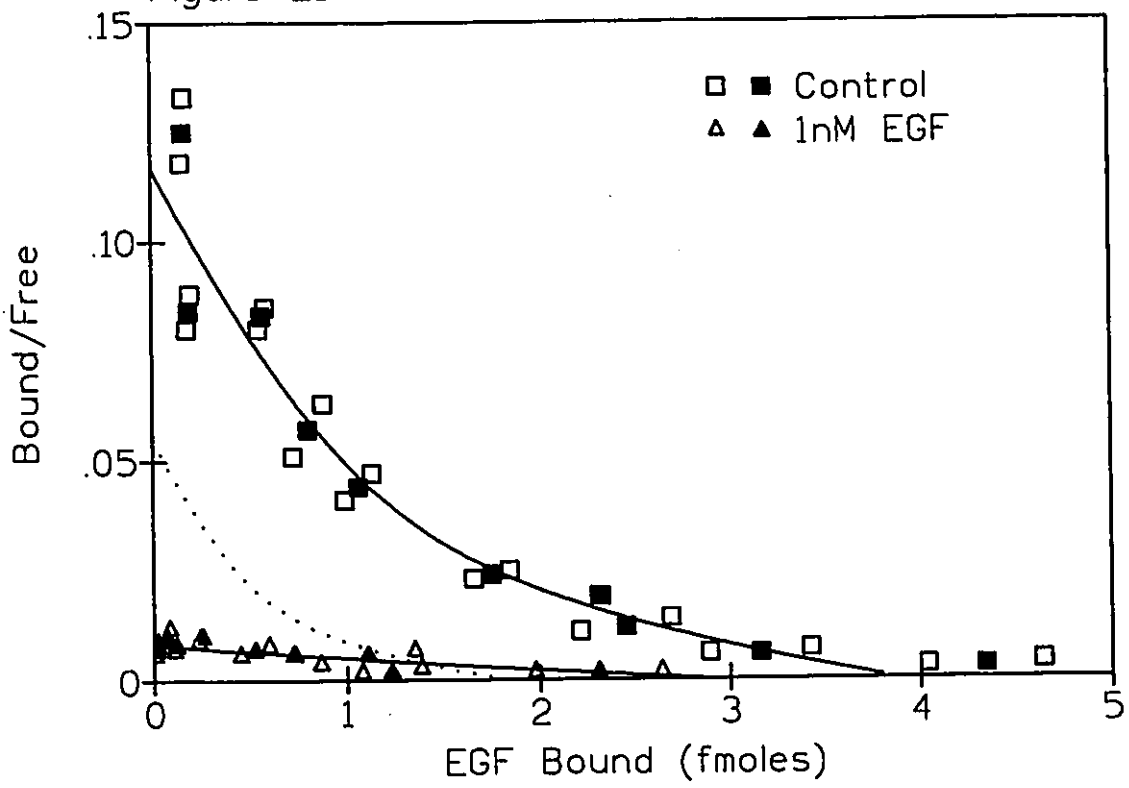

1.5
1.0
0.5
0.0
EGF Bound (fmoles/10^5 cells)
Control
1nM EGF
0
1
2
3
4
5
EGF concentration (nM)

Figure 21: Effect of growing cells with 1nM EGF for 24 hours on receptor binding Scatchard curve.

This figure shows the Scatchard transformation of the EGF receptor binding association data plotted in Figure 20. The transformed data are plotted as the mean (filled symbol) of duplicate measurements and the range (open symbols) for each EGF binding concentration used. The solid lines represent the lines of best fit to the Control and EGF-treated data points generated from the computer assisted ENZFITTER program assuming that there are two binding sites and one binding site respectively. The dotted line represents a theoretical line drawn to indicate that the EGF-treated data correspond to a one binding site model. The line was generated mathematically by reducing the total number of binding sites determined from the Control culture to the number of binding sites determined for the EGF-treated culture. The ratio of high affinity to low affinity sites and the Kd's of the two sites were not changed. Comparison of the theoretical line and the experimental data for the EGF-treated culture strongly suggest that these data represent EGF binding to a single site on the EGF receptor.

Figure 21



binding affinity of the low affinity binding site or not. Kd values do vary from one experiment to another and a doubling in value is not usually taken as significant. Technically this experiment was very difficult to perform because of the extremely low level of binding achieved at low EGF concentrations. This experiment was only performed once and would have to be repeated a number of times in order to determine the true Kd of the low affinity site under these experimental conditions.

Discussion

The first part of the results section clearly demonstrates the effects of adding 1nM EGF to proliferating rat liver epithelial T51B cells. There is an initial period of hyperplasia lasting 48 to 72 hours where the cell numbers reach double the control values. This is followed by a decrease in DNA synthesis and an increase in cell death. The cells die through apoptosis and the cell numbers return to around the control level five days after the initial EGF addition. This type of growth curve with EGF is also described by Armato et al., 1986 in primary cultures of neonatal rat liver cells. However, while EGF is mitogenic for most cell types, the hyperplasia seen with the T51B cells is not universal. Lagarrigue, Seillen-Heberden, Martel and Gaillard-Sanchez, 1992 show that normal rat liver epithelial (REL) cells do not increase their growth rate in the presence of 0.7nM EGF. This can be explained, to some extent, by the findings of Tsao and Liu, 1988 who showed that the *in vitro* proliferation of normal REL cells may be either inhibited or unaffected by EGF depending on the passage number. The proliferation of newly derived or early passage cells was inhibited by EGF. However, this growth inhibition was an unstable phenotype that could be diminished or lost after multiple passages if the conditions for culture and the times of subculture were not rigorously controlled. Lagarrigue et al., 1992 used late passage cells which did not respond to EGF. Other workers have found the addition of nanomolar doses of EGF to some tissue culture systems to be growth inhibitory, but this does seem to be a peculiarity of neoplastic cells. For example, it is fairly well

known that high doses of EGF inhibit the growth of A431 epidermoid carcinoma cells (Barnes, 1982) but EGF has also been found to inhibit the growth of the human hepatoma Li-7 A cell line (Knowles, 1988) and of the MKN-28 human gastric carcinoma transplanted into nude mice (Murayama, Onishi and Mishima, 1992). In most instances growth inhibition of neoplastic cells by EGF has been correlated with the presence of a large number of EGF receptors per cell (Kawamoto, Mendelsohn, Le, Sato, Lazar and Gill, 1984; Filmus, Pollak, Cailleau and Buick, 1985). However, it is not yet clear why this should result in growth inhibition in the presence of the growth factor.

Angello, 1992 has shown that the length of the cell cycle of human fibroblasts grown with 2nM EGF is decreased compared to controls and that their mitotic cell volume is reduced. This is of interest because the T51B cells grown with EGF appear to be smaller than the controls during the growth phase (Figure 2.2). It is also interesting that the morphology of the T51B cells changes and that they are able to grow over one another in the presence of EGF. Exactly how the growth factor reduces or removes the normal contact inhibition of the cells is not known. Lau, Kanemitsu, Kurata, Danesh and Boynton, 1992 have shown that short term exposure (30 minutes) to 1nM EGF disrupts gap-junctional communication (gjc) in T51B cells. This disruption does not seem to be long lasting (gjc is re-established within 3 hours) and so it may not affect cell morphology and/or contact inhibition. It is becoming apparent that the EGF structural motif occurs in numerous proteins, including some found in the extracellular matrix (ECM) (Browne, 1991).

It is possible that the relatively high concentration of EGF used in this system interferes with the normal cell-cell and/or cell-ECM interactions. Once these interactions are removed or reduced the T51B cells are able to grow over one another.

One of the main objectives of this project was to set up an *in vitro* model that could be used to study the early events in apoptosis. Having described a system where cells died in large numbers it was then necessary to show that this death was via apoptosis. Unfortunately there in no definitive marker for this mode of cell death so I used a variety of possible markers in order to determine that the cells were dying through an active form of cell death as opposed to necrosis.

Apoptosis was first described in terms of cell morphology (Wyllie, Kerr and Currie, 1980) so experiments were carried out to determine whether any dying T51B cells exhibited the morphology typical of apoptosis. Under the light microscope cells with convoluted surfaces (blebs) could be seen (Figure 2.4) indicating the start, in morphological terms, of apoptosis. The formation of these blebs represents the early stage of the fragmentation of a cell into apoptotic bodies. Under the electron microscope phagocytosed apoptotic bodies were seen in healthy cells (Figure 3.2), indicating the end of apoptosis. These two morphological studies were performed in a qualitative manner and serve to show that T51B rat liver epithelial cells are capable of dying through apoptosis.

It is interesting that the healthy cells are capable of phagocytosis and some recent papers have shed some light on changes in the apoptotic cell that allow this. Fadok, Voelker, Campbell, Cohen, Bratton and Henson, 1992 have shown that apoptotic lymphocytes lose membrane phospholipid asymmetry and expose phosphatidylserine (PS) on the outer leaflet of the plasma membrane. It appears that macrophages specifically recognise the exposed PS and phagocytose the apoptotic lymphocytes. Savill, Dransfield, Hogg and Haslett, 1990 showed that the vitronectin receptor on the surface of macrophages mediated the phagocytosis of apoptotic cells, although they were unable to identify the relevant ligand. It would appear that various markers could label a cell for phagocytosis and that these markers could be specific or non-specific. For example, in the liver it has been proposed that the asialoglycoprotein receptor mediates the clearance of apoptotic cells (Dini, Autuori, Lentini, Oliverio and Piacentini, 1992). Immature glycan structures are exposed on the surface of apoptotic cells as the endoplasmic reticulum and Golgi dilate and fuse with the plasma membrane. These structures then interact with the sugar recognition systems of the liver and the apoptotic bodies are phagocytosed. A similar idea is put forward by Duvall, Wyllie and Morris, 1985 who propose that macrophages (or other cells) might recognize a variety of proteins that become abnormally presented on the surface of dying cells. It is not yet clear whether there is a general mechanism common to all systems. In the nematode, *Caenorhabditis elegans*, 7 genes have been identified whose protein products are required by the phagocytosing cells for the engulfment of dying cells (Ellis, Jacobson and Horvitz,

1991). Nematodes that have one or two of these genes mutated still appear to be able to extend pseudopodia. This suggests that the mutations affect the recognition of dying cells rather than the phagocytotic mechanisms of the engulfing cells.

The earliest biochemical marker taken to be specific for apoptosis was the activation of an endogenous endonuclease (Wyllie, 1980). This enzyme, or enzymes, digests DNA into multiple nucleosome length fragments which gives a characteristic DNA "ladder" under agarose gel electrophoresis. Cohen and Duke, 1984 found that the endonuclease was Ca^{2+}/Mg^{2+} dependent and Cidlowski's group have spent many years trying to identify, purify and characterize it (Caron-Leslie, Schwartzman, Gaido, Compton and Cidlowski, 1991; Gaido and Cidlowski, 1991). The candidate endonuclease purified by this group is an 18 kDa protein which was called NUC18. It was found to be inactive in the absence of calcium, and known inhibitors of apoptosis such as zinc and aurintricarboxylic acid inhibited its activity. Gel filtration analysis revealed that NUC18 was present as a high molecular weight complex (>100 kDa) in both control thymocytes and those induced to undergo apoptosis with glucocorticoids. However, it also existed as a low molecular weight form in glucocorticoid-treated cells. Whether this lower molecular weight form could be the activated enzyme is not clear from the papers. Other workers previously suggested that the putative endonuclease(s) discovered by Cidlowski's group were in fact histones (Baxter, Smith and Lavin, 1989; Alnemri and Litwack, 1989) and a recent paper proposes that the endonuclease is DNase I which gains access to

the nucleus due to the breakdown of the endoplasmic reticulum and the nuclear membrane during apoptosis (Peitsch, Polzar, Stephan, Crompton, MacDonald, Mannherz and Tschopp, 1993). Thus some controversy still remains in this area.

I carried out DNA electrophoresis in my system and Figure 4 shows that an endonuclease(s) is activated in T51B cells. DNA ladders are seen in both of the attached cultures which indicates that this phenomenon is not limited to the cells grown with 1nM EGF. There is also a faint DNA ladder pattern seen with EGF-treated cells which have lifted off the culture plate and are floating in the media. It has been reported in other systems, such as with nutrient-starved human melanoma MM96 cells (Sheridan, Bishop and Simmons, 1981), that cells dying by apoptosis in tissue culture become detached from the substratum and eventually undergo secondary necrosis. However, in my system the cell death appears well controlled, without large numbers of floating cells. Consistent with this the DNA nucleosome ladder from the floating cells is very faint. The lack of floating cells seen in this system also ties in with the electron microscopy work which shows that apoptotic bodies are engulfed by neighbouring cells. Unfortunately this technique is not particularly quantitative so it is difficult to determine the amount of apoptosis occurring in any one sample. Recently Tilly and Hsueh, 1993 have published a method that allows more quantitative analysis of apoptotic DNA fragmentation. The method involves adding one molecule of $[\alpha^{32}P]$-dideoxynucleotide to the 3'-end of DNA fragments. Following gel electrophoresis and autoradiographic analysis the total amount of

radiolabel incorporated into the low molecular weight DNA fraction can be quantified. This method could be useful for future studies on this system.

Although at one time the presence of a DNA "ladder" would have been taken as proof of apoptosis in a particular system it is not clear whether it is a definitive marker for all systems (Lockshin and Zakeri, 1990). It has been correlated with apoptosis (Wyllie, 1980) but has not been universally associated with ultrastructural changes, for example, in target cells killed by granules derived from cytolytic T lymphocytes (Gromkowski, Brown, Masson and Tschopp, 1988) and in the intersegmental muscles of the moth *Manduca sexta* at the end of metamorphosis (Schwartz, Smith, Jones and Osborne, 1993). This also leads to speculation as to whether cell death can be described solely under the three headings necrosis, differentiation and apoptosis outlined in the introduction. A review by Clarke in 1990 examines the manner in which cells are dismantled during developmental cell death. He argues that in morphological terms there are three ways in which this can occur. These are termed apoptosis; in which the fragments of the dying cell are destroyed in secondary lysosomes of other cells; autophagic degeneration, where the cell is destroyed to a great extent within its own lysosomes, and non-lysosomal vesiculate degradation, where cells are destroyed without the lysosomes playing any detectable role. Comparative studies on cell death in mouse T cells and moth intersegmental muscle cells by Schwartz et al., 1993 show that these two cell types differ in most aspects of their apoptotic cell deaths. The

only common features are that both types of cell death are initiated by specific physiological signals and both require *de novo* gene expression. Thus it is becoming clear that there is more than one type of cell death that currently comes under the heading "apoptosis". The problem at the present time is to agree on the characteristics of the different types of cell death and to name them consistently. If the different morphological types of cell death described by Clarke, 1990 represent different underlying mechanisms then it seems likely that there would also be a diversity of signals. If we are to understand cell death it is essential that this diversity be recognised and not confused.

One obvious marker for a form of cell death that requires *de novo* gene expression would be the increase in specific mRNA transcripts. Unfortunately, at present, the genes responsible for mediating apoptosis are largely unknown. An increase in the gene expression of polyubiquitin is seen during cell death in the moth intersegmental muscles (Schwartz, Myer, Kosz, Engelstein and Maier, 1990) but not in dying T cells (Schwartz et al., 1993). Piacentini, Autuori, Dini, Farrace, Ghibelli, Piredda and Fesus, 1991, have shown that there is an increase in "tissue" transglutaminase enzyme activity in neonatal rat liver cells undergoing apoptosis but it is not clear whether there is also an increase in gene expression because suitable controls were not performed. An increase in the activity or expression of ubiquitin and "tissue" transglutaminase is relevant to apoptosis. Ubiquitin can be covalently linked to cellular proteins to mark them for degradation (Finley and Chau, 1991) and "tissue" transglutaminase covalently cross-

links proteins at the plasma membrane forming a protein shell which presumably helps to stabilize the apoptotic bodies (Fesus, Thomazy, Autuori, Ceru, Tarcsa and Piacentini, 1989). During the involution of the prostate in rats after castration there is induction of a gene, termed TRPM-2, which coincides with the start of apoptosis (Léger, Montpetit and Tenniswood, 1987). TRPM-2, or its protein product, has also been identified from a number of other biological systems in which it is expressed constitutively, for example, as serum protein 40,40 (SP-40,40) from human serum (Kirszbaum, Sharpe, Murphy, d'Apice, Classon, Hudson and Walker, 1989) and as Glycoprotein III from bovine adrenal medullary chromaffin granules (Palmer and Christie, 1990). SP-40,40 is a complement associated protein (Kirszbaum et al., 1989) and it has been suggested that TRPM-2 may be necessary for maintaining the integrity of the cell membrane and for preventing complement fixation during the formation of apoptotic bodies (Wong, Pineault, Lakins, Taillefer, Léger, Wang and Tenniswood, 1993). Whilst TRPM-2 may be a good marker for apoptosis in the regressing rat prostate it is not so useful in T51B cells. Work on an earlier cell death model developed with these cells, using the calcium ionophore A23187, showed that while TRPM-2 expression did increase during apoptosis the gene was also expressed constitutively and expression increased during cell proliferation (Brabyn and Kleine, 1990). Thus the expression of TRPM-2 was not used as a marker for apoptosis in this system.

In order to show categorically that the decrease in cell numbers seen 3 to 5 days after EGF treatment was due to an increase in apoptosis

over the control levels I decided to fix the cells and stain them with Hoechst dye. I was then able to determine the number of cells undergoing apoptosis at any particular time by counting the number of fragmented nuclei (FN). Figure 7 shows that the percentage of FN starts to increase in the EGF-treated cultures 3 days after EGF addition and that it increases to around 4% by day 5 of the experiment. Although the percentage is low it is sufficient to account for the decrease in cell numbers. Reports on the duration of the histological stages of apoptosis in liver suggest that the visible stages could be complete in 3 to 4 hours (Bursch, Paffe, Putz, Barthel and Schulte-Hermann, 1990b; Faa, Ambu, Congiu, Costa, Ledda-Columbano, Coni, Curto, Giacomini and Columbano, 1992) which means that only a small number of cells need to be going through apoptosis at any one time for there to be a significant drop in cell numbers. To emphasize this point Bursch et al., 1990a state quite clearly that because of the rapid destruction time it is only necessary for 2-3% of cells to be undergoing apoptosis at any one time to obtain a very substantial regression of a tissue; in the order of 25% per day. The standard error associated with the percentage of FN is very large at day 5. This can be attributed to the variation in the exact growth curve obtained from one experiment to another. Sometimes the cell death would slow down at day 4 and the cell numbers would reach the control values at day 5, whereas at other times there would still be a lot of cell death at day 5 and the cell numbers would fall off rapidly. This is borne out by the cell growth curve in Figure 1. I am not sure why this variation occurs but feel that it is fairly common in tissue culture systems.

Setting up the double labeling technique allowed me to monitor DNA synthesis as well as the number of cells undergoing apoptosis. In the results section I mentioned that very few of the fragmented nuclei had incorporated tritiated thymidine, which suggests that the dying T51B cells could be in G_1 or even G_0. Originally I had hoped that apoptosis might be linked to a specific phase of the cell cycle for all cells because then it might prove easier to determine the initial signal transduction pathway(s). However, recent work on different systems shows that cells can enter apoptosis from any point in the cell cycle. Darzynkiewicz, Bruno, Del Bino, Gorczyca, Hotz, Lassota and Traganos, 1992 suggest that apoptosis could be selective to different cells at different stages of the cell cycle. They found that human leukemic HL-60 cells exposed to DNA topoisomerase II inhibitors entered apoptosis in S-phase, whereas with rat thymocytes the same drugs resulted in apoptosis when the cells were in G_0. On the other hand Cotter, Glynn, Echeverri and Green, 1992a have shown that A1.1 T-cell hybridoma cells exposed to chemotherapeutic agents can enter apoptosis in all phases of the cell cycle. Other reports have shown cells entering apoptosis in G_1 (Ijiri and Potten, 1990; intestinal crypt cells from irradiated mice), at G_2/M (Kruman, Matylevich, Beletsky, Afanasyev and Umansky, 1991; Murine BW 5147 thymoma cells induced with dexamethasone and γ-irradiation) and mid-S-phase (El Alaoui, Mian, Lawry, Quash and Griffin, 1992; met B hamster fibrosarcoma cells under normal growth conditions).

Combining the results from the morphological studies, the DNA electrophoresis and the quantification of nuclear fragmentation leads to

the conclusion that the decrease in T51B cell numbers over days 3 to 5 after EGF addition is due to an increase in apoptosis. The blebbing of the plasma membrane, the nuclear condensation and the existence of a DNA internucleosomal ladder along with the phagocytosis of apoptotic bodies by neighbouring cells correspond with the classic features of apoptosis as described by Wyllie et al.. 1980 and Clarke, 1990. Interestingly in this model it is the addition of a growth factor to a cell culture system that results in apoptosis. Most other cell culture models rely on withdrawal of the growth factor to induce apoptosis, for example, serum deprivation of C3H 10T 1/2 fibroblasts (Kanter, Leister, Tomei, Wenner and Wenner, 1984); IL-2 withdrawal from CTLL-2 cells (Rodríguez-Tarduchy and López-Rivas, 1989) and granulocyte macrophage colony-stimulating factor or IL-3 withdrawal from hemopoietic cells (Rajotte, Haddad, Haman, Cragoe and Hoang, 1992).

* * * * * * *

The second part of the Results section describes the results from cell growth experiments carried out to explore the model system further. I changed various conditions in the system and observed how it responded. This was done in order to try to explain why the hyperplasia and apoptosis were occurring and to see which events, if any, were EGF-specific.

The first two experiments in this part of the results section involved trying to reproduce the growth curve obtained with EGF by other means. Liver is the main source of production for IGF-1 (Quin, 1992)

and there is an increase in IGF-1 binding in regenerating rat liver compared to normal liver (Caro et al., 1988). Thus it seemed possible that IGF-1 might also prove to be a good mitogen for T51B cells. However, there was very little increase in cell numbers compared to controls even at high doses of IGF-1 (Figure 8.1 and 8.2). It is possible that non-parenchymal liver epithelial cells do not respond to IGF-1 *in vivo* or that T51B cells have somehow lost their responsiveness. On the other hand, the IGF-1 receptor might have a Kd higher than the 1nM given by Werner et al., 1991 which could mean that the concentrations I was using were too low to generate hyperplasia. However, 10nM would be a high concentration for most growth factors and the levels that I used were consistent with those reported in the literature. It is possible that other growth factors relevant to liver or epithelial cells might reproduce the EGF growth curve, for example hepatocyte growth factor, but no others were tried. I did not use TGF α because this growth factor utilizes the EGF receptor and I was interested in reproducing the growth curve through another receptor pathway. Next I tried changing the cells' media and serum daily to see if hyperplasia could be induced by refeeding. Figure 9 indicates that there was no change in the cell growth curve. Although the use of one other growth factor (IGF-1) and refeeding is a rather limited trial it does suggest that the EGF growth curve is not a general phenomenon.

When I set up this model system I found that I could generate the growth curve by adding EGF to the cells just once. However, in the system set up by Armato et al., 1986 they had changed the media and

replenished the EGF daily. I found that changing the media daily had no effect on the control growth curve (see above) and that adding EGF daily had no effect on the EGF growth curve (Figures 10.1 to 10.4). This shows that it is the initial dose of EGF that is important in generating the hyperplasia. Assuming that the EGF is taken up by the cells and the ligand or the receptor/ligand complex is degraded fairly rapidly (Carpenter and Cohen, 1976) then after this initial dose further exposures to EGF do not alter the cells' growth pattern. In addition, since only one exposure to EGF is required to generate the hyperplasia, which persists for several days, one can hypothesize that some long term changes might be occurring in the cells to sustain it. Whether the changes could represent a continuous message being sent down a signal transduction pathway or new gene transcription is not certain and would require further investigation.

One attempt to determine whether the cell death was specifically due to the addition of EGF was to add the EGF either 1 day or 3 days after plating. If the cell death was due to the EGF then it was hypothesized that the cells would start to die at a certain interval after EGF addition. This experiment involved plating the cells at half the usual density, as well as adding the EGF at different times, in order to try to distinguish between EGF and density-related events. Figures 11.1 to 11.4 show that adding EGF at 1 day or 3 days after plating had no significant effect on the growth curve; the cells reached a density of 5.5×10^6 cells per plate 7 or 8 days after plating and then started to die. This would suggest that the increase in apoptosis is

not specifically caused by EGF, but that it is related to cell density. Perhaps a growth inhibition signal is generated once the cells reach a certain density.

I also wanted to determine whether the cell phenotype was being changed by exposure to high doses of EGF. I investigated the growth characteristics of the cells after they had been exposed to EGF for either 2, 4 or 7 days by replating them. This represents proliferating, dying and surviving populations of cells respectively. Figures 12.2 and 12.3 indicate that the control and EGF-treated cells at Days 2 and 4 grow to the same density once replated. The Day 2 cells do not continue to proliferate past the usual density of $5x10^6$ cells/dish, nor do they proliferate and then die. The Day 4 cells also proliferate to the usual density and do not die. This suggests that the growth characteristics of the cells have not been changed by their initial exposure to EGF and that perhaps it is the cell density that results in the increase in apoptosis. The surviving cells (Day 7) were replated and re-treated with EGF to see if they represented a resistant population of cells. Figure 12.4 shows that these cells are quite capable of responding to 1nM EGF in the normal manner; comparing Figure 12.4 to the growth curve for the original exposure to EGF, Figure 12.1. Although this experiment was only performed twice it does suggest that the growth phenotype of the cells is not being changed by exposure to 1nM EGF.

The final experiments in this section involved growing the T51B cells with different doses of EGF and this produced some very interesting results. Figures 13.1 to 13.6 plot the growth curves of

cells cultured in a wide range of EGF concentrations; from 10pM to 10nM. Figure 13.6 summarizes the results and shows that there is a distinct cut-off in the EGF-response at 0.5nM EGF. This, and higher concentrations, produce the characteristic hyperproliferation/apoptosis growth curve seen with 1nM EGF. In fact this growth curve is maintained even at the very high dose of 100nM EGF (data not shown). The relatively low dose of 10pM EGF does not produce this growth curve and the cell numbers follow the control curve. However, at 0.1nM EGF the hyperplasia is initiated but for some reason is not sustained. This is interesting because the cells only reach a density of $4x10^6$ cells per plate before dying back down to the control level. This brings into question the conclusion above that a growth inhibition signal could be generated once a certain cell density is reached. Perhaps the lower dose of EGF can only generate a lower cell density before such a signal is initiated.

In order to try to understand why there would be a distinct cut-off in the EGF-response I looked to the literature on EGF receptor binding affinities. There appear to be two binding affinities for EGF, for example, in freshly isolated adult rat hepatocytes (Gladhaug, Refsnes and Christoffersen, 1992); in rat liver after partial hepatectomy (Vesey et al., 1992a) and in Swiss 3T3 cells (Brown, Blay, Irvine, Heslop and Berridge, 1984). Using the Kd values shown in these papers I have calculated the EGF receptor occupancy levels at the various EGF concentrations used (Table 2). This shows that the high affinity binding site is almost saturated at 0.5nM EGF whereas the low affinity

Table 2: EGF receptor occupancy levels (%) at various ligand concentrations using binding parameters from three sources.

This table provides a summary of the EGF receptor occupancy levels at various EGF concentrations using the Kd's from A: Gladhaug et al., 1992; B: Vesey et al., 1992a and C: Brown et al., 1984. The occupancy levels are calculated as [EGF]/([EGF]+ Kd).

Table 2: EGF receptor occupancy levels (%) at various ligand concentrations using binding parameters from three sources.

	A		B		C	
Kd:	20pM	600pM	5.2pM	3.6nM	9.0pM	1.8nM
[EGF]						
10 pM	33.3	1.6	16.1	0.3	10.0	0.6
0.1 nM	83.3	14.3	65.8	2.7	52.6	5.3
0.5 nM	96.2	45.5	90.6	12.2	84.7	21.7
1.0 nM	98.0	62.5	95.1	21.7	91.7	35.7
10 nM	99.8	94.3	99.5	73.5	99.1	84.7

binding site is just starting to be occupied at this concentration. This suggests that perhaps it is occupancy of the low affinity binding site which leads to hyperplasia in the T51B cells.

* * * * * * *

The final part of the Results section describes the results from EGF binding studies carried out in T51B cells. These were done to test the hypothesis that the EGF receptor's low affinity binding site is involved in the generation of the hyperplasia/apoptosis growth curve seen when the cells are grown with high concentrations of EGF.

The first experiments set out to determine the EGF receptor binding characteristics in T51B cells. The non-specific binding of the (3-[^{125}I]iodotyrosyl)EGF, and presumably of the unlabeled EGF, was found to be consistently low at less than 10% of the total cpm bound (Figure 14). All binding assays were carried out at 4°C to reduce receptor-mediated endocytosis of the EGF and the proteolytic degradation of the free and bound hormone (Gammeltoft, 1990). It was found that equilibrium binding was achieved by 8 hours. The binding remained steady for 24 hours so this incubation time was used for all experiments (Figure 15).

Figure 16 shows the results from a binding study carried out over a very wide concentration range (6pM to 10nM). The curve shows that the binding has reached saturation at around 5nM EGF. This is consistent with work carried out on primary rat hepatocytes (Gladhaug and Christoffersen, 1987). The data from this experiment were then

transformed into a Scatchard plot in order to calculate the binding parameters of the EGF receptor in the T51B cells (Figure 17). The cells displayed a curvilinear plot indicating that there is more than one binding affinity for EGF. Based on the literature it was assumed that there are two binding affinities for EGF and the results from the computer analysis of the binding data are shown in Table 1. The Kd values obtained for the EGF receptor in the T51B cells are consistent with those obtained using freshly isolated adult rat hepatocytes (A in Table 2). The two binding affinities for EGF are quite distinct in the T51B cells since the Kd's differ by an order of magnitude. The results also show that there are about six times as many low affinity sites as high affinity sites. Scatchard analysis was carried out on data from two separate experiments and the binding parameters obtained indicate that these results are reproducible.

Table 3 shows the EGF receptor occupancy levels calculated for the T51B cells at key EGF concentrations using the Kd values obtained in the two separate experiments. Despite the variations in Kd values from one experiment to another the only difference in the calculated occupancy levels between experiments A and B is for the high affinity site at 10pM EGF. The receptor occupancy levels suggest that while the high affinity site might be responsible for the control of cell proliferation (10pM EGF) it is the occupancy of the low affinity site which leads to the hyperproliferation (0.1nM and 0.5nM EGF).

The final set of EGF binding experiments was carried out on cells which had been grown with EGF in order to investigate how the high and

Table 3: EGF receptor occupancy levels (%) in T51B cells at key EGF concentrations.

This table provides a summary of the EGF receptor occupancy levels in T51B cells at key EGF concentrations identified during the dose response experiments (Figures 13.1 to 13.6). The Kd values are those obtained from two separate experiments (A and B). The results from experiment A are shown in Figures 16 and 17. The results from experiment B are not shown. The occupancy levels are calculated as [EGF]/([EGF]+Kd).

Table 3: EGF receptor occupancy levels (%) in T51B cells at key EGF concentrations.

[EGF]	A		B	
	High 13.8pM	Low 387pM	High 26pM	Low 320pM
10pM	42	3	28	3
0.1nM	88	21	80	24
0.5nM	97	56	95	61

low affinity binding sites varied during the growth curves. Full five day growth curves were set up at 1nM and 10pM EGF and the binding was carried out at two EGF concentrations (20pM and 3nM). This was done in order to obtain a qualitative assessment of the binding due to the high and low affinity sites respectively.

The growth curve with 1nM EGF (Figure 18.1) shows the usual hyperproliferation and cell death observed at this EGF concentration. The EGF binding curves (Figures 18.2 and 18.3) suggest that the receptor is down-regulated within the first 24 hours after EGF addition and that there is only a slight recovery of the low affinity site by Day 5 (Figure 18.3). The Scatchard analysis (Figure 21) of the binding data obtained from cells which had been grown with 1nM EGF for 24 hours indicates that the high affinity binding site has almost completely disappeared and that the residual binding obtained at 20pM EGF (Figure 18.2) is due to the remaining low affinity sites. The Scatchard analysis also suggests that the decreased EGF binding is due both to down-regulation and desensitization of the low affinity binding sites since there is a reduction in the number of sites and an increase in the Kd compared to the control values shown in Table 1. This experiment was only performed once at a single time point and so care must be taken in interpreting the data, particularly the changes in the numbers and the Kd of the low affinity binding site.

The loss of high affinity binding sites has been reported in other systems, for example for dexamethasone binding to rat liver nuclear envelopes (Howell and LeFebvre, 1989). Gladhaug, Refsnes, Sand and

Christoffersen, 1988 have shown that there is a loss of high affinity EGF binding sites during culturing of primary rat hepatocytes. Other workers have reported a loss of high affinity binding to an insulin-EGF receptor chimera when cells are incubated with high concentrations of insulin or phorbol ester (Tartare, Ballotti, Lammers, Filloux, Chauvel, Schlessinger, Ullrich and Van Obberghen, 1992). The binding patterns seen in these papers are very similar to those observed with the T51B cells which have been grown with 1nM EGF for 24 hours. Interestingly Gladhaug et al., 1988 report that the proliferative response to EGF is stronger in the rat hepatocytes once their high affinity binding sites have disappeared.

Additional experiments would have to be performed to determine the cause of the decrease in low affinity binding when the cells are grown with 1nM EGF. Repeating the Scatchard analysis at the 24 hour time point as well as carrying out the analysis at other time points would indicate whether the Kd of the low affinity site really has increased. These experiments would also show whether the high affinity site has disappeared at other time points as is expected. Western blotting experiments using T51B plasma membranes and antibodies to the EGF receptor could be used to corroborate these data since they would indicate whether there is a down-regulation of the receptor. There are some reports which suggest that a reduction in EGF binding in some systems is solely due to a decrease in receptor affinity (increase in Kd) and that there is no significant change in the number of cell surface receptors (Gray, Wei and Macara, 1989; Gray and Macara, 1988).

The binding experiments carried out on cells grown with 1nM EGF help to explain why there is no difference in cell numbers when the EGF is added to the cells daily or once, at Day 0, as shown in Figure 10.3. The rapid down-regulation/desensitization of the receptor in the first 24 hours after EGF addition means that further additions of EGF can produce no significant effect on the cells. The replating experiment, where EGF-treated cells are regrown with 1nM EGF (Figure 12.4), suggests that the receptor reappears when the cells are replated because the cells are once again responsive to EGF. This would also suggest that EGF is fairly stable at 37°C for at least five days because there is no significant reappearance of the receptor during that period (Figures 18.2 and 18.3). However, a bioassay or radioimmunoassay would need to be performed to determine whether this is correct.

The dose response curves show that when the cells are grown with 10pM EGF there is no difference in the growth curve between control cells and EGF-treated cells (Figure 13.1). The binding experiments indicate that there is also no difference in EGF binding between control and EGF-treated cells (Figures 19.2 and 19.3). This suggests that there is no down-regulation of the receptor in cells grown with 10pM EGF. Table 3 shows that at 10pM EGF the high affinity binding site is occupied in the T51B cells whereas the low affinity site is only just occupied (3%). Thus my results do seem to suggest that it is the occupancy of the low affinity binding site which leads to the hyperproliferation of the T51B cells. However, the fact that there is no change in cell numbers or EGF binding compared to the controls could

mean that the EGF does not interact with the receptor at this concentration. It is possible, for example, that EGF could be bound by serum factors in the cell media and that at low EGF concentrations very little EGF is free to bind to the receptor. To determine whether 10pM EGF does bind to the receptor when serum is present one could ascertain whether the receptor's inherent protein tyrosine kinase activity has increased. This could be done by looking for an increase in the amount of phosphotyrosine present in EGF-treated cells versus control cells.

There are some reports in the literature which indicate that some cell types can respond to 10pM EGF when low amounts of serum are present, for example there is an increase in DNA synthesis over control levels when human foreskin fibroblasts are grown with 10pM EGF and 1% serum (Carpenter and Cohen, 1975). The levels of EGF in sera are in the range 25pM to 90pM for humans and mice (Hirata, Moore, Bertagna and Orth, 1980; Fisher, Salido and Barajas, 1989; Byyny, Orth, Cohen and Doyne, 1974) which suggests that 10pM EGF could be a very low dose which would not produce any significant effects on cells *in vivo*.

Assuming that serum factors do not significantly affect the concentration of "available" EGF in the growth media then my results implicate a role for the low affinity binding site on the EGF receptor in generating hyperplasia in the T51B cells. This is a rather controversial conclusion because, as stated in the introduction, the favoured model of EGF receptor activation describes the low affinity binding site as the single receptor molecule, having no physiological relevance. Böni-Schnetzler and Pilch (1987) isolated EGF receptor

monomers and noncovalently associated dimers by sucrose density centrifugation. They showed that for solubilized receptors the monomers were the low affinity receptors and the dimers were the high affinity receptors. A more recent study was carried out using the soluble, extracellular ligand binding domain of the EGF receptor (Zhou, Felder, Rubinstein, Hurwitz, Ullrich, Lax and Schlessinger, 1993). This study showed that soluble receptor dimers stabilized with a covalent cross-linking agent bound EGF with a higher affinity than did soluble receptor monomers. While both of these studies support the hypothesis that receptor dimers represent the high affinity binding sites they both ignore the effects of the plasma membrane on receptor binding.

Two studies using antibodies to the EGF receptor suggest that signal transduction events and early responses are generated through the high affinity binding site (Defize, Boonstra, Meisenhelder, Kruijer, Tertoolen, Tilly, Hunter, van Bergen en Henegouwen, Moolenaar and de Laat, 1989; Bellot, Moolenaar, Kris, Mirakhur, Verlaan, Ullrich, Schlessinger and Felder, 1990). The first study involved the use of an antibody which specifically blocks EGF binding to the low affinity site. The authors claim that the antibody had almost no effect on EGF-induced activation of the receptor's protein tyrosine kinase activity. The second study used an antibody to reduce the binding of EGF to the high affinity site. This resulted in a reduction, but not a complete cessation, of early responses to EGF such as tyrosine-specific phosphorylation of the receptor, turnover of phosphatidyl inositol,

elevation of cytoplasmic pH and release of calcium from intracellular stores.

However, there are also some reports in the literature which suggest a role for the low affinity EGF binding sites. Wollenberg, Harris, Farber and Hayes (1989) showed that the high affinity EGF binding sites almost completely disappear during the first 24 hours of culturing of primary rat hepatocytes and that the cells only start to respond to EGF between 16 and 24 hours after isolation. They conclude that the high affinity EGF receptors do not elicit the proliferative response to EGF. A similar conclusion has been reached by Gladhaug, Refsnes and Christoffersen (1992), again working with freshly isolated rat hepatocytes. These authors go further and even suggest that the high affinity binding site could be involved in differentiation, whereas the low affinity binding site is involved in mitogenesis.

Attempting to reconcile roles for both the high and low affinity binding sites in EGF-mediated signal transduction with the current models of what these two sites represent is very difficult. The favoured model of allosteric aggregation suggests that only the high affinity binding sites could have physiological relevance (Yarden and Schlessinger, 1985). The model proposed by Wofsy et al., 1992, and described in the introduction, suggests that both sites represent aggregated receptors and thus that both sites could be involved in signal transduction. However, this model does not accommodate the fact that in all systems examined there are always far more low affinity sites than high affinity sites. It is possible that the two binding

affinities represent sites for different ligands. However, this seems unlikely for the known ligands because it has been shown that the binding affinities and capacity of receptors in A431 cell membranes and placental membranes for EGF and TGF α are strikingly similar (Massagué, 1983).

Taking all the available evidence into account I favour the model where receptor monomers represent the low affinity sites and receptor dimers represent the high affinity sites. However, I disagree with the notion that the low affinity sites have no physiological role. It has been shown by Koland and Cerione (1988) that single, solubilized receptors are capable of intramolecular activation of the protein tyrosine kinase activity and the work by Wollenberg et al., 1989 with primary cultures of rat hepatocytes is quite compelling. The model proposed by Wofsy et al., 1992 would produce negative cooperativity on binding but it is possible that the pattern seen experimentally is due to some form of receptor heterogeneity. This heterogeneity could be produced by differences in the numbers of autophosphorylated sites, by different interactions with membrane and cytoskeletal factors or even by different degrees of receptor glycosylation. Wofsy et al., 1992 did try to remove many sources of receptor heterogeneity but admit that they could not account for them all.

To determine whether the low affinity binding sites really are responsible for the hyperproliferation obtained when T51B cells are grown with high doses of EGF it would be necessary to carry out further experiments. For example, using antibodies, such as those described by

Defize et al., 1989 and Bellot et al., 1990, to block the low affinity or the high affinity binding sites while the cells were exposed to EGF and determining whether the hyperproliferation still occurred.

The EGF binding experiments carried out on cells growing with 1nM EGF indicate that the receptor is rapidly down-regulated and that it does not reappear to any significant extent during the time course (Figures 18.2 and 18.3). This suggests that the increase in apoptosis induced over Days 3 to 5 could be due to receptor down-regulation. This is analogous to apoptosis being induced by removal of a trophic hormone, for example in the prostate after castration (Kyprianou and Isaacs, 1988) or in CTLL-2 cell cultures after removal of IL-2 (Rodriguez-Tarduchy and López-Rivas, 1989). There appears to be a time lag between the receptor down-regulation which occurs within 1 day of EGF addition, and the increase in apoptosis 3 days after EGF addition. However, in all the growth curves presented in the results section the maximum cell numbers achieved by the EGF-treated cells are double those of the corresponding confluent control cultures. This suggests that the high doses of EGF (0.5nM and higher) produce, on average, one additional cell division in the treated cells compared to the controls. Thus it is possible that the initial exposure to EGF, during Day 1, initiates this additional cell division and that when it is complete the new cells cannot respond to EGF again because their receptors are down-regulated. The cells then die via apoptosis.

It is also possible that the increase in apoptosis is initiated by other factors such as some form of contact inhibition

occurring when the cells reach a very high density. Indeed this would account for the fact that not all of the cells die and that the cell density returns to around the confluent control level by Day 5. However, it is interesting that the cells reach a lower density before the increase in cell death when they are grown with 0.1nM EGF (Figure 13.2). Jiang, Lu, Garcia and Thompson (1993) reported spontaneous apoptosis in murine leukemic L1210 cells grown to a high density. Their results support a model in which the gradual depletion of a serum factor(s), and not contact inhibition or the production of a "death factor", leads to the activation of a cell death program. The control T51B cells can remain confluent for about 10 days in culture without the need for media and serum changes suggesting that the depletion of serum factors might not account for the increase in apoptosis in this system. However, it is always possible that there are insufficient serum factors available to sustain the cells at the very high densities which result when they are treated with high doses of EGF.

Conclusion

The results clearly show that EGF is mitogenic for T51B rat liver epithelial cells. Doses of 0.5nM EGF and more result in hyperplasia which is characterized by a doubling in cell numbers compared to control cultures and a change in cell morphology. This is followed by a decrease in cell numbers as the cells die by apoptosis and the cell density returns to around the confluent control level. The fact that this growth curve is produced in a well controlled manner over a period of five days means that this system represents a very good *in vitro* model of both liver hyperplasia/regression and liver homeostasis.

The finding that healthy T51B cells are capable of phagocytosing the apoptotic bodies from the dying cells suggests that this model is more physiological than some *in vitro* models of apoptosis. It also means that this system could be used to study the phagocytosis of apoptotic bodies. The large numbers of cells going through the mitotic cycle and apoptosis means that the model will facilitate the study of early signaling events occurring in cell proliferation and active cell death.

The results from the EGF dose response experiments and from the EGF binding studies suggest that the hyperproliferation of the T51B cells at high doses of EGF is due to occupancy of the low affinity binding site of the EGF receptor. These studies also suggest that the increase in apoptosis could be initiated by the down-regulation of the EGF receptor once the mitogenic signal has been processed. However, I have not ruled

out the possibility that the increase in apoptosis is due to contact inhibition at high cell densities or to the depletion of a serum factor(s) by the treated cells, alone or in combination with the effective removal of the trophic hormone.

This work not only achieves the major objective of setting up an *in vitro* model of liver homeostasis it also provides a good system for studying the role of EGF in cell proliferation and cell death.

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Curriculum vitae

Name:	Caroline Jane Brabyn
Date of birth:	1st December 1960
Place of birth:	Taplow, England
Citizenship:	British and Canadian

Education:

Holt School for Girls
Wokingham, England
Age 12-18

University College, Cardiff
Cardiff, Wales
BSc. (Hons) Biochemistry, 1983

Institute of Chartered Accountants in England and Wales (ICAEW)
Admission examinations, 1986

Canadian Institute of Chartered Accountants (CICA)
Admission examinations, 1989

University of Ottawa
Ottawa, Ontario
PhD. (Biochemistry) registered 1988-present

Awards:

Ontario Graduate Scholarship, 1990

University of Ottawa Entrance Scholarship, 1990 and 1991

Experience:

Research technician - ICI Plant growth regulator division
Jealotts Hill, UK, 1980

Auditor - Grant Thornton
Oxford, UK, 1983-1986

Auditor - Deloitte Touche
Ottawa, Ontario, 1987-1988

Lab Demonstrator - Biochemistry 2936
University of Ottawa, 1990-1993

Lab Demonstrator - Biochemistry 3946
University of Ottawa, 1989-1992

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