Development and Evaluation of a Novel Explant Culture Technique for Studies on Growth Regulation in Human Breast Cancer

by

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For the Degree of
Doctor of Philosophy

School of Biomedical Sciences
University of St. Andrews
November 1999

ACKNOWLEDGEMENTS

It is with sincere gratitude that I give thanks to Mr. Paul Preece, Consultant Surgeon, Ninewells Teaching Hospital, for the generous donation of precious human tissue for this study and to the courageous women who underwent surgery.

I thank the staff in the Ludwig Institute for Cancer Research, London and the Royal Victoria Hospital, Newcastle upon Tyne, for their invaluable instruction in tissue culture and immunohistochemistry, respectively.

I thank my supervisors, Professor C. Michael Steel and Dr. John McLachlan, for their help in reading and compiling the thesis.

Many thanks to all my colleagues in the lab for their help and support, and for providing a happy working environment.

And last, but not least, thank you to my parents for their unfaltering faith, enduring support and unconditional love for me, now and always.

ABSTRACT

Breast cancer is the commonest female cancer in western countries. Anti-estrogens, e.g. tamoxifen, are used to combat the mitogenic effects of estrogens on breast tissue. Expression of Transforming Growth Factor β1 (TGFβ1), a negative growth factor in many epithelial cell systems, can be upregulated by tamoxifen in breast cells *in vitro* and *in vivo*. Controversy exists over the site of TGFβ1 production and its regulation of cell growth in 'real' carcinomas.

Most studies have focused on the MCF-7 cell line, which displays an estrogen receptor (ER)-mediated growth-inhibitory response to tamoxifen by upregulating TGFβ1. Similar studies on fresh-fixed tissue and *in vivo* trials have produced varied results.

This study aimed to develop and evaluate a culture system for the maintenance of fresh breast cancer explants. Stromal-epithelial interactions in the breast are believed to be central to growth regulation. It was essential, therefore, to preserve breast tissue architecture in culture. The value of the culture system was analysed by a time course of apoptotic rate and expression of TGFβ1.

Even in hormone-depleted medium, tissue was maintained with reasonable preservation of cell viability and histological appearance for about 30 days. No significant differences in apoptosis were observed between ER-positive and ER-negative tumours. The system was simple to set up but multiple replicates would be required because the tissue content of small tumour explants is highly variable.

Explants were cultured for 8 days in estrogen, tamoxifen and control conditions. The pattern of TGFβ1 mRNA could not be established by *in situ* hybridisation, indicating mRNA degradation, which highlights the need for experimental optimisation.

TGFβ1 protein was localised, by immunohistochemistry, to stromal and epithelial cells. Epithelial cells displayed much stronger staining intensities. No consistent relationship was established between TGFβ1 expression and apoptosis, drug treatment or ER status. The culture system has clinical and research potential for determining the effects of therapeutic manipulation on fresh biopsy tissue.

In breast cancer explants that reflect *in vivo* by preserving stromal-epithelial interactions, the simple correlation between estrogen withdrawal and TGFβ1 induction, demonstrated *in vitro* by MCF-7, is not consistently observed.

DECLARATIONS

I, Laura Evelyn McLeod, hereby certify that this thesis, which is approximately 45,000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

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I was admitted as a research student in October 1994 and as a canditate for the degree of PhD in October 1995; the higher study for which was carried out in the University of St. Andrews between 1994 and 1999.

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CHAPTER 1

INTRODUCTION

The introductory chapter of this thesis aims to give the reader an overview of normal and tumourigenic processes, with particular emphasis on the breast. The influence of hormones and growth factors will be discussed, especially the effects of the Transforming Growth Factor β family. The therapies used to combat breast cancer will be considered, concentrating on the anti-cancer drug, tamoxifen, and its effect on breast tissue. Finally, the aims of the thesis will be debated with reference to previous research.

1.1 NORMAL GROWTH AND CARCINOGENESIS

1.1.1 Normal Growth

Most normal adult cells have a finite lifespan. They must reproduce themselves by undergoing mitosis during the cell cycle (refer to Figure 1.1). Each cell type has a characteristic number of doublings which results in a constant and controlled turnover of cells in any tissue or organ.

Normal adult cells reproduce only when instructed to do so by external signals. The well-orchestrated communication between cells and their environment ensures that proliferation of normal cells is tightly regulated. Following their set number of divisions, cells will enter senescence. A system exists for counting the number of divisions. Each time a cell divides, the telomeres of the chromosomes shorten. When they reach a critically short length, the cell is led into senescence (Weinberg, 1996).

1.1.2 Carcinogenesis

Carcinogenesis is the process by which normal cells become cancerous. It is a progressive, multi-step process, involving an accumulation of genetic errors in vital regulatory pathways. Aberrations in a single cell can be passed to daughter cells, giving them a growth advantage over their normal neighbours. One of several new aberrations within one cell in the second generation will give further growth advantages and so growth enhancements are compounded. Figure 1.2 illustrates a

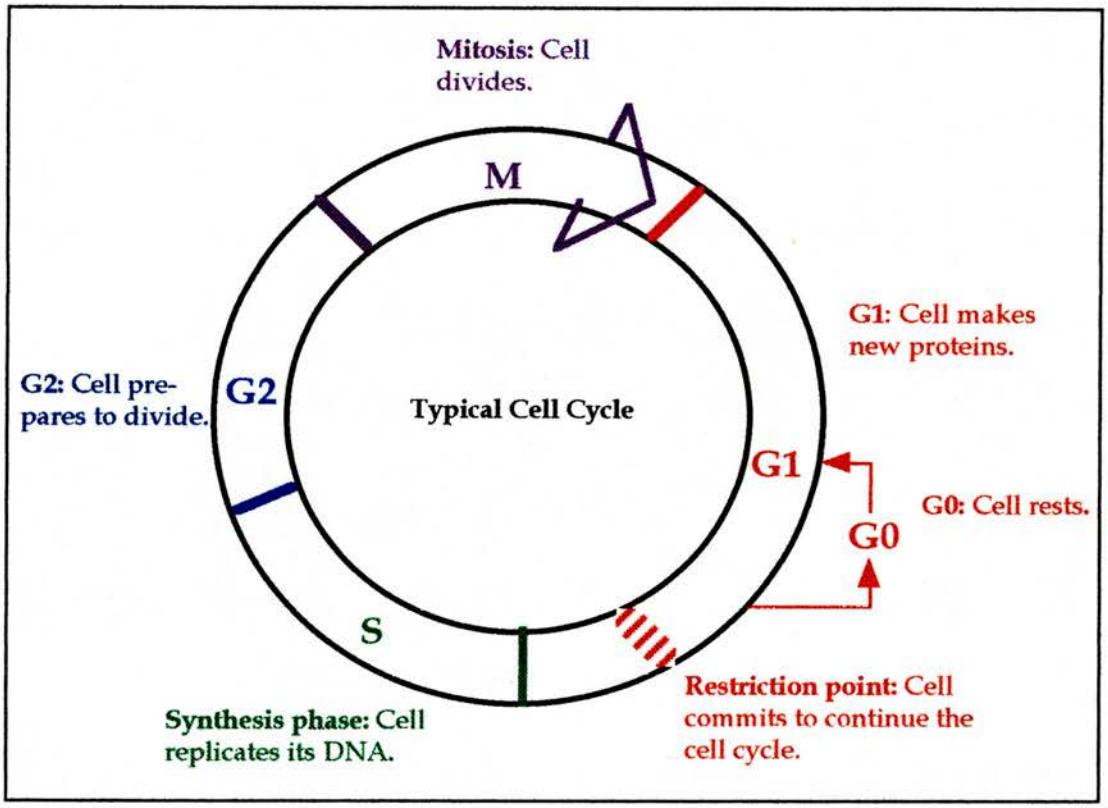


Figure 1.1 Diagram of the Cell Cycle.

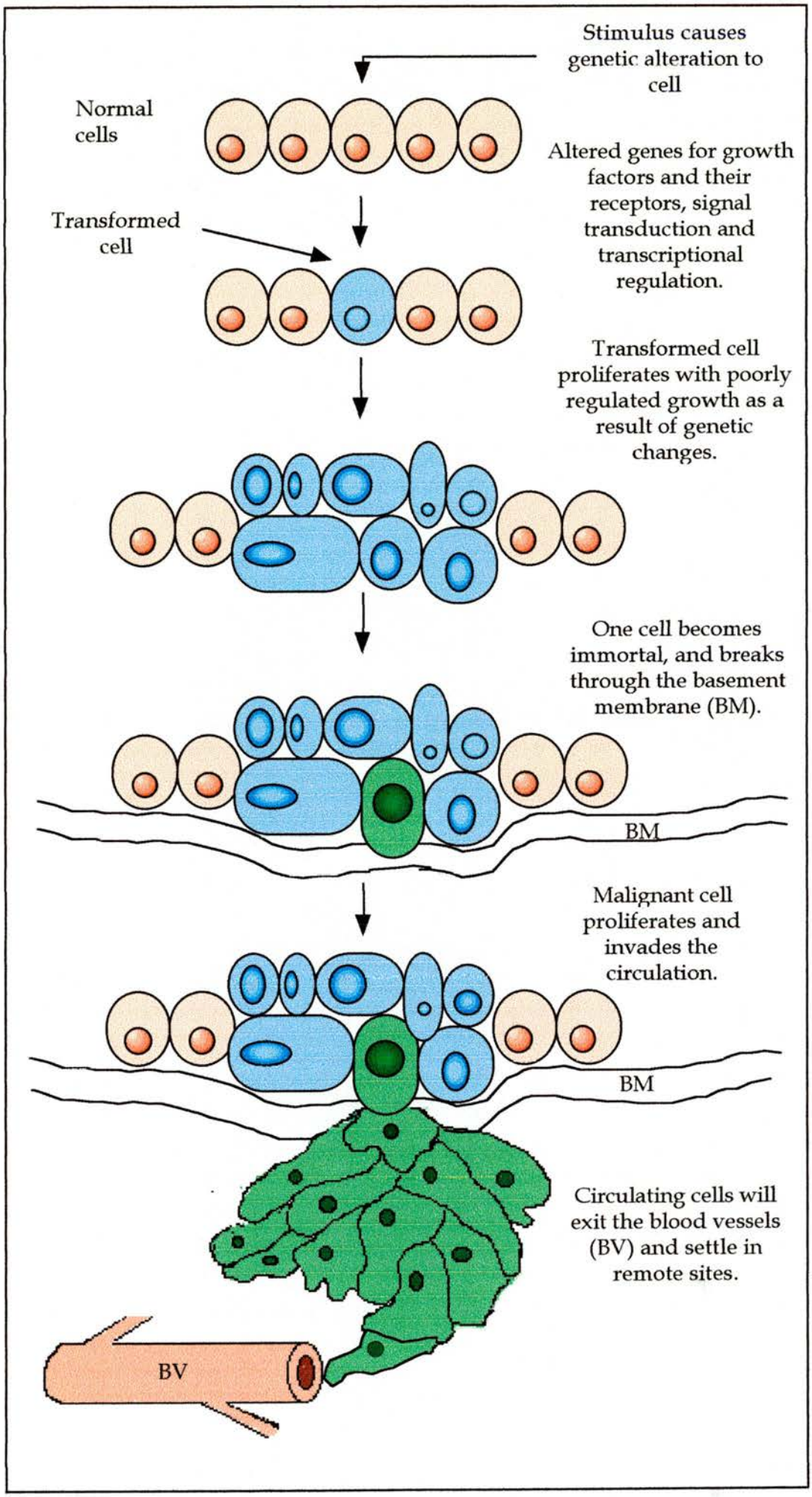


Figure 1.2 Key Events in Carcinogenesis

simplified version of carcinogenic events. Millions of these cells would have to be generated in this way in order to form a recognisable tumour. Many years can pass between initial exposure to a carcinogen and the recognition of a tumour. This period is known as latency but, in fact, the carcinogenic process will be very slowly continuing. A long latency period allows tumours to be well established and even to have metastasised by the time of primary diagnosis.

The carcinogenic process can be divided into five major stages (Underwood, 1996). *Initiation* is the introduction of neoplastic potential into a cell's genome i.e. transformation and *promotion* is the phase during which the single transformed cell will proliferate. Promoters can take the form of external factors or further genetic change. When clonal proliferation no longer requires initiators or promoters (i.e. when the tumour cells can grow independently and at a faster rate than their non-transformed counterparts), the tumour has entered the *persistence* stage. This is characterised by dedifferentiation, autonomous growth and aggressive behaviour. Changes in sensitivity to neighbouring cells, local growth factor production, receptor status and signal transduction occur during this stage. By now, the tumour is well established but due to its increase in size, more nutrients are required to sustain the core cells. The tumour secretes diffusible activators to initiate *angiogenesis*, the process by which new blood vessels are created from existing ones. The first soluble inducers to be identified were acidic and basic Fibroblast Growth Factors (aFGF and bFGF, respectively) and then Vascular Endothelial Growth Factor (VEGF) was identified for its ability to permeabilise vessel walls (Hanahan and Folkman, 1996). They have shown that angiogenesis is a rate-limiting step in tumour establishment; an essential step if the tumour must persist. The final stage is *metastasis*, when primary tumour cells gain the ability to break away from the parent tumour and invade the newly synthesised blood vessels on their journey to a secondary site of invasion (Welch *et al.*, 1990). Metastasis occurs through 4 routes - local invasion of neighbouring tissue; via the lymphatic system from where they settle in lymph nodes; through the vascular system (metastasising commonly to the brain, lungs, bone marrow and adrenal glands) and via transcoelomic spread where primary tumours metastasise into coelomic spaces, for example pleural cavities, and spread along the surface of other tumours (Stevens and Lowe, 1995). Tumour cells achieve metastasis by losing contact inhibition, secreting proteases that digest basement membranes and

endothelial linings or dedifferentiating to a migratory phenotype (seen in melanoma where the melanocytes become like their embryonic progenitors from the neural crest).

i. Carcinogens

Carcinogens are agents capable of initiating neoplastic growth. They cause mutations in DNA, often in conjunction with other factors, and there is good evidence that the whole process consists of several discrete steps (Thompson *et al.*, 1995; Kinzler & Vogelstein, 1996). Most carcinogens act only to initiate a cascade of events; they are not required throughout the entire process of transformation which explains why some tumours may present clinically years after exposure, as seen in tobacco smoke or asbestos-induced tumours.

The major categories of carcinogen are chemical, viral, ionising and non-ionising radiation, hormones, mycotoxins, parasites and others. Influences like race, diet, gender, familial predisposition, age, premalignant lesions and transplacental exposure to carcinogens are also factors to be considered in assessing cancer risk.

Many people are exposed to carcinogens daily through their work. Common associations are myelogenous leukaemia from benzene, lung cancer from diesel fumes, skin cancer from mineral oils and bone marrow cancer from ionising radiation (Trichopoulos, 1996).

ii. Molecular Events during Carcinogenesis

Disruption of the cell cycle and genetic alterations are essential for tumorigenesis to occur (Polyak *et al.*, 1996). A statistical study of sporadic and hereditary Retinoblastoma, a rare childhood tumour of the retina, by Knudson in 1971, demonstrated that as few as 2 mutations are required for the carcinogenic process to begin. This has become known as the 'two-hit hypothesis', though some cancers require more than 2 insults. People may inherit, or develop, a mutation in one regulatory gene, thereby requiring only one further insult in the 'healthy' locus to initiate growth dysregulation. These insults can be unrepaired chance mutations, arising during normal DNA processing, or mutations induced by carcinogens, both of which can be repaired to some extent by normal regulatory

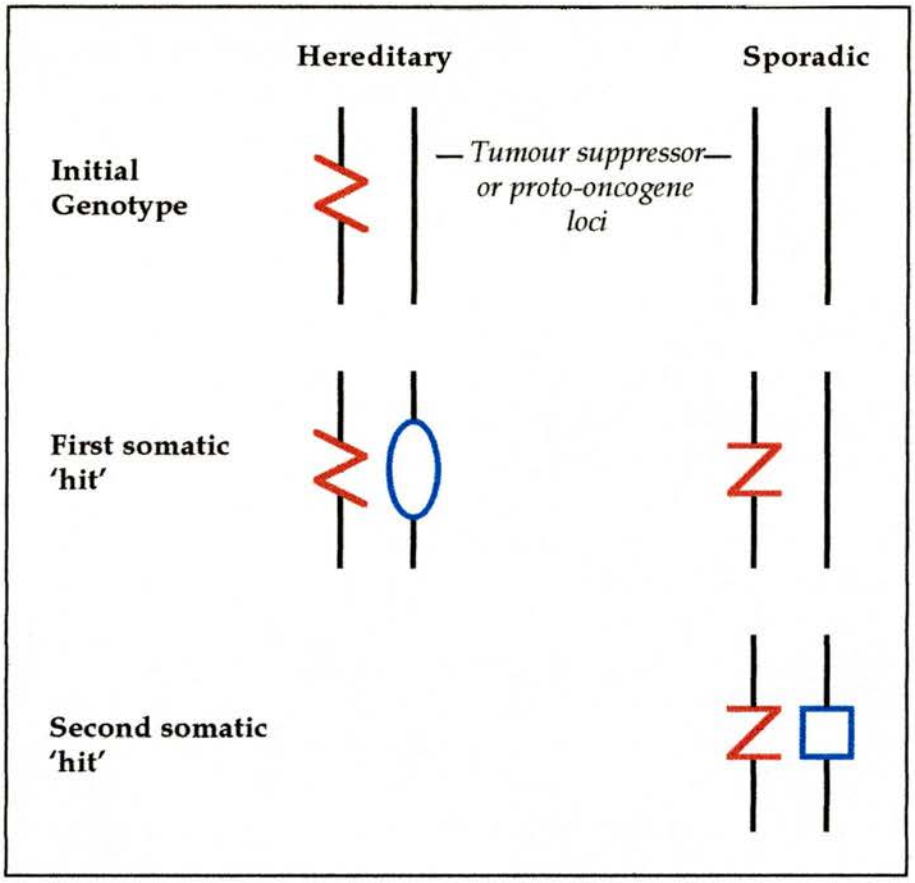


Figure 1.3 An Illustration of Knudson's '2-hit' Hypothesis (from Eccles and Houlston, 1995).

Two genetic insults on corresponding loci of tumour suppressors genes or proto-oncogenes are required for the initiation of carcinogenesis. Inherited tumours require only one somatic insult whereas sporadic tumours require 2 separate somatic 'hits'.

functions. This theory is demonstrated in Figure 1.3. Combinations of both genetic and environmental factors contribute to the initiation of cancer. Transformation of a cell arises from the accumulation of several discrete mutations in regulatory genes, as illustrated in colorectal cancer (Kinzler & Vogelstein, 1996).

Mutations result in transformed cells which divide without undergoing senescence, which normal cells do. A crisis stage is reached when the cells outgrow their environment and they die in large numbers. One cell may escape this mass apoptosis, becoming immortal, and multiplying indefinitely.

To escape the telomeric counting system, almost all malignant cells express an enzyme, telomerase, which is not detected in normal cells. This enzyme is used by cancer cells to repair shortened telomeres, therefore by-passing senescence and crisis (Weinberg, 1996). Telomerase activity is correlated with more aggressive breast tumours (Clark *et al.*, 1997; Hoos *et al.*, 1998) and is associated with the epithelial cells rather than the stromal cells (Yashima *et al.*, 1998).

Mutations are occurring all the time during routine cell cycling. Normally, permanent mutations are avoided because DNA repair mechanisms either correct the mistake or lead the cell into programmed cell death if the damage is too great to repair. Problems occur when these regulatory genes which control normal cell cycling become mutated. There are 2 genetic mechanisms leading to tumour growth - loss of tumour suppressor gene function which is generally a recessive event and abnormal expression of oncogenes, a dominant occurrence (Weinberg, 1996). The normal proteins of both types of gene are involved in the regulation of cell growth and differentiation so the former must be inactivated and the latter must be over-activated for malignancy to occur.

Sporadic tumour formation can arise from mutations in either tumour suppressors or proto-oncogenes through processes such as repeated cell division, i.e. ageing, and cumulative exposure to carcinogens. Predisposition to cancer formation is thought to be accounted for mainly by inherited mutations in tumour suppressor genes (Weinberg, 1996).

Tumour suppressor genes usually act in a recessive manner so that both alleles must be inactivated or lost before cell growth is affected (Prosser *et al.*, 1991). Their normal function is to control processes or proteins that would otherwise promote growth. An example of a classic tumour suppressor gene is the Retinoblastoma (Rb1) gene. The loss of both Rb1 alleles causes retinal tumours

almost exclusively in children. p53 is also a tumour suppressor gene and is located on chromosome 17p. p53 deviates from the normal pattern by requiring the loss of only one mutated allele to inactivate the wild-type protein (Eccles and Houlston, 1995). This makes it the most frequently observed mutational cause of human cancers (Lemoine, 1994). p53 corrects any DNA imperfections in the newly synthesised DNA strand, slowing the cell cycle until repair is complete or driving the cell to apoptosis if the damage cannot be corrected (Eccles and Houlston, 1995). The recently identified Breast Cancer susceptibility gene (BRCA1) is known to be a tumour suppressor. Mutations in this gene are thought to account for about 45% of hereditary breast cancer (Miki *et al.*, 1994).

Oncogenes were first discovered as components of oncogenic retroviruses then found to have equivalents in normal cells. The transcription of these genes is tightly controlled and the functions of the normal proteins provide an insight into how their mutated counterparts can cause growth dysregulation.

About 100 oncogenes have been identified and many of them code for helper proteins that transduce growth signals from the cell membrane to the nucleus. If these signalling proteins are mutated, the signal will not be relayed properly and a continuous stimulatory signal to grow may be transmitted to the nucleus. Examples of oncogenes which are commonly mutated in cancer are *c-fms* which codes for the receptor of Colony Stimulating Factor (CSF) (Maher *et al.*, 1998), *erbB2* which mimics the EGF receptor causing enhanced epithelial growth stimulation, *myc* which alters the activity of transcription factors in the nucleus and is often found in very high quantities in tumours even in the absence of growth factors and *Bcl family* (B-cell leukaemia-lymphoma) which is usually involved in cell survival and prevention of apoptosis (Johnston *et al.*, 1994) but when over-expressed can inhibit p53-induced apoptosis (Schott *et al.*, 1995).

1.2 CLASSIFICATION OF TUMOURS

Tumours can be classified as either malignant or benign. However, only malignant tumours are described as cancer. In general, tumours arise from one ancestral cell that has gained the ability to divide faster than its neighbours,

possibly decades before the tumour becomes symptomatic. The term 'cancer' is used to describe a collection of over one hundred diseases. Almost every body tissue can yield at least one type of cancer (Weinberg, 1996). Primary tumours are rarely fatal; it is the metastasised secondary tumours that are clinically more dangerous, since they can rarely be excised and cause physical and functional disruption of vital organs.

Benign tumours exist as overgrowths within their parent tissue and retain the characteristics of their original cell type. They do not invade neighbouring tissue nor do they metastasise. Benign tumours are usually curable since the surface types tend to spread non-invasively and the solid tissue types are normally encapsulated within a defined border. There is a range of premalignant tendency associated with benign tumours - some are almost never associated with cancer, e.g. uterine fibroids or subcutaneous tumours, while others carry a significant risk of becoming malignant, for example, bowel polyps or bladder papillomas.

Malignant tumours also originate as cellular overgrowths. The growth is followed by changes affecting extracellular matrix and growth factor production, enabling these overgrowths to invade neighbouring tissue, gain entry into the circulation and metastasise to remote sites in the body where they establish new tumours.

There are 4 major classes of malignant tumour. Epithelial cancers are described as *carcinoma* (non-glandular tissue) and *adenocarcinoma* (glandular tissue). Cancers of mesodermal tissue origin are known as *sarcomas* and those of lymphoid and haematopoietic origin are *lymphomas or leukaemias*. Minor classes of malignancy are teratomas (tumours of germ cell origin), blastomas (tumours in the very young which resemble embryonic forms of the affected organ), melanomas (tumours involving melanocytes in the skin) and mixed tumours (combination of cells with different origins).

Malignant tumours can be either well-differentiated (the tumour cells closely resemble the parent cell type) or poorly differentiated (the tumours cells bear little resemblance to the parent tissue). Tumours which display unidentifiable parent cell origin are said to be anaplastic. Aggressive tumours are usually poorly differentiated or anaplastic while well-differentiated tumours are considered to be less threatening.

An 'in-between' stage of malignancy is carcinoma *in situ*, a term used to describe a tumour which displays all the features of malignancy but has yet to break through the basement membrane and invade its neighbouring tissue and blood vessels. Excision at this stage can guarantee a cure and one aim of screening projects is to increase the proportion of tumours detected at this stage.

1.3 BREAST DEVELOPMENT

The human breast undergoes physiological and pathological changes throughout the various phases of life. Growth and development of the breast is governed by an interaction of hormones, paracrine factors and environmental influences (Gilbert, 1997). Hormone levels vary before, during and after reproductive life, causing changes in breast structure and function whenever necessary. The 4 major changes during life can be categorised as follows:-

1.3.1 Prenatal Development

The mammary ridge in human embryos is a band of epidermal tissue which arises on both sides of the ventral mid-line about week 7 of gestation (Sadler, 1990). Concentrations of cells appear in each ridge, forming one mammary bud per ridge. Development, at this stage, is identical in male and female embryos. The epithelial cells begin to proliferate and branch inwards but, in normal males, the mammary cord is pinched off from the skin by condensing mesenchyme (under the influence of testosterone which is being produced by the Leydig cells of the testes). In female embryos, the mammary cord continues to proliferate under the influence of estrogen. The mammary stroma is also essential for the appropriate development of breast epithelial growth (Kratochwil, 1969).

Between 16 and 24 buds sprout from the cord; these form the mammary lobes in the adult. By birth, the epithelial sprouts have canalised, forming early ducts. The ducts from each small lobe unite into one lactiferous duct beneath the nipple. The nipple is a simple epithelial pit at birth which is soon transformed into the mature nipple by proliferation of underlying mesenchyme (Sadler, 1990). The

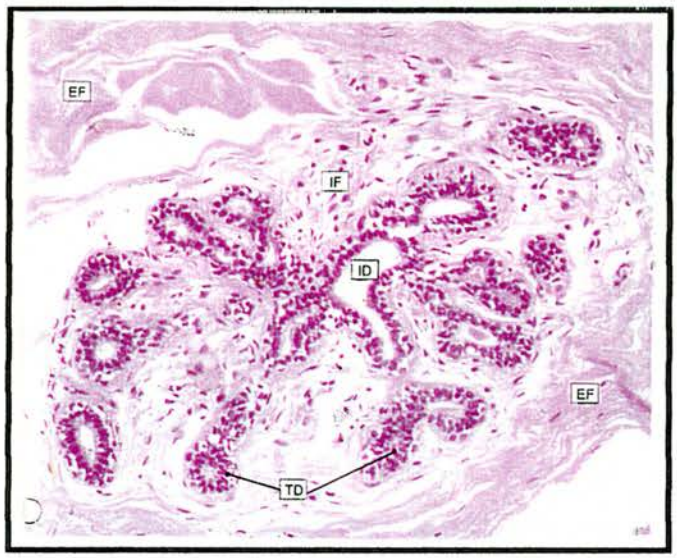


Figure 1.4 Typical Mammary Lobule (from Stevens and Lowe, 1993, pp 366).
The epithelial components of the lobule (terminal ductules-TD and intralobular duct-ID) are embedded in loose intralobular fibres (IF) which in turn is surrounded by dense extralobular connective tissue (EF).

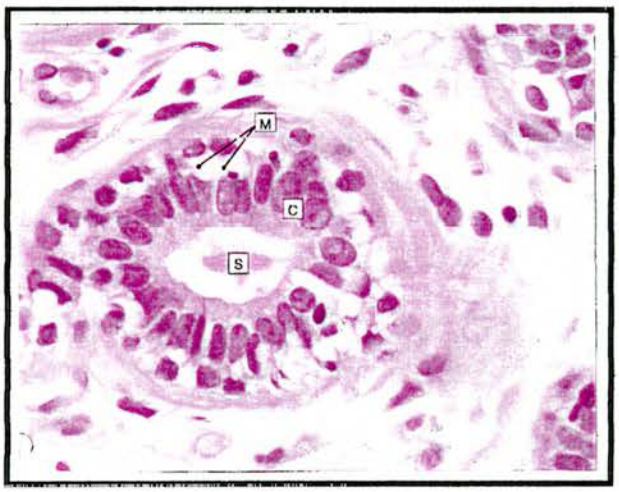


Figure 1.5 High Power Micrograph of Terminal Ductule (from Stevens and Lowe, 1993, pp366).
The columnar cells of the inner epithelial are shown (C) surrounded by the outer myoepithelial cells (M). There is a small amount of secretion (S) in the duct lumen.

mammary gland in this state is non-functional but occasionally, due to the high levels of maternal hormones during pregnancy, new-born babies can release breast secretions, known as 'witch's milk'.

1.3.2 Pubertal Development

The breast remains inactive until puberty when surges of estrogens cause extensive branching of lactiferous ducts with the formation of solid spheres at the ends which will eventually become true alveoli. Ductal and myoepithelial cell growth is concentrated at the tips of the ducts under the control of estrogen and growth factors, namely EGF (Coleman *et al.*, 1988). Lobulo-alveolar development may be controlled by prolactin and/or growth factor, as revealed by murine studies (Plaut *et al.*, 1993).

As menstrual cycles continue, the mammary tissue is exposed to more and more estrogen and growth factors, causing gradual lobular and alveolar growth. In addition, the breasts increase in size with deposition of fat and expansion of connective tissue. Cyclical changes in the breast occur in non-pregnant women due to the changes in hormone levels, resulting in breast volume increase and even some secretory activity in the pre-menstrual period.

It is unusual for an organ of the body not to be fully differentiated by the time of birth and the breast is an example of an organ which requires specialised growth and differentiation signals at puberty. It is these same signals, namely EGF, TGFα and insulin, that can be stimulatory for transformed breast epithelial cells in later life.

Figures 1.4 shows a typical breast lobule and figure 1.5 shows a terminal duct alveolus.

1.3.3 Pregnant Development

The breast only reaches maximal functional activity during pregnancy. During early pregnancy, the partially developed ductular-lobular-alveolar system undergoes considerable hypertrophy under the influence of estradiol, progesterone and possibly insulin (Gilbert, 1997). This results in prominent lobules forming in the breast and the dilatation of the lumina of the alveoli. Differentiation of the

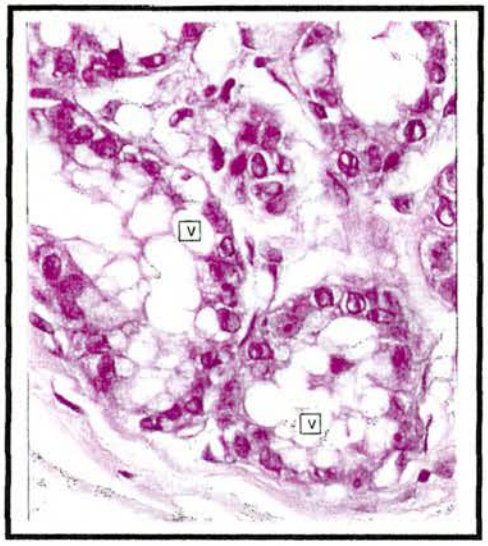


Figure 1.6 High Magnification of the Mammary Lobules during Pregnancy (from Stevens and Lowe, 1993, pp367).
Note the hyperplastic cells of the terminal ductules and the clear vacuoles (V) which contain a lipid-rich secretion.

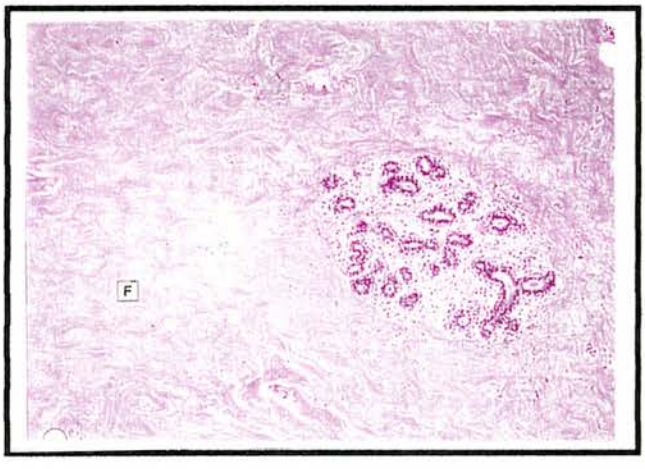


Figure 1.7 Menopausal Breast (from Stevens and Lowe, 1993, pp368).
Note the paucity of lobules and the density of the fibrous connective tissue (F).

alveolar cells into true secretory cells occurs around mid-pregnancy, after the initial period of hypertrophy. The epithelial cells of the alveoli are full of secretory material from this point onwards. They require specific endocrine signals which will stimulate the release of milk into the ducts post-partum. With the cessation of lactation, the breast involutes over several months with the mechanical atrophy of alveoli and lactiferous ducts due to an accumulation of unused milk.

Figure 1.6 shows how the cells of the alveoli have expanded and filled with lipid-rich secretion.

1.3.4 Menopausal Development

During the menopause, the ovaries become non-functional, resulting in a decrease in circulating estrogens. Biosynthesis of estradiol from adrenal androgens and other estrogens occurs in peripheral tissues, including the breast itself. Changes in the menopausal breast are due to altered ratios of plasma sex steroids. The fibrous component of the breast thickens and the epithelial cells of the alveoli are lost (refer to Figure 1.7). These changes often occur at different rates, resulting in lumpiness which is often misconstrued as breast disease.

Oddly, the concentration of estrogen in breast tissue remains as high as the level observed in the pre-menopausal breast. This could be due to a selective uptake and concentration of circulating estrogens and/or local biosynthesis of estrogen by aromatisation of androgens or intraconversion of estrone (Miller, 1991). This, perhaps, explains why the breast is still vulnerable to mitogenic influences later in life.

1.4 FUNCTIONAL HISTOLOGY OF THE BREAST

1.4.1 Histology

The main function of the breast is the production and secretion of milk. The breast has evolved from a sweat gland to suit this function. There are 16-24 distinct lobes in the breast, each with its own duct system and opening to the

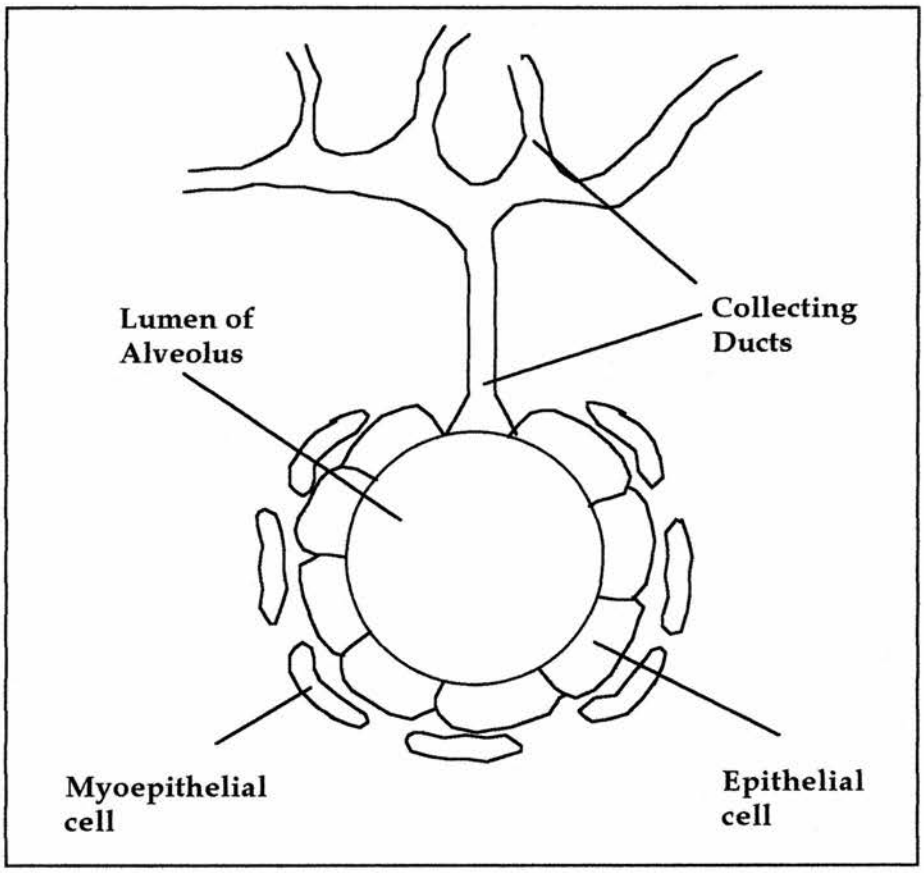


Figure 1.8 Schematic Representation of a Terminal Duct Alveolus.
Milk proteins and lipids accumulate in the epithelial cells and are ejected into the lumen of the alveolus by the contraction of the myoepithelial cells. Milk flows into the collecting ducts which open into larger lactiferous ducts.

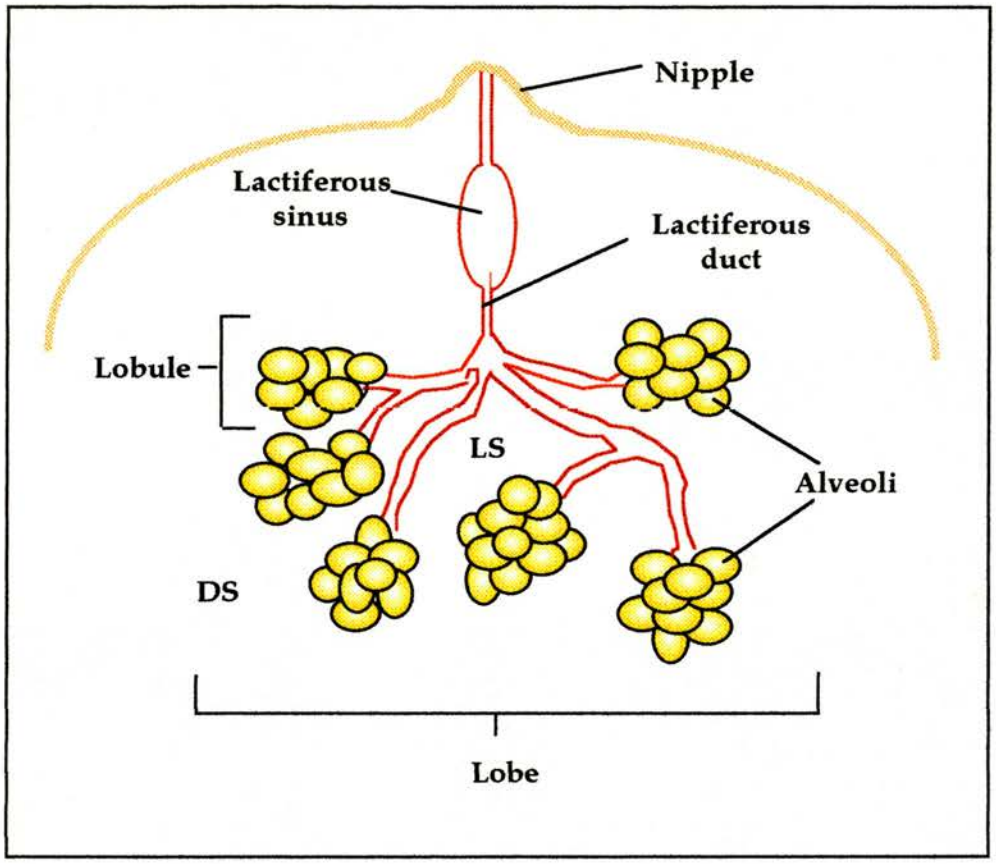


Figure 1.9 Schematic Representation of the Adult Breast.
One of the 16-24 lobes is shown here with lobules of terminal alveoli. The lobe is embedded in dense fibroadipose stroma (DS) while looser stroma (LS) cushions the individual lobules.

surface. Each main duct in the lobes divides many times and ends in bunches of alveoli, each bunch known as a lobule. The ducts are embedded in loose, intralobular connective tissue which contains many fibroblastic cells. These fibroblasts respond to the menstrual cycle hormones and there is signalling between the epithelial cells and the stromal cells (Schor *et al.*, 1991). Each alveolus is a 2-layered epithelium (refer to Figure 1.8) - one continuous layer of columnar or cuboidal epithelium (secretory) and a discontinuous layer of myoepithelial cells (contractile). The alveoli secrete milk into their lumina and the myoepithelial cells contract, forcing the milk into communal ducts which feed into larger lactiferous ducts and then onwards to the nipple surface. The supporting tissue of the breast is fibro-adipose tissue in which circular and longitudinal muscles are embedded. The ducts of the breast, like the alveoli, are lined with a single layer of epithelial cells and one layer of myoepithelial cells but unlike the alveoli, are set in dense connective tissue (refer to Figure 1.9).

1.4.2 Function

Although the pregnant breasts are ready to produce milk before giving birth, high levels of progesterone and estrogen from the placenta are inhibitory to lactation. This inhibition is lost with the placenta at birth. The suckling stimulus causes the release of 2 hormones - prolactin, which causes the secretion of more milk into the alveoli cells, and oxytocin, which allows the release of milk from these cells into the ducts.

Initially, the breast produces a yellow liquid, colostrum, which contains high levels of protein and maternal immunoglobulins (IgG). Antibody levels in new-born babies are low until approximately 3 months of age so breast-fed babies gain extra immune protection passively from their mothers. True milk, which begins to flow a few days post-partum, is a blue-white colour with a very thin consistency containing a mixture of protein, carbohydrate and fat as well as minerals, growth factors and hormones. An effect of breast-feeding is the partial suppression of ovulation due to prolactin feeding back to inhibit the release of Luteinising Hormone.

1.5 BENIGN & MALIGNANT BREAST DISEASES

1.5.1 Benign Diseases of the Breast

The continuous changes in the breast during menstrual cycles and pregnancies can lead to disproportionate growth of the mammary components. Common breast disorders can either be non-proliferative, namely inflammatory disease and fat necrosis, or proliferative, due either to increased ductal tissue (adenosis) or increased fibrous tissue (fibrosis); a combination of these is called fibroadenosis. Benign tumours do not usually precede malignancy themselves but can indicate a tendency for the development of more sinister breast disease later in life. An overview of breast diseases is given in 'Pathology' by Stevens and Lowe (1995).

i. Non-proliferative Benign Breast Diseases

Inflammatory breast disorders, or infections, are usually caused by micro-organisms gaining entry to the breast tissue during breast-feeding. The subsequent mastitis is characterised by swelling and pain, and if untreated can develop into breast abscesses. *Fat necrosis* of the breast tissue is the result of inflammation following physical trauma. The adipose tissue becomes necrotic and fibrous tissue replaces the dead fat cells, resulting in a lump, which can be mistaken for carcinoma if not properly examined.

ii. Proliferative Benign Breast Diseases

Fibroadenoma is the commonest type of benign breast disease, accounting for approximately 12% of all palpable masses (Dixon, 1991) and is most often seen in younger women (Symmers, 1978). Fibroadenoma arises from both the fibrous and the epithelial components of the breast, both of which respond to hormonal stimulation. The tumours are usually no bigger than 40mm in diameter and are mobile in the breast (Stevens and Lowe, 1995). They display duct-like structures and overgrown fibrous masses. Generally, it is thought that fibroadenoma is not a pre-cancerous disease since the tumours neither become cancerous themselves nor

Year	Cases	Year	Cases
1920	4488	**1950**	7892
1925	5372	**1955**	8449
1930	6052	**1960**	9059
1935	6768	**1965**	9670
1940	7058	**1970**	10 677
1945	7291	**1975**	11 637

Table 1.1 Deaths from Breast Cancer in England and Wales between 1920 and 1975

predispose a patient to cancer elsewhere in the breast (Symmers, 1978). *Duct papillomas* are most common in middle-aged women and rarer than fibroadenoma. They arise from ductal epithelium and most patients present with blood-stained nipple discharge. The tumours consist of fibro-vascular cores surrounded by epithelium and are often not readily palpable. There are contrasting views on whether or not duct papilloma is pre-cancerous, which affects the way the tumours are treated clinically.

1.5.2 Malignant Diseases of the Breast

In the United Kingdom, breast cancer accounts for 20% of all female cancers and is the most common cause of death amongst women aged 35-55 years of age. The age-standardised incidence and mortality from breast cancer in the UK are both among the highest in the world (McPherson *et al.*, 1994). With around 25,000 new diagnoses and 15,000 deaths each year, it is estimated that any British woman has a 1 in 12 lifetime chance of developing the disease. Diagnoses of breast cancer have risen steadily over the last 7 decades. This is partially attributed to an increase in average lifespan and improvements in early clinical diagnosis of the disease but there has been a true increase in the incidence. Table 1.1 illustrates the increase in breast cancer deaths between 1920 and 1975. Despite major advances in cancer therapy, deaths from breast cancer have not fallen in recent years. The UK death toll from breast cancer is now over 15,000 women annually (McPherson *et al.*, 1994).

Breast cancer arises most commonly in the epithelia of the intra- or extra-lobular ducts (ductal carcinoma) and in the terminal alveoli (lobular carcinoma). Because the breast is served by many blood and lymphatic vessels, local growth and metastasis of tumours are common in breast cancer and often lead to a poor prognosis. The axillary lymph nodes are usually the first site to which cancer spreads and later, the bones and lungs are commonly affected.

Prevention of breast cancer is almost impossible because there are so many endogenous factors involved. These factors are difficult to manipulate and different for each woman. Breast cancer is a collective term for many malignant diseases of the breast. Different clinical parameters (for example, age at diagnosis

and menopausal status) affect the type of tumour likely to present, the response to therapy and the patients' prognosis.

The commonest category of ductal carcinoma in both pre- and post-menopausal patients is of 'no special type', indicating a tumour of indefinable origin. Some of the rarer breast diseases can be coupled to particular groups of patients. For example, mucinous carcinomas are generally seen in post-menopausal women while lobular carcinomas *in situ* are more commonly seen in pre-menopausal women.

i. Non-invasive Breast Carcinomas

Non-invasive cancers remain in place, or *in situ*, without breaking through their basement membranes and invading neighbouring tissue. In breast cancer, tumours *in situ* are confined to the ducts or the alveoli where they first arose, resulting in ductal carcinoma *in situ* and lobular carcinoma *in situ*, respectively.

Ductal Carcinoma in situ (DCIS) can arise in both pre- and post-menopausal women, most often between the ages of 40 and 60 years. About 5% of palpable breast lumps and 20% of radiologically-detected lumps will be DCIS (Stevens and Lowe, 1995). Small and medium sized ducts are generally involved in this disease but many patterns of abnormality are known. The cells are irregularly-shaped and divide to fill the ductal lumina, often resulting in central necrosis. DCIS is usually unilateral, i.e. only one breast is affected, and due to the presence of micro-calcifications, the condition can often be detected by mammography. The treatment for DCIS is surgical excision as this malignant condition becomes invasive in about 50% of cases. *Lobular Carcinoma in situ* affects mainly pre-menopausal women, accounting for around 6% of all breast cancers. It does not present as a lump so is often only diagnosed coincidentally with other breast symptoms e.g. cystic disease. The alveolar cells divide to fill the lumina with similarly-shaped cells but do not normally cause necrosis. Around 20% of cases of this pre-malignant lobular disease will paradoxically progress to invasive ductal carcinoma in one or both breasts (Stevens and Lowe, 1995).

ii. Invasive Breast Carcinomas

Invasive breast tumours gain the ability to break down their basement membranes, which otherwise prevent movement, and spread into neighbouring tissue. Symptoms of carcinoma are unusual lumps (often 20-30mm at presentation), tethering of the lump to skin or muscle, dimpling of the skin due to infiltration of the lymphatic system and changes in the nipple. They are staged pathologically and clinically, according to a universal system (refer to Figure 1.10). Breast tumours are staged by the TNM system, using presence of Tumour, lymph Nodal involvement and evidence of Metastasis as prognostic indicators. Histologically, invasive carcinomas are highly heterogeneous. Most are adenocarcinomas and are classified by being ductal or lobular (Harris *et al.*, 1992).

Invasive ductal carcinomas are the most common type of breast cancer, accounting for around 85% of pre- and post-menopausal patients. They vary in their histological presentation but are generally fibrous in nature with the tumour cells grouped together in gland-like structures. *Invasive lobular carcinomas* usually affect younger women and account for about 10% of all breast cancers in the UK. They are almost always very fibrous and often multi-focal throughout the entire breast. The tumour cells are usually dispersed or in strings throughout the stroma, giving a very different appearance from ductal carcinoma. *Mucinous carcinomas* constitute around 2% of cases in post-menopausal women. They are well-encapsulated without dense stroma. The tumour cells form cords of similar cells which secrete glycoproteins into the surrounding area. The prognosis for sufferers of mucinous carcinomas is much better than that for ductal or lobular carcinomas. *Tubular carcinomas* are well-differentiated tumours, the cells of which are arranged into small tubule-like structures. They make up 1-2% of all invasive breast cancer cases and tend to be associated with very good prognoses. *Paget's Disease of the Nipple* presents as a roughening of the nipple skin but is associated with a sub-cutaneous carcinoma, accounting for 2% of all breast cancers. *Other invasive breast carcinomas* include papillary carcinoma and medullary carcinoma, seen in post-menopausal women and a juvenile disease called secretory carcinoma which affects young women and girls (Louvet *et al.*, 1996). The incidences of different types of breast cancers are illustrated in Figure 1.11.

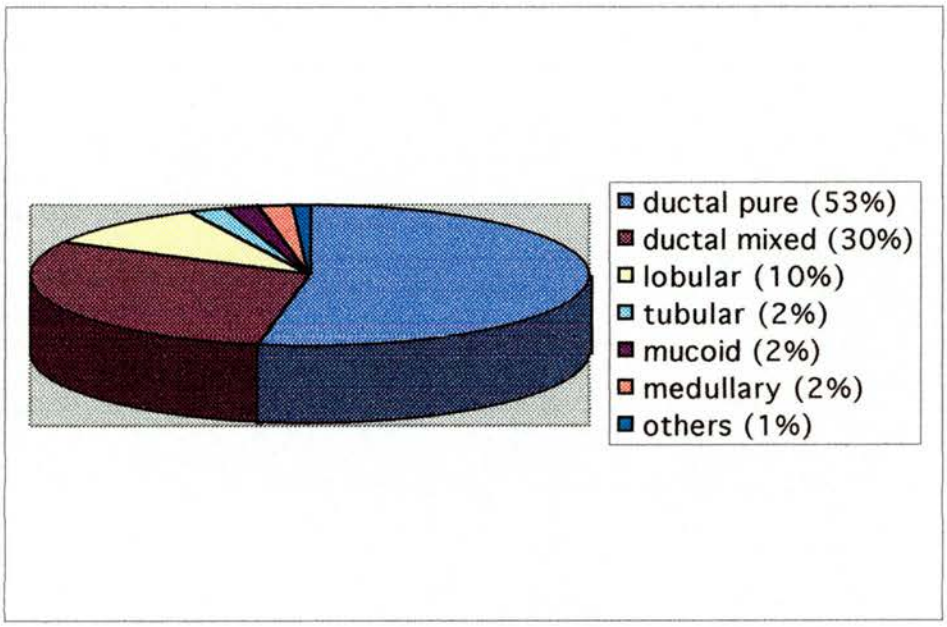


Figure 1.11 Incidence of Different Types of Breast Cancers.

iii. Risk Factors in Breast Cancer

Many studies have been carried out over centuries to establish links between breast cancer and environmental influences but so many variables exist within the groups of patients and control volunteers, that there is conflicting evidence for almost every suspected risk. It is clear that the internal hormonal environment has influence during the development of breast cancer but it is not known where.

The risk factors which are thought to increase the chances of developing breast cancer are gender, age, early menarche and/or late menopause, number of pregnancies and breast feeding, diet, personal history, socio-demographics and family history of the disease.

a. Gender: Women are the obvious sufferers of breast cancer but 1 in 100 of all breast cancer patients is male. In most countries, about 15% of male breast cancer is associated with mutations in breast cancer susceptibility gene 2 (BRCA2) (Wooster *et al.*, 1994) and is usually ductal in origin as the male breast rudiment contains no lobular units (Stevens and Lowe, 1995).

b. Age: The risk of breast cancer rises as a woman gets older, as with most cancers, but age incidence curves of breast cancer show that the most likely time of presentation is around the menopause. If the disease presents in the thirties, a hereditary factor is often suspected. Breast cancer is rare before the age of 25 but there is a form of the disease that presents in childhood called secretory carcinoma (Louvet *et al.*, 1996).

c. Menstrual History: Many studies have been carried out to establish a link between longer than average reproductive life and risk of breast cancer, the theory being that longer exposure to hormonal cycles could adversely affect breast epithelium. Michels *et al.* (1996a) showed from a large study that late menarche (≥15 years of age) was associated with a reduced risk of breast cancer development. However, there is dispute about the evidence (reviewed in Moore *et al.*, 1983) that women who experience both early menarche and late menopause may be at a higher than normal risk of breast cancer.

d. Parity: Studies in the 18th and 19th centuries reported that the incidence of breast cancer in nuns was significantly higher compared to the incidence in married women (reviewed by Moore *et al.*, 1983). It is proposed that nulliparous women (no pregnancies) have an increased risk of breast cancer because the breast never realises its full growth potential and the epithelial cells do not become

completely differentiated. Lambe *et al.* (1996) found that mothers of 1 child had a 12% higher breast cancer risk than multiparous women.

The age at which women first become pregnant is also a factor to be considered in breast cancer risk. It is thought that late first pregnancies correlate with a higher risk of breast cancer. There have been many studies to show whether or not breast-feeding influences the risk of breast cancer (Brinton *et al.*, 1995; Michels *et al.*, 1996; Enger *et al.*, 1997). The majority of studies reveal a weak protective effect of breastfeeding. Others find no effect. No study has shown a deleterious effect so there are no contraindications for breastfeeding with respect to the development of breast cancer. Perhaps the protective effect stems from being pregnant rather than actually breastfeeding. Lambe *et al.* (1996) note that the protective aspects of pregnancy may be due to the inhibitory effects of human Chorionic Gonadotrophin and α-Fetoprotein.

e. Diet: In post-menopausal women, an accumulation of body fat to the point of obesity may relate to an elevated chance of developing breast cancer but studies are ongoing at present to show whether or not these observations are conclusive (Hulka and Stark, 1995). Studies to determine the benefits of fruit and vegetables in the diet support a protective effect but no significant relationship has been established. Alcohol intake has also been studied and found to contribute to the risk of contracting breast cancer if consumed in quantities greater than 15g per day (Hulka and Stark, 1995).

f. Personal Clinical History: A history of certain types of benign breast disease, for example, polycystic disease or severe atypical hyperplasia, can indicate a higher than normal personal risk of breast cancer (Page *et al.*, 1996) and a previous malignant lesion of the breast carries a risk of a subsequent primary disease.

g. Socio-demographic Factors: Unlike most other cancers, breast cancer is more often seen in the upper rather than lower social class. In the USA, women of Jewish descent are more likely to suffer breast cancer than non-Jewish women and those with the lowest risk are of Asian ancestry. Caucasian women have the highest overall risk of all ethnic groups, except in the early-onset age group when African-American rates are the highest (Hulka and Stark, 1995). Japanese women and other Oriental groups have the lowest world-wide risk of cancer (McPherson *et*

al., 1994) but immigrants reared in westernised countries develop a risk similar to the indigenous population, indicating dietary and environmental influences.

h. Cancer Predisposition: Through studies on family history, it has become clear that when breast cancer affects several family members, there is a hereditary factor or a specific environmental factor affecting all members. Between 5% and 10% of breast cancers have an inherited basis. In real terms, this is a small number of cases but if predictive tests were available, women with mutated predisposition genes could have their tumours detected early or even avoided. Young age at onset of breast cancer is the clearest genetic indicator of genetic predisposition (Hulka and Stark, 1995). The genes responsible for hereditary breast cancer are discussed in 1.6.1/2.

Syndromes exist which confer a higher than normal predisposition to breast cancer. Ataxia Telangiectasia (AT) is a autosomal, recessive syndrome of progressive cerebellar ataxia and oculocutaneous telangiectasia. AT homozygotes are extremely sensitive to ionising radiation and are 100-fold more susceptible to developing cancers. Heterozygotes of the AT mutant gene may be predisposed to a higher than normal risk of breast cancer and in some studies, it has been estimated that 2-7% of breast cancer cases may be attributable to AT heterozygosity. Li-Fraumeni syndrome is characterised by the development of multiple soft tissue cancers, particularly lung cancers, osteosarcomas, lymphoid tissue cancers, adrenocorticoid cancers and breast cancers, due to inherited p53 mutations. Peutz-Jeghers syndrome is an autosomal dominant disorder which causes abnormal cutaneous pigmentation and gastrointestinal polyposis with an increased rate of hormone-related cancers. The gene has been localised to a region on chromosome 19p13.3 (Nakagawa *et al.*, 1998). Cowden Syndrome is a variable collection of tumorous diseases with an increased risk of breast and thyroid tumours (Eccles and Houlston, 1995).

i. Prenatal Factors: There is growing evidence from case-controlled studies that intrauterine influences may contribute to development of cancer in later life. A study by Michels *et al.* (1996a) for breast cancer risk showed that high birth-weight babies (≥ 4000g) appear to experience a higher risk of breast cancer later in life while low birth-weight babies (≤ 2500g) have almost half has much risk as the heavier birth-weight. The middle range of birth-weights proved less conclusive for an increased or decreased risk. Further evidence for this comes from compromised

pregnancies, for example with pre-eclampsia, where estrogen levels are lower and babies tend to be in the lower birth-weight category. The explanation for this is that the undifferentiated fetal mammary gland is exposed to elevated concentrations of hormones during pregnancy, including mammotrophic estrogens.

1.6 MOLECULAR GENETICS OF BREAST CANCER

Genes have been identified that are commonly mutated in sporadic and inherited cancers, including breast cancer. Strong familial patterns of diseases, such as breast cancer and colorectal cancer, sparked the search for heritable genes that confer a predisposition to the disease in early life. The discovery of such genes has widened the scope for molecular research and clinical screening for the disease.

Cancer susceptibility genes have normal cell functions until they are mutated, when a loss of, or reduction in, protein function causes an abnormal growth pattern. The mutated genes have an autosomal dominant pattern of inheritance (Lemoine, 1994) but the alleles which confer the predisposition act recessively in somatic cells. Loss or damage to the wild-type (i.e. healthy allele) in conjunction with an inherited mutation can lead to malignancy.

Between 5% and 10% of all breast cancer patients have a familial history of the disease (Kainu *et al.*, 1996), which relates to between 1 in 200 and 1 in 400 women being predisposed to a higher risk of breast cancer than the general population (Karp and Broder, 1995).

1.6.1 Breast Cancer Susceptibility Gene 1 (BRCA1)

In 1994, Miki *et al.* identified a gene by positional cloning methods and proposed it as the familial breast/ovarian cancer predisposition gene. BRCA1 confers up to a 95% certainty of developing breast and/or ovarian cancer in those women who have mutations in the BRCA1 exons. BRCA1 was mapped to chromosome 17q21 in 1990 by Hall *et al.* (1990) and Narod *et al.* (1991) and it appears to encode a tumour suppressor in normal cells. BRCA1 is a large gene of 5,592 nucleotides, spread over more than 100 kilobases. The open reading frame

suggests a protein of 1,863 amino acids (Miki *et al.*, 1994). BRCA1 gene gives at least 2 distinct mRNA species and proteins of varying weights, 210kD, 185kD, 135kD and 85kD (Gudas *et al.*, 1996). The nature and identity of the full protein remains tentative.

Mutations in the breast cancer susceptibility gene seem to cause the early onset cancer. BRCA1 mutations are responsible for about 25% of breast cancers in the under-30 age group compared to an estimated 5% in the older population (Miki *et al.*, 1994). So far, over 40 mutations have been detected in the BRCA1 gene, most of which are frameshift or non-sense aberrations (Karp and Broder, 1995). BRCA1 accounts for 40-50% of all familial breast cancers (Kainu *et al.*, 1996). The other cases are due to mutations in BRCA2 (Wooster *et al.*, 1994), p53, PTEN (Li *et al.*, 1997) and perhaps breast cancer genes that are as yet uncharacterised. BRCA1 mutations are reported to elevate the risk of prostate and colon carcinomas (Karp and Broder, 1995) but are not responsible for familial male breast cancer (Wooster *et al.*, 1994).

In BRCA1 tumours, the healthy allele is invariably lost, leaving only the mutated allele (Miki *et al.*, 1994). The authors concluded that BRCA1 acts as a tumour suppressor in normal cells and becomes altered, reduced or absent in breast and ovarian epithelial tumour cells. Kainu *et al.* (1996) demonstrated that in BRCA1-induced tumours, mRNA levels of the gene were significantly lower than in normal and non-BRCA1 tumour breast tissue. They concluded that somatic inactivation of the wild-type allele was responsible for the reduction in BRCA1 mRNA.

Several breast cancer cell lines (normal breast, ER-positive, ER-negative) have been examined for their levels of BRCA1 mRNA. The results have been variable (Gudas *et al.*, 1996). The only pattern they found was that ER-positive breast cancer cells lines, for example MCF-7, T-47-D, had higher than average amounts of BRCA1 mRNA and that non-tumourigenic immortal cell lines had lower than average levels of BRCA1 mRNA.

Gudas *et al.* (1996) also found that BRCA1 levels in normal cells, which had been exposed to TGFβ, were low for 6-12 hours and dramatically reduced after 48 hours of continuous exposure. They concluded that there is a strong correlation between BRCA1 mRNA and proliferative status of cultured human breast epithelial cells. Conditions that led to cell cycle arrest, e.g. TGFβ addition or

growth factor withdrawal, resulted in a downregulation of BRCA1 mRNA levels so BRCA1 transcription falls when growth slows. Conversely, conditions that increase the number of cycling cells were associated with increased BRCA1 mRNA levels.

A study to determine differences between BRCA1 associated breast cancer and sporadic cancers was undertaken by Robson *et al.* (1998). BRCA1 tumours tended to be more poorly differentiated with a higher proliferative rate in comparison with the non-BRCA1 tumours. The heritable cancers were usually ER negative but no differences were found in EGFR, cathepsin-D or bcl-2 expression. The 2 groups of tumour did not vary in tumour type, histological grade, stage or size or presence of nodal metastases.

Work done by Marquis *et al.* (1995) on the murine form of the BRCA1 gene (Brca1) revealed that the gene is expressed in fast-growing cells, especially when they are undergoing differentiation. They found predominant Brca1 mRNA expression in the thymus, testis, breast, ovary, uterus, spleen, lymph node and liver of adult mice. Lower expression was found in the stomach, small intestine and lung. Specifically in the mammary gland, Brca1 mRNA was detected in the epithelial cells and peaked during puberty and pregnancy. Marquis and colleagues tested the theory that Brca1 expression during pregnancy may be sustained by ovarian hormones. Treatment of ovariectomised mice with estrogen or progesterone alone did not affect Brca1 levels but with the 2 hormones together, expression was elevated 4-5 fold compared to untreated mice.

1.6.2 Breast Cancer Susceptibility Gene 2 (BRCA2)

Breast cancers not linked to BRCA1 led to the identification of a second breast cancer susceptibility gene, BRCA2. BRCA2 was localised to human chromosome 13q12-13 by Wooster *et al.* (1994). Loss of heterozygosity (LOH) of this gene region is often observed in human cancers, possibly indicating tumour suppressor activities for both BRCA2 and the Retinoblastoma gene which also lies on the long arm of chromosome 13 (Cooney *et al.*, 1996). The BRCA2 gene is very large with 10,254 nucleotides, encoding a protein of 3,418 amino acids (Gayther and Ponder, 1998). The function of the gene has not been fully established but there is evidence for the protein belonging to a family of DNA mismatch repair proteins and having a similar function to BRCA1 (Gayther and Ponder, 1998).

Mutations in BRCA2, like BRCA1, confer a predisposition to familial early onset breast cancer. Mutations are spread over the entire gene and most of these result in a truncated protein (Ellison and Haber, 1998). Allelic loss in the region of the BRCA2 gene has been observed in 48% of sporadic prostate cancers (Cooney *et al.*, 1996) and in 34% of breast cancers (VandenBerg *et al.*, 1996). The linkage of mutations in BRCA2 with ovarian cancer is not as strong as that for BRCA1 but male BRCA2 carriers are more likely to develop breast cancer than their BRCA1-carrying counterparts (Wooster *et al.*, 1994).

1.6.3 Retinoblastoma (Rb1)

In normal retinal cells, the protein product of the Retinoblastoma gene acts as a tumour suppressor and is instrumental in cell cycle regulation. The retinoblastoma gene is positioned on chromosome 13q14 (Black, 1994). It is a recessive gene so both alleles must be inactivated for the initiation of tumorigenesis. Mutations and LOH of the gene region have been noted in lymphoid cancers (Liu *et al.*, 1995), prostate cancers (Cooney *et al.*, 1996), colorectal cancers (Poller *et al.*, 1997), squamous cell carcinomas of the head and neck (Li *et al.*, 1997) and breast cancers (VandenBerg *et al.*, 1996). Structural rearrangement mutations in the Rb1 gene have been found in 25% of breast cell lines and 7% of primary tumours (T'Ang *et al.*, 1988). VandenBerg *et al.* (1996) analysed 84 primary breast tumours for loss of heterozygosity (LOH) at the Rb1 locus. They found LOH of Rb1 in 27% of tumours. However, this did not serve as a marker for either hormone receptor status or clinical prognosis. Poller *et al.* (1997) also found that in colorectal cancers, Rb1 protein expression was of no value as a marker of prognosis.

There seems to be an association between Rb1 and p53 mutations. Loss of Rb1 is compensated for by a functional p53 gene product so, in human tumours, the loss of both Rb1 and p53 function is often observed (White, 1994).

1.6.4 p53

Normal p53 protein is expressed at low levels in almost all adult mammalian cells (Prosser *et al.*, 1991). It is a DNA-binding, cell cycle regulating

transcription factor (Karp and Broder, 1995). It acts as a tumour suppressor (Prosser *et al.*, 1991) and a transcriptional regulator (Kovach *et al.*, 1996). p53 normally promotes repair of damaged DNA, induced by ionising radiation, heat shock, spindle damage or viral infection. Genomic stability is maintained because p53 arrests the cell cycle in G1 until the repair is complete or causes apoptosis if the damage is too extensive to repair (Kovach *et al.*, 1992). Thus, p53 prevents proliferation of mutations under normal circumstances.

The early mouse embryo expresses p53 in all tissues until 12.5 days post coitum when expression becomes tissue-specific. p53 is not essential for development as p53 null mice develop normally but are susceptible to premature tumorigenesis (Stuart and Gruss, 1995).

Mutations in the p53 gene are the most frequent genetic alterations found in human cancers (Coles *et al.*, 1992). For example, more than 80% of colorectal cancers have p53 mutations, though these alone do not cause the subsequent cancer (Kinzler and Vogelstein, 1996). The majority of p53 mutations have been identified in the core of the gene (exons 5-8 of the gene). The mutations are usually missense, the products of which interfere with the normal functioning of the wild-type protein (Miki *et al.*, 1994). The mutations give cells a selective growth advantage and perhaps, resistance to ionising radiation and certain chemotherapies making mutant-p53 cells more aggressive.

Certain tumours often display characteristic mutations of p53. For example, p53 mutations in skin cancer caused by ultraviolet light are usually at dipyrimidine sites; lung cancer displays p53 base pair transitions (indicative of tobacco and alcohol); colon cancers are characterised by many C-T changes (Coles *et al.*, 1992). Aberrations in p53 are found in 25-40% of breast cancers, fewer than seen in other cancers (Lemoine, 1994). In an unselected group of sporadic breast cancers, Coles *et al.* (1992) found p53 mutations in 40% of tumours; a higher than expected incidence of GC-TA transversions and guanosine mutations was observed. In breast cancers, loss of heterozygosity is most often observed with loss of homozygosity and gross genetic rearrangement being very rare (Thompson, 1992).

p53 abnormalities can be detected by immunohistochemistry because mutations usually result in protein conformation change. Overexpression of p53 protein has been detected in some, not all, *in situ* breast cancers and it may be that

while p53 mutations add to the early events in some breast cancer carcinogenesis, they are not essential for tumour progression (Lemoine, 1994). Subsequent effects of p53 mutations can aid tumour progression. For example, wild-type p53 controls the expression of Thrombospondin (TSP), which is an angiogenic inhibitor. Mutations in p53 cause a decrease in TSP and therefore, a more conducive environment for tumour progression (Hanahan and Folkman, 1996).

It is now estimated that approximately 1% of familial breast cancer susceptibility is due to germline p53 mutations (Black, 1994).

An inherited familial pattern of p53 mutations has been identified and called Li-Fraumeni Syndrome which is characterised by soft tissue embryonal malignancies and early onset breast cancer. However, Prosser and co-workers (1991) reported that mutations in p53 rarely account for heritable breast cancer. They studied 5 affected families and found that mutations in p53 did not contribute to those patients' susceptibility to cancer.

p53 can be used as a prognostic marker. Somatically acquired mutations in p53 are related to poor prognosis in breast cancer patients (Kovach *et al.*, 1996; Li *et al.*, 1998) and in other cancers, for example, chronic lymphoblastoid leukaemia (Cordone *et al.*, 1998).

1.6.5 c-erbB2, c-myc, c-ras and cyclin D1

At least 4 proto-oncogenes are thought to be commonly involved in the pathogenesis of breast cancer - c-erbB2, c-myc, c-ras (Benz *et al.*, 1989) and cyclin D1 (Hui *et al.*, 1996). In human breast cancer, the most frequently amplified chromosomal regions are the c-erbB2 and c-myc proto-oncogenes (Bièche *et al.*, 1994).

The *c-erbB2* proto-oncogene is located on chromosome 17p21-22 and encodes a 190kD transmembrane glycoprotein. The extracellular domains of c-erbB2 and the EGF receptor (EGFR) have 40% sequence homology (Gullick, 1990a). The homology between their amino acid sequences of the intracellular tyrosine kinase domains is 80% (Ellis, 1991). Levels of this oncoprotein were immunoassayed in normal and tumour breast tissue by Koschielny *et al.* (1998). They found that overall levels of expression were comparable in both tissue types but that tumour tissue displayed a wider range of expression. Very low levels of

c-erbB2 protein expression in breast tumour tissue (compared to the median for both tumour and normal) was correlated with poor prognosis in this study. Conversely, other workers have found that overexpression of c-erbB2 is correlated to poor prognosis (Sainsbury *et al.*, 1987; Costa *et al.*, 1988). An increase in EGFR-like c-erbB2, as reported by Sainsbury *et al.* (1987) and Costa *et al.* (1988), would result in an increase in EGF signalling and phosphokinase activity which, in turn, would promote growth. A tentative association has been established between elevated c-erbB2 and increasing tumour grade, i.e. undifferentiated breast tumours display an overexpression of the protein more often than differentiated tumours (Gullick, 1990a).

The *c-myc* proto-oncogene transcribes to a 62kD nuclear phosphoprotein which functions as a transcription factor. It is highly expressed in embryonic and adult proliferating cells but seen at low levels in quiescent and differentiating cells (Ellis, 1991). c-myc is overexpressed in many breast cancer cell lines (Gullick, 1990) and in about 30% of breast tumours. A high level of c-myc is not sufficient to cause transformation but is a necessary component in the multi-step process. Its overexpression appears to be associated with LOH at the 1p32-pter region, a putative tumour suppressor region (Bièche *et al.*, 1994). These 2 events - LOH of 1p32 and overexpression of c-myc - may collaborate in the development of breast cancer.

The *ras* family consists of closely related proto-oncogenes and proteins. They encode GTP-binding proteins, transducing signals from ligand-bound growth factor receptors to the nucleus. The h-ras-1 proto-oncogene lies close to a highly polymorphic region on chromosome 11p15. A study by Krontiris *et al.* (1993) found a 2-fold increased risk of breast cancer in association with rare alleles at this region (Ellisen and Haber, 1998). c-ras^{H} p21 oncoprotein is the most extensively studied of the ras family. Point mutations in the amino acid sequence are sufficient to induce transformation and are detected in about 15% of breast tumours.

Cyclin D1 is coded for on chromosome 11q13, a region which is also frequently amplified in breast cancer. Cyclin D1 is a cell cycle regulator, critical in normal breast development. It is induced early in breast cancer by mitogens, so the gene has been proposed as an oncogene since mutations in it result in cell cycle dysregulation and possible disease progression (Hui *et al.*, 1996).

1.6.6 Ataxia Telangiectasia (AT)

Ataxia Telangiectasia is an autosomal recessive disorder, characterised by cerebellar degeneration which leads to ataxia, together with dilation of the conjunctival blood vessels (telangiectasia). The disease also carries a 100-fold increase of cancer susceptibility (Zakian, 1995) and is further complicated by immunological defects (Eccles and Houlston, 1995). The ATM gene is related to signal transducer genes and other genes involved in responding to DNA damage (Lavin and Shiloh, 1996) and has homology with other checkpoint genes from different organisms (Meyn, 1995).

Heterozygotes for the AT mutation, estimated to be 1% of the US population, also have an increased susceptibility to developing cancer. It is suggested that ATM heterozygotes could make up about 9% of breast cancer patients in the United States (Zakian, 1995). Another estimate is between 2 and 7% by Easton *et al.* in 1993 (Eccles and Houlston, 1995). Studying the AT gene, therefore, maybe as valuable as BRCA gene research from the view of detection of susceptibility and prevention of breast cancer.

1.7 TRANSFORMING GROWTH FACTORS

The Transforming Growth Factors are highly versatile proteins with roles in growth , differentiation, motility and cellular organisation. In the breast, both types of transforming growth factor influence growth and development in normal and malignant breast tissue. Most vertebrates express transforming growth factors, both alpha and beta isoforms.

1.7.1 Transforming Growth Factor Alpha (TGFα)

TGFα is 50-amino acid secreted polypeptide of 5.6kD which shares 35% sequence homology with EGF (Matsui *et al.*, 1990). TGFα is derived from a 160 amino acid precursor molecule (Barrett-Lee *et al.*, 1990). It binds to the EGFR and like EGF, is a potent mitogen for several cell types, for example, basal cells of the epidermis (Gilbert, 1997). TGFα has mitogenic roles in embryogenesis and is

produced by a variety of normal adult cells, for example, keratinocytes, macrophages and gastric mucosal cells. It is also an angiogenic stimulator, an inducer of bone resorption and an inhibitor of gastric juice secretion. Because of its mitogenic properties, it is associated with the promotion of cancer (Matsui *et al.*, 1990).

In the normal mouse mammary gland, TGFα and EGF promote the proliferation and formation of ductal structures (Gullick, 1990a). In breast cancer cell lines, TGFα is one of the most extensively studied autocrine growth factors and it has been found to promote the growth of several cell lines (Karey and Sirbasku, 1988). It is detectable in some breast tumours *in vivo* (Gullick, 1990). A tissue biopsy study by Walker and Dearing (1992) showed that TGFα was detected in 22% of non-malignant tissue and 38% of tumour tissue fragments. Tumours displaying elevated concentrations of TGFα and EGF receptors may grow more rapidly and more aggressively than others (Gullick, 1990a). TGFα is found most commonly in ER-negative tumours in conjunction with the EGFR, suggesting an autocrine pattern of secretion (Travers *et al.*, 1988). It acts as a true growth stimulator in MCF-7 cells and as a mediator of estrogen-induced growth in ER-positive cells, being induced itself by estrogen (Ethier, 1995). However, the cell line models may not accurately reflect the *in vivo* situation.

The growth promoting abilities of TGFα in mammary cancer were confirmed by Matsui *et al.* (1990) when they examined the effects of systemic TGFα over-production in normal mouse mammary glands. They found a variety of abnormalities from simple hyperplasia to true adenocarcinoma. Male mice, similarly treated, had no obvious defects in their body organs, showing that TGFα is directed at breast tissue. However, its tumorigenic properties can be abrogated by TGFβ1 in transgenic mouse mammary epithelium (Serra and Moses, 1996).

TGFα levels have been measured in breast cancer patients before and after tamoxifen treatment. In ER-positive patients, TGFα was downregulated but the level remained constant in ER-negative patients showing that tamoxifen caused a decrease of TGFα through the ER (Noguchi *et al.*, 1993).

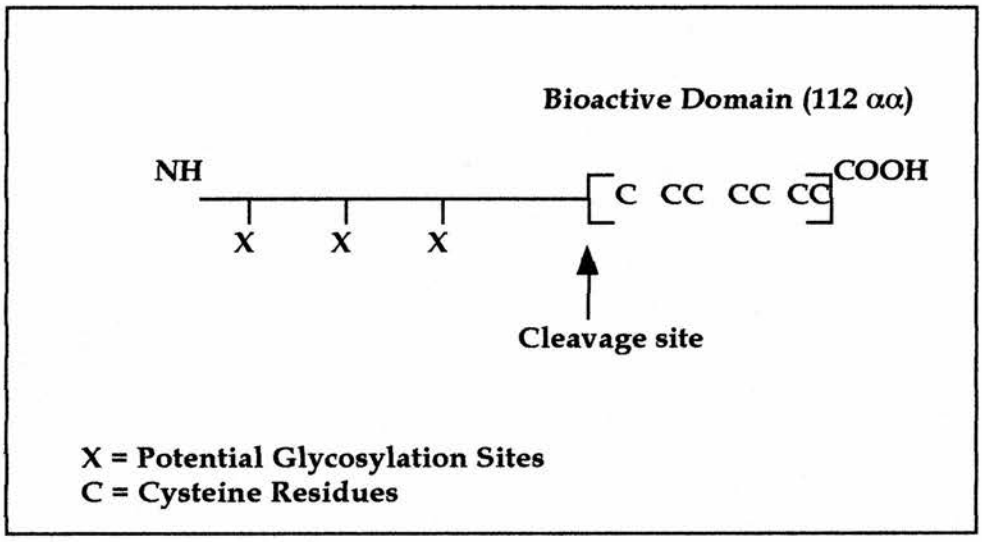


Figure 1.12 Precursor Structure of TGFβ1 (390αα). (From Massagué *et al.*, 1992).

1.7.2 Transforming Growth Factor Beta (TGFβ) Superfamily

Recent years have seen the recognition of a multifunctional family of growth factors which have become the central group of regulators in embryonic and adult life. They were discovered, initially, for their ability to transform normal fibroblasts on a substrate to cells that would grow in suspension (Colletta, 1990) and so were named the Transforming Growth Factors beta (TGFβ) superfamily.

The TGFβ family is composed of pleiotropic cytokines which have both inhibitory and stimulatory effects on a variety of cell types. They have roles in cell cycle regulation, development, differentiation, immunomodulation, fibrinogenesis and wound repair. The distribution of these proteins, their receptors and their target tissues is widespread but roles in development and morphogenesis are common to all.

There are about 30 structurally related members in the family. Their common features are their precursor structure, 7-9 conserved cysteine residues in the mature sequence, homo- or heterodimer structure as bioactive factors and the 25-70% homology in their amino acid sequences (Massagué *et al.*, 1992). Crystallography of TGFβ2 revealed that 6 of the conserved cysteines are grouped to form a cysteine knot which is held together with 3 disulphide bonds. The knot is probably responsible for the strong resistance to heat, denaturants and pH that TGFβ family members display (Kingsley, 1994).

i. TGFβ Precursor

The gene for the human TGFβ precursor, characterised by Derynck *et al.* (1987), has 7 distinct exons and 6 large introns. The structures of the other related genes are highly conserved. TGFβ precursor proteins are of approximately 400 amino acids long with an N-terminus, a pro-domain and a mature c-terminus. The cDNA sequence of TGFβ confirms that each mature polypeptide in the TGFβ family is synthesised from the C-terminal portion of the same large precursor (refer to Figure 1.12). The bioactive peptides are between 110 and 140 amino acids long (Massagué *et al.*, 1994).

The precursors dimerise and then are proteolytically cleaved at basic residues by a furin peptidase, allowing the release of the bioactive domains

(Massagué *et al.*, 1994). TGFβ dimers can exist as homodimers or as heterodimers, giving diversity in protein structure and function.

Protein activity remains latent during secretion. The pro-region of the precursor (also known as the latency associated peptide or LAP) can remain covalently attached to the bioactive peptide to ensure that the protein folds correctly and that its disulphide bonds are properly formed (Massagué *et al.*, 1992). Latent molecules of TGFβ cannot bind to their receptors until they are activated extracellularly by enzymatic proteolysis which results in the removal of the LAP.

ii. Related Proteins in the TGFβ Superfamily

Activins are activators of Follicle Stimulating Hormone (FSH) and red blood cell development (Gilbert, 1993). Activin A is composed of 2 βA TGFβ chains and Activin B is a homodimer of 2 βB TGFβ chains. In *Xenopus laevis* embryos, activins induce the formation of dorsal mesoderm.

Inhibins are potent and specific inhibitors of FSH (Mason *et al.*, 1985). They are stimulated by estrogen from the follicular granulosa cells of the ovary and feed back to stop the production of FSH from the pituitary. They are heterodimers of TGFβ chains - Inhibin A is composed of an α chain and a βA chain while Inhibin B is made up from an α chain and a βB chain.

Bone Morphogenic Proteins (BMP) were originally identified as inducers of endochondral bone formation, but have since been found to have developmental roles in proliferation, cell death and morphogenesis. There are 12 isoforms in the family and they differ from the other families of Transforming Growth Factors as they have 9 conserved cysteine residues compared with the usual 7 cysteines.

Anti-Müllerian Hormone (AMH) is expressed in embryonic male gonadal tissue by the Sertoli cells of the differentiating testes. It is a non-steroidal hormone which causes regression of the Müllerian duct (which in females, becomes the uterus, oviducts, cervix and upper vagina). Structurally, AMH is a homodimer of 70-72kD glycosylated subunits and its C-terminus is homologous with TGFβ (Massagué, 1987).

Decapentaplegic Complex (DPPC) is a gene found in *Drosophila* with many developmental functions. Its protein product regulates dorso-ventral

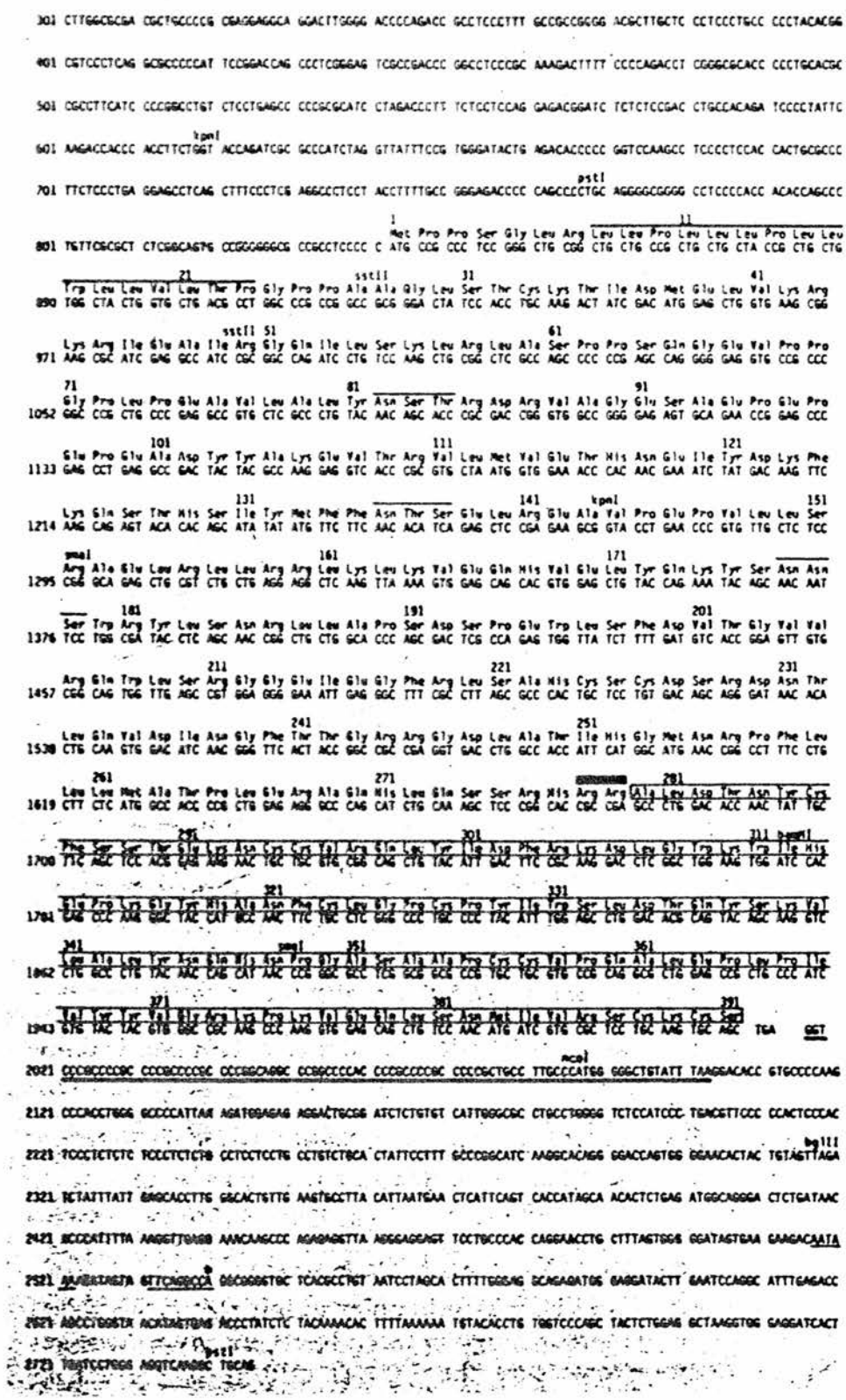


Figure 1.13 Gene Sequence of TGFβ1 (from Derynck *et al.*, 1985)

specifications and controls morphogenesis of the larval imaginal discs (Massagué, 1987). The C-terminus amino acid sequence is thought to share 36% homology with human TGFβ. It is suspected that the secreted polypeptide of DPPC is a disulphide-linked dimer of 22-26kD with autocrine and paracrine abilities. Functionally, DPPC is very similar to BMP4.

Vg1 protein is expressed exclusively in vegetal cells of *Xenopus* embryos. Vg1 mRNA transcripts are present in oocytes and become translocated to the vegetal pole of the embryo by microfilaments and microtubules as development continues. The resultant peptide participates in embryo development by inducing dorsal mesoderm formation and a homologous protein can be found in equivalent sites in chick embryos.

1.8 TRANSFORMING GROWTH FACTOR BETA

The archetype of the TGFβ superfamily is Transforming Growth Factor beta type 1. It is a 25kD, disulphide-linked dimeric protein originally recognised because of its ability to reversibly transform anchorage-dependent fibroblasts into cells which required no substratum to grow (Colletta, 1990). There are about 25 other factors related to TGFβ (Massagué *et al.*, 1994) by function and/or structure. Vertebrate TGFβ exists as 8 isoforms with only 3 being identified so far in mammals (Kondaiah *et al.*, 1990). TGFβ4 has been discovered in chickens and TGFβ5 in *Xenopus laevis* (Massagué *et al.*, 1992). They have regulatory functions in cell proliferation, recognition, differentiation and death as well as pivotal roles in development, tissue recycling and repair.

For the purposes of this introduction, only the mammalian isoforms of TGFβ, especially TGFβ1, will be considered.

i. TGFβ Genes

The gene for human TGFβ1 is on chromosome 19q, position 13.1-13.2 (Dijkhuizen *et al.*, 1996). It was cloned and characterised by Derynck *et al.* (1985). The sequence is shown in Figure 1.13.

ii. TGFβ mRNA

The approximate length of TGFβ was determined as 2.5kb once a cDNA became available. Derynck *et al.* (1985) used the cDNA to analyse tumour cell lines for the presence of TGFβ mRNA. It was detectable in all lines tested, including solid tumours such as Wilms tumour, glioblastoma A172, bladder carcinoma T24 and MCF-7 as well as haematopoietic cancer cell lines like Burkitt lymphoma B lymphoblast and T-cell leukaemia. The mRNA pattern does not always correlate to that of the protein, possibly due to the accumulation of protein away from synthesis site or the non-translation of the message.

iii. TGFβ Protein

The 3 mammalian TGFβ isoforms are disulphide-linked homodimers and heterodimers. Different subunits can bond to form mixtures of the proteins. The isoforms of TGFβ have 70-80% sequence homology and their functions are similar but not identical. The transcription of each isoform is controlled by its own unique transcriptional regulatory element, which may account for their diverse functions. The bioactive peptide is 112 amino acids long after cleavage by proteases or glycosidases (Massagué *et al.*, 1992). It is this peptide that is recognised by the TGFβ receptors (Colletta, 1990).

Sequence similarity to the other related proteins ranges from 30-60% (Massagué *et al.*, 1994). The homology is especially evident in the active domain of the proteins with 7 conserved cysteines, 6 of which form the cysteine knot. The 7th cysteine forms a disulphide bond with the corresponding cysteine from the other half of the dimer. The monomers lie flat and anti-parallel to one another, forming hydrophobic bonds (Massagué *et al.*, 1994).

In the adult, bone is the major production site of TGFβ1 (Akhurst *et al.*, 1990) but it is detectable in most adult and embryonic tissues. It can be extracted from both normal and tumour tissues, including kidney, placenta and blood platelets (Derynck *et al.*, 1985). The expression of bioactive TGFβ has to be strictly regulated because so many cells can produce and respond to it. Production is regulated at several levels - transcriptional, mRNA stability, translational, storage of latent protein, latent protein secretion, amount delivered to receptor by

betaglycan, i.e. receptor type III, and by sequestration of protein in ECM and circulating protein in the plasma (Massagué *et al.*, 1994).

Most cells in culture produce a latent form of TGFβ. Production of biologically active TGFβ has been reported in 3 cell lines - fetal fibroblasts in response to tamoxifen (Colletta *et al.*, 1990), MCF-7 breast cancer cells in response to tamoxifen (Knabbe *et al.*, 1987) and rat keratinocytes treated with retinoic acid (Glick *et al.*, 1991).

iv. TGFβ Receptors

Most normal and neoplastic cells have receptors, or at least binding proteins, for TGFβ (Tucker *et al.*, 1984; Frolik *et al.*, 1984). There are 3 types of TGFβ receptor, identified by photoaffinity and cross-linking experiments (Cheifetz *et al.*, 1987; Massagué & Like, 1984). TGFβ types 1, 2 and 3 all bind to each of the 3 receptors but more distant members of the TGFβ family do not (Mason *et al.*, 1985). All 3 receptors bind with high affinity to their ligands, TGFβ1 creating the strongest bond (Cheifetz *et al.*, 1987). There are about 10,000-50,000 receptors per cell (Tucker *et al.*, 1984). The cloning of the receptor gene sequences revealed that TGFβ receptors type I and II are serine-threonine kinase transmembrane proteins.

Receptor type I is 53kD with a different set of kinase domains to receptor type II. It has a glycine-serine rich (GS) domain (29 amino acid motif with a GSGSG sequence in the middle) and forms stable ternary complexes with ligand-bound type II receptors. Type I alone can neither bind ligand or initiate signalling but may increase ligand-binding affinity and is necessary for the correct signalling by type II.

Receptor type II is a 70-85kD membrane protein containing a functional serine-threonine kinase. By itself, type II receptor can recognise and bind to TGFβ but like type I receptor, it is insufficient to cause signalling alone (Massagué *et al.*, 1994). The cytoplasmic domain of the type II receptor interacts with TGFβ receptor-interaction protein I (TRIP-I). The role of TRIP-I may be as an adapter protein in TGFβ signalling (Ravitz and Wenner, 1997).

Receptor type III has a weight of between 200-400kD and is known as betaglycan (Massagué *et al.*, 1994). It has no actual signalling domain but it can regulate access of TGFβ to the other receptors in a positive and a negative way.

The signalling receptors bind more efficiently to TGFβ that is bound to type III as opposed to TGFβ alone; this is especially true for TGFβ2 (Massagué *et al.*, 1994). Type III receptors are the largest of the 3 types and 50% of the receptor molecule is composed of heparin sulphate and chondroitin sulphate (Segarini *et al.*, 1988).

Each of the receptors has a small extracellular region, a single trans-membrane domain and an intracytoplasmic Serine/Threonine (ser/thr) kinase domain. TGFβ receptors are different from other hormone receptors because they are serine/threonine kinases (rather than tyrosine kinase), making them a unique family. All membrane ser-thr receptors identified in animal cells so far belong to the TGFβ family. Different isoforms of receptor exist, allowing different type I receptors to interact with different type II receptors, which could contribute to the variability and flexibility of TGFβ response (Massagué *et al.*, 1994). Ligand binding specificity is determined by receptor type II and the pattern of the extracellular cysteines, which differ depending on the ligand they bind. Receptor type I may increase the affinity with which each ligand binds to receptor type II and may determine the biological response (Massagué *et al.*, 1994).

1.8.1 TGFβ Signalling

Signalling requires the formation of a trimeric, perhaps even a heterotetrameric, complex made of TGFβ and both type I and type II receptors. For TGFβ signalling to occur, both receptor types are required in mammalian tissues (Wrana *et al.*, 1994) and in Drosophila (Letsou *et al.*, 1995). TGFβ1 protein binds to receptor type II , which is constitutively phosphorylated so the binding of TGFβ1 does not increase the phosphorylation. Bound TGFβ1 is recognised by receptor type I which binds to the ligand (i.e. the TGFβ1-bound type II receptor) only when TGFβ is in place. It does not associate with unoccupied type II receptor. Type II phosphorylates type I and a signal can then be generated to the cell so long as the type I has an active kinase domain.

Type III is widely distributed throughout the cell membrane and binds to TGFβ directly with high affinity. TGFβ-bound type III receptors are capable of binding to the type II receptor which in turn binds to the type I as before. Type III then dissociates itself from the I and II complex. Cells deficient in type III are

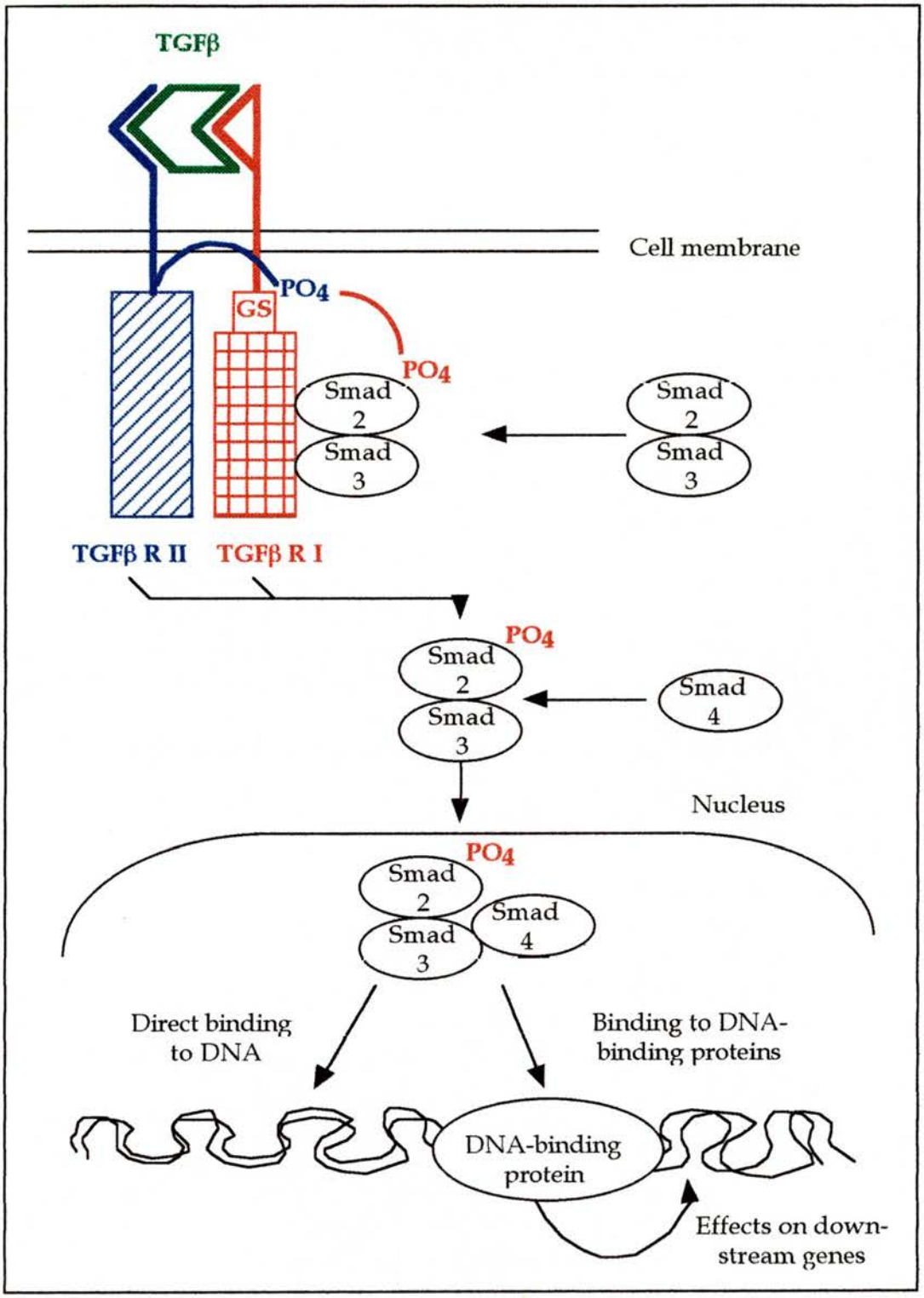


Figure 1.14 TGFβ Signalling through the Smad Pathway.
TGFβ1 binds to receptor type II. The resultant complex recruits and phosphorylates receptor type I in the GS domain. Smads 2 and 3 associate with activated receptor type I and become phosphorylated by it. Smad 4 is recruited and the Smad 2/3/4 complex is translocated to the nucleus, binding directly to DNA or to DNA binding proteins.

insensitive to TGFβ2 as the affinity of the isolated type II receptor for TGFβ2 is very low and normally type III receptors would enhance that affinity.

Type II acts upstream of type I and their differences lie in the way that they bind ligands. Type II for TGFβ and Activin recognise their ligands free in the intercellular medium whereas their type I receptors recognise the ligand bound type II as its target site. The entire complex then is believed to be a heterotetramer.

i. Signal Transducers Involved in TGFβ Signalling

Smad genes derive their name from *Drosophila* genes, Mothers against DPPC (Mad genes) and homologous genes in *Caenorhabditis elegans* (Sma genes) (Heldin *et al.*, 1997). Vertebrate homologues of these genes were identified by Derynck *et al.* (1996). Smads are encoded on human chromosome 18q and loss of gene function from this position has been implicated in a variety of tumours (Powell *et al.*, 1997). Mutations in these genes have also been associated with tumour promotion (Riggins *et al.*, 1997).

Smad genes encode proteins which transduce signals from ligand-bound TGFβ receptors on the cell membrane to the nucleus. Heldin *et al.* (1997), Massagué *et al.* (1997), Kretzschmar and Massagué (1998), Padgett *et al.* (1998) and Attisano and Wrana (1998) have reviewed TGFβ signalling through the Smad pathway. Each presented a hypothetical TGFβ signalling pathway from the published evidence. Based on the data given, the signalling pathway is emerging as follows - TGFβ binds to Receptor type II, inducing hetero-oligomerisation with Receptor type I and transphosphorylation of the GS domain of Receptor type I by the Receptor type II kinase (Liu *et al.*, 1997). In the cytoplasm, pathway-restricted Smads 2 and 3 are phosphorylated by the heteromeric receptor complex. The Smads 2/3 then phosphorylate a common mediator, Smad 4, forming another heteromer. The Smad 2/3/4 complex is translocated to the nucleus where it either binds directly to DNA or binds to DNA-binding proteins, for example a transcription factor named FAST-1, which in turn affect transcription of specific genes (refer to Figure 1.14).

Inhibitory Smads have been discovered. Smads 6 and 7 are the most divergent members of the Smad family and can inhibit the transcriptional activity of TGFβ under certain conditions. If overexpressed, Smad 6 can associate with

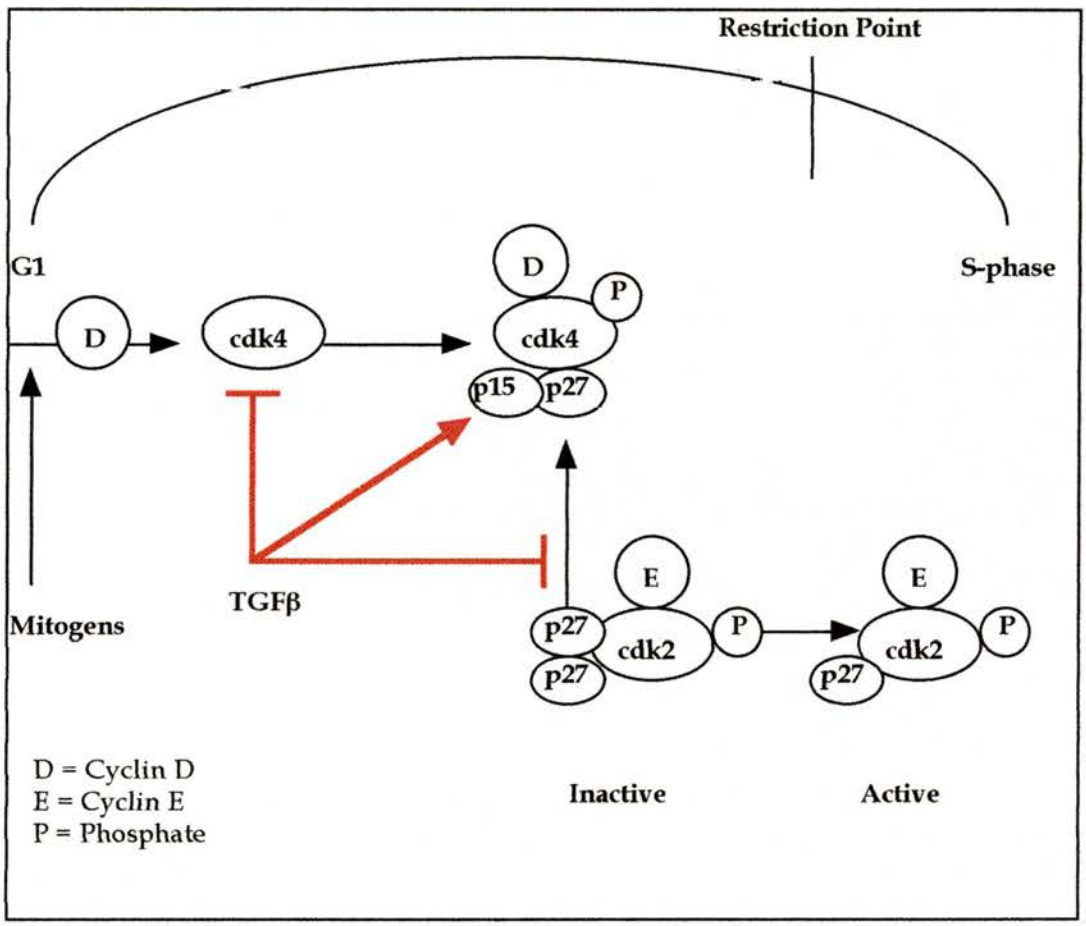


Figure 1.15 Schematic Representation of Inhibitory TGFα Signalling through the Cyclin-cdk Pathway.

TGFβ inhibits cell proliferation via the regulation of G1-phase cdks. In epithelial cells, TGFβ depresses the synthesis of cdk4. TGFβ treatment also induces certain cells to increase steady state levels of p15. Together, these effects of TGFβ prevent the sequestration of p27 from cyclin E-cdk2 by the cyclin D-cdk4 holoenzyme, either by preventing, or rendering ineffective, the CAK-mediated phosphorylation and activation of these kinases. (From Ravitz and Wenner, 1997)

receptor type I and can inhibit phosphorylation of Smad 2. Smad 7 associates with activated TGFβ receptor complexes, blocking the interaction with and phosphorylation of Smad 2 or 3 (Kretzschmar and Massagué, 1998).

Cyclin dependent kinases (cdk) are cell cycle regulators which govern cell cycle transitions in mammalian cells (Reynisdóttir and Massagué, 1997) by driving the cell cycle in space and time (Morgan, 1997). The G1 progression is controlled by cyclin-D dependent kinases, cdk4 and/or cdk6, and the cyclin-E dependent kinase, cdk2 (Reynisdóttir *et al.*, 1995). Cdks are regulated by positive effectors, cyclins, and by negative effectors, cdk inhibitors.

Cdk inhibitors are small proteins, identified for their ability to bind to and regulate the cyclin-cdk complexes involved in the G1→S-phase. They have a negative effect on their corresponding cdk as the binding results in the cessation of the cell cycle. There are 2 families of cdk inhibitor - the kinase inhibitor protein (KIP) family, comprising p21, p27 and p57 and the INK4 family, of which p15, p16, p18, p19 and p20 are members. In mammalian cells, 5 of these regulators have been identified, p15, p16, p21, p24, and p27. Because these molecules have a negative effect on the cell cycle, they are of interest in tumorigenesis. Mutations in the cdk inhibitors may contribute to the loss of growth control seen in tumour cells. Some are induced by and participate in the signalling of growth factors.

p16 (also known as cdkN2, INK 4A or MTS1) was identified as a cdk inhibitor in 1993 by Serrano *et al.* The same research group isolated a related factor, p15 (Hannon and Beach, 1994). Both p16 and p15 bind to cdk4/6, causing an arrest of the cell cycle in G1 (Walker *et al.*, 1995). The association of p15 with cdk4 and 6 is increased following treatment by TGFβ in HaCaT epithelial cells (Hannon and Beach, 1994). Levels of p16 were unaffected by the same treatment. Hannon and Beach demonstrated that the increase in p15-cdk4/6 association corresponded with a 30-fold rise in p15 mRNA. Li *et al.* (1995) also demonstrated that TGFβ induces p15 transcription and its subsequent association with cdk4/6. TGFβ-induced cell cycle arrest can occur through the cdk inhibitor pathway (refer to Figure 1.15) as well as the Smad pathway.

Because of their ability to cause cell cycle arrest, it is believed that p16 and p15 are tumour suppressors. They are both situated on chromosome position 9p21, a region which is often deleted or abnormal in tumours (Devlin *et al.*, 1996). Chang *et al.* (1997) examined 30 malignant skin tumours and found a highly

significant relationship between loss of p16 expression and metastasis. Loss of p16 and p15 function could be responsible for TGFβ insensitivity and subsequent tumour progression.

DNA damage causes cell cycle arrest by inducing p53, which in turn induces the cdk inhibitor, p21 (Darbon *et al.*, 1995). p21 is also detected in quiescent and senescent cells. Datto *et al.* (1995) showed that TGFβ can cause a rapid transcriptional induction of p21 via a p53 independent pathway, suggesting that p21 can be stimulated by both intracellular and extracellular signals and that a single signal like TGFβ can cause growth arrest through many signalling pathways. TGFβ increases p21 mRNA due to transcriptional activation of p21 promoter (Datto *et al.*, 1995a). When induced, p21 binds to cdk2 causing cdk2 inhibition and subsequent growth arrest (Reynisdóttir *et al.*, 1995).

p27 causes G1 arrest in response to cell contact, TGFβ or cAMP. It normally resides in combination with cdk4/6 but is freed from these complexes in the presence of TGFβ. The release of p27 correlates with a surge in p15 in mink lung epithelial cells showing that TGFβ-induced arrest can occur through co-operation of p15 with p21 or p27 (Reynisdóttir *et al.*, 1995).

Reynisdóttir *et al.* (1995) review the current understanding of cell cycle mechanisms with respect to studies on TGFβ. Previous explanations as to how TGFβ inhibits proliferation include suppression of cdk4 synthesis (Ewen, 1993), downregulation of cyclin and cdk inhibition (Geng and Weinberg, 1993; Slingerland *et al.*, 1994), inhibition of cdk2 and cdk4 complexes by p27 (Slingerland *et al.*, 1994) and inhibition of cdk4 and cdk6 by p15 (Hannon and Beach, 1994). The results presented by Reynisdóttir *et al.* (1994) suggested that TGFβ induces cell cycle arrest through the co-operation between p15 and p27/p21. A decline in cdk levels was observed after TGFβ-induced arrest as the cells become quiescent.

Retinoblastoma (Rb): The protein product of a normal Retinoblastoma gene suppresses tumorigenicity. However, Rb mutations occur in many tumours. Rb protein is normally phosphorylated by cdk4 and cdk6. In its phosphorylated state, Rb will allow progression of the cell cycle from G1→S-phase. TGFβ inhibits the phosphorylation of Rb, preventing it from allowing G1 progression which results in cell cycle arrest. Additionally, p15, which is upregulated by TGFβ, suppresses phosphorylation of Rb by inhibiting cdks (Serra and Moses, 1996).

It is very rare for a cell not to express TGFβ receptors but the transformed Retinoblastoma cell line is one exception. The lack of TGFβ receptors appears to explain how these cells escape the growth confines of normal cell division. Its non-transformed counterparts, fetal retinal cells, express all 3 TGFβ receptors and can be growth inhibited by TGFβ (Kimchi *et al.*, 1988).

1.8.2 Biological Effects of TGFβ

The effects of TGFβ *in vitro* are diverse. Depending on cell type and growth conditions, TGFβ can stimulate or inhibit growth, block or allow entry into pathways, promote or inhibit migration and stimulate extracellular matrix. TGFβ proteins mediate major events in development and growth in species as diverse as *Drosophila, Xenopus* and mammals.

i. Effects on Cell Growth - Stimulation and Inhibition

TGFβ was discovered through its ability to stimulate anchorage-independent growth in non-transformed fibroblasts but very few cell types actually grow in the presence of TGFβ (some fibroblasts and osteoblasts do). TGFβ affects the eukaryotic cell cycle during G1-phase, promoting or inhibiting cyclin-dependent kinases (cdk) as described in 1.8.1i. The effect of TGFβ depends very much on cell type, differentiation state , growth conditions and presence of other growth factors. While TGFβ generally causes reversible inhibition of normal epithelial cells (Silberstein and Daniel, 1987; Shipley *et al.*, 1986), it stimulates growth of normal mesenchymal cells.

ii. Effects on Cell Differentiation

TGFβ levels have been measured in myoblastic cell lines which irreversibly differentiate into myotubes (Ewton *et al.*, 1988). The results showed that the number of TGFβ binding sites decreased as differentiation progressed, which correlated to a loss of sensitivity to the protein. No change in growth rate was observed. Rizzino (1988) reviewed the effects of TGFβ on cell differentiation. Stimulation of differentiation by TGFβ is observed in, for example, bronchial epithelium, keratinocytes, and intestinal epithelium while inhibition of

differentiation has been noted in several cell types, including embryonic mammary epithelium, adipocytes and skeletal muscle satellite cells. Characteristics of differentiated cells can also be modified by TGFβ. For example, growth factor production is enhanced in monocytes and FSH release is increased from pituitary cells by TGFβ. In contrast, production of surfactant is inhibited in pulmonary cells and steroidogenesis in Leydig and adrenocortical cells is halted by TGFβ.

iii. Stimulation of Extracellular Matrix (ECM)

The stroma of tumours is believed to be important in malignant growth because its composition differs from that of normal tissue. TGFβ affects the synthesis and deposition of various ECM proteins (Streuli *et al.*, 1993). For example, TGFβ causes an increase in production of collagen types I and III in human lung fibroblasts and in fibronectin and collagen I from human dermal fibroblasts. TGFβ, produced by MCF-7 cells, acts on fibroblasts to produce tenascin (Chiquet-Ehrismann *et al.*, 1989).

The expression of integrins (proteins that act as receptors for cell-ECM interactions) is increased by TGFβ treatment resulting in increased cell-ECM adhesion, an environment which is not conducive to metastasis. TGFβ can decrease the expression of proteases while increasing the expression of protease inhibitors so maintaining the integrity of the ECM which otherwise would be digested by the proteases (Roberts and Sporn, 1990). The ECM can act as a reservoir for TGFβ by binding to it; TGFβ can then be retrieved when required (Streuli *et al.*, 1993). Streuli *et al.* have also shown that TGFβ is negatively regulated by the ECM because TGFβ promoter levels are high in the absence of ECM proteins and low in the presence of basement membrane. This could explain the uniform expression of TGFβ in cultured cells (where there is no ECM) and the well-defined expression *in vivo* where it will be tightly controlled by the ECM products. Once ECM is deposited, the TGFβ gene is strongly downregulated so a feedback loop exists between TGFβ and ECM.

Walker *et al.* (1994) investigated the relationship between TGFβ1 and the extracellular matrix. They found an abundance of fibronectin in breast carcinomas and correlated this with metastatic potential. The authors previously found higher TGFβ expression in invasive tumours compared with *in situ* carcinomas (Walker

and Dearing, 1992) and suggested that TGFβ might have an effector role in tumour invasion. Because TGFβ influences the ECM, the study considered TGFβ in invasive tumours with regard to stromal components, lymphocytic/macrophage infiltration and tumour characteristics to establish any role for TGFβ and ECM proteins in invasion. They studied 86 fresh-frozen invasive carcinomas and used immunohistochemistry to detect TGFβ, fibronectin, tenascin, macrophages and T-lymphocytes. All of the tumours with prominent TGFβ staining had metastasised, which was statistically significant. This was only true for TGFβ1, not TGFβ2, which concurs with a study by Gorsch *et al.* (1992). Strong immunoreactivity for TGFβ tended to be associated with nodal metastases, higher amounts of fibronectin with different staining patterns and higher amounts of tenascin. Tenascin is also expressed to a higher degree in tumour tissue than normal tissue and can be used as a marker of epithelial malignancy (Mackie *et al.*, 1987). MCF-7 cells react to tenascin by losing cell contacts and becoming invasive.

iv. Angiogenesis

Angiogenesis is the process through which new blood vessels are formed from existing vessels. It occurs during embryogenesis, menstrual cycles, wound repair and tumorigenesis (Hanahan and Folkman, 1996). Many growth factors are required during this multi-step process, including Vascular Endothelial Growth Factors (VEGF) types A, B and C, acidic and basic Fibroblast Growth Factors, TGFα and TGFβ.

In vitro, TGFβ inhibits endothelial cell growth but it stimulates angiogenesis *in vivo* (Relf *et al.*, 1997). TGFβ, injected subcutaneously into neonatal mice, causes both angiogenesis and stimulation of collagen production from fibroblasts (Roberts *et al.*, 1986)

During tumourigenesis, angiogenesis must occur before metastasis can be achieved. The advantages of gaining a blood supply are the ability to utilise exogenous nutrients from the blood supply rather than rely on self-production and the facility to nurture the centre of the tumour. The paradox in breast cancer is that while TGFβ may slow the rate of epithelial growth, it may also be enhancing the ability of the tumour to create blood vessels.

v. Involvement in Immune System

TGFβ null mice die a few weeks after birth from widespread inflammation (Christ *et al.*, 1994) so TGFβ appears to have some role in immunity. Research has shown that the development of T and B cells in the immune system is suppressed by TGFβ. TGFβ also inhibits the production of immunoglobulin by B cells and the cytotoxicity of natural killer cells. Monocyte migration by chemotaxis and growth factor production is enhanced by TGFβ (Rizzino, 1986).

vi. Wound Healing

TGFβ is provided by platelets in large quantities at wound sites. Rodent experiments showed that the presence of TGFβ increases the tensile strength of healing lesions and an increase in infiltrating inflammatory response cells (Mutsaers and Laurent, 1995). The expression of matrix proteins is also upregulated at wound sites because of TGFβ. Topical application of TGFβ improves the rate of wound healing but an excess of the growth factor can cause fibrosis.

vii. Involvement in Embryogenesis

In mice, the TGFβ1 gene is activated soon after fertilisation, remaining active through post-implantation development. It is expressed in defined places, at precise times (Akhurst *et al.*, 1990). Using *in situ* hybridisation, Akhurst *et al.* (1990) found high expression of TGFβ1 mRNA in areas of haematopoiesis, angiogenesis and vascularisation. Millan *et al.* (1991) studied the gene expression of TGFβ1, TGFβ2 and TGFβ3 in mouse embryos. They found that the genes had different expression patterns usually relating to distinct individual functions. For example, TGFβ1 alone was involved in haematopoiesis, angiogenesis and osteogenesis, confirming the data presented by Akhurst *et al.* (1990). TGFβ2 figured strongly in neuronal tissue and epithelial structures, sometimes in conjunction with TGFβ1, while TGFβ3 was involved in mesothelial tissue but more often as a partner to either TGFβ1 or β2.

Despite the many developmental roles of TGFβ1, knockout mice defective for the TGFβ gene in the germ-line show no gross abnormalities at birth (Massagué *et al.*, 1994) but go on to develop a wasting syndrome, characterised by weight loss

and multi-focal inflammation (Christ *et al.*, 1994). The mice die at about 3 weeks of age due to an overwhelming infiltration of inflammatory cells into major organs such as the heart, lungs (Christ *et al.*, 1994) and stomach (Shull *et al.*, 1992).

viii. Effect on Steroid Production

Estrogens are aromatised from testosterone. Harada (1997) analysed aromatase mRNA expression in normal and malignant breast tissue. The expression of aromatase was a characteristic of stromal cells and expression levels were much higher in malignant tissue than in normal tissue, indicating that the biosynthesis of estrogen is elevated in cancer. Addition of TGFβ into a breast stromal cell culture system caused a reduction in aromatase mRNA expression but in co-culture with epithelial cells, aromatase mRNA remained high. The author suggested that the epithelial cells must be sequestering TGFβ so that it cannot affect the expression of aromatase from the stromal cells.

1.8.3 TGFβ in Normal Breast Tissue

TGFβ mRNA transcripts are detected at lower levels in normal breast tissue than in malignant breast tissue (Travers *et al.*, 1988; Barrett-Lee *et al.*, 1990). An immunohistochemical study by McCune *et al.* (1992) revealed that intra- and extralobular normal breast epithelium reacted strongly with antibodies against all 3 isoforms while stromal fibroblasts and myoepithelial cells were negative or weakly stained. The intralobular stroma stained strongly for TGFβ1 and β3 but interlobular stroma and fatty tissue was negative for all isotypes.

Introduction of exogenous TGFβ into pre-pubertal murine mammary glands results in inhibition of development and secretory function of the gland during sexual maturity and pregnancy, demonstrating that mammary epithelial cells do not develop properly in the presence of excess TGFβ.

i. Site of TGFβ Production in Normal Breast Tissue

McCune *et al.* (1992) examined the immunoreactivity of TGFβ1, β2 and β3 along with the TGFβ latency associated peptide (LAP) in paraffin-embedded breast carcinomas. They found that normal epithelial cells reacted strongly to anti-

TGFβ antibodies, producing all 3 isoforms of the protein. The stromal fibroblastic cells stained weakly, if at all. The fibrous intralobular stroma stained positively for TGFβ1 and TGFβ3 mature proteins, showing that they have been secreted into the matrix. The LAP proteins are normally cleaved off the protein as it becomes activated. TGFβ1-LAP and TGFβ3-LAP proteins were localised to the mammary epithelium, suggesting that they are manufactured there. Gomm *et al.* (1991) found that TGFβ1 protein was localised to the periductal and intralobular fibrous tissue.

1.8.4 TGFβ in Malignant Breast Tissue

Members of the TGFβ family have many functions in tumorigenesis. At early tumour stages, BMP and TGFβ can act as tumour suppressors, inhibiting the formation of chemically-induced murine skin tumours (Serra and Moses, 1996).

McCune *et al.* (1992) found that, in all the infiltrating carcinomas they tested, malignant epithelial cells stained positively for TGFβ1, β2, β3, TGFβ1-LAP and TGFβ3-LAP, while the myoepithelial cells remained negative, as seen in the normal tissue. The conclusion of the study was that particular TGFβ staining patterns were specific to cell types regardless of the pathological lesion, suggesting that TGFβ itself may not be involved in the malignant progression of breast cancer.

Gorsch *et al.* (1992) also looked retrospectively at the expression pattern of TGFβ1, β2 and β3 in paraffin wax embedded breast tumour tissue and found that it correlated with disease progression. Their analysis of the antibody staining was more specific than that of McCune *et al.* (1992). From 42 breast biopsy specimens stained immunohistochemically for all 3 isoforms, TGFβ1 reactivity was observed mainly in the cytoplasm of the epithelial cells of ductal tumours, especially at the cytoplasmic membrane. TGFβ2 stained primarily in the stromal cells and on luminal surface of ducts with only weak affinity to the epithelial cells. TGFβ3 was restricted to the surrounding myoepithelial cells, which differs from the findings of McCune *et al.* (1992). The association of disease progression with TGFβ staining did not exist for TGFβ2 or TGFβ3, so TGFβ1 was of particular interest. This may be because of a preferential activation of TGFβ1 from the latent complex or an effect of TGFβ1 that allows positive feedback to increase its own production.

Walker and Dearing (1992) also found a positive association between TGFβ staining and the metastatic potential of tumours. They used immunohistochemistry to examine the expression of TGFβ in DCIS and invasive carcinomas of the breast. Only 11% of the non-invasive tumours showed prominent TGFβ staining with 55.5% being negative. In comparison, 20% of the true carcinomas showed prominent TGFβ stain with 33.3% being negative. There were no differences in staining between lobular and ductal carcinomas and no significant correlation between histological differentiation and TGFβ expression which concurs with McCune *et al.* (1992) who said that the staining pattern was cell-specific rather than lesion-specific. Production of TGFβ may augment certain aspects of the malignant phenotype i.e. sustain the tumour-like characteristics. The ways in which TGFβ could enhance tumorigenesis and metastatic potential are regulation of humoral and cellular immunity, alteration of the ECM and basement membrane and promotion of angiogenesis.

Gorsch *et al.* (1992) and Walker and Dearing (1992) are contradicted by data from Mizukami *et al.* (1990) which showed that TGFβ positivity was correlated to longer disease-free periods and Barrett-Lee *et al.* (1990) and Thompson *et al.* (1991) who both found that high TGFβ expression was coupled with the absence of nodal metastasis.

Travers *et al.* (1988) extracted total RNA and probed for TGFβ. They found that TGFβ mRNA was elevated to significantly higher levels in all types of breast tumour compared with normal breast epithelium, suggesting that transcription of TGFβ is upregulated in malignant breast cells. The amount of TGFβ mRNA was not associated with ER content or the presence or absence of other growth factors. However, as Travers *et al.* noted, it became important to establish the site of TGFβ production.

TGFβ mRNA has been detected in a wide variety of tissues with low or undetectable amounts in normal tissue and higher levels in tumour compared to adjacent normal tissue. Walker and Gallacher (1995) carried out an *in situ* hybridisation study of TGFβ1 mRNA expression in fresh fixed, wax-embedded breast carcinoma. Their technical findings were that RNA preservation in routinely fixed tissue, as opposed to optimally fixed tissue, was poor, which resulted in problems with interpreting the pattern of expression due to background staining. From 16 carcinomas studied, 9 of them were positive for mRNA in epithelial

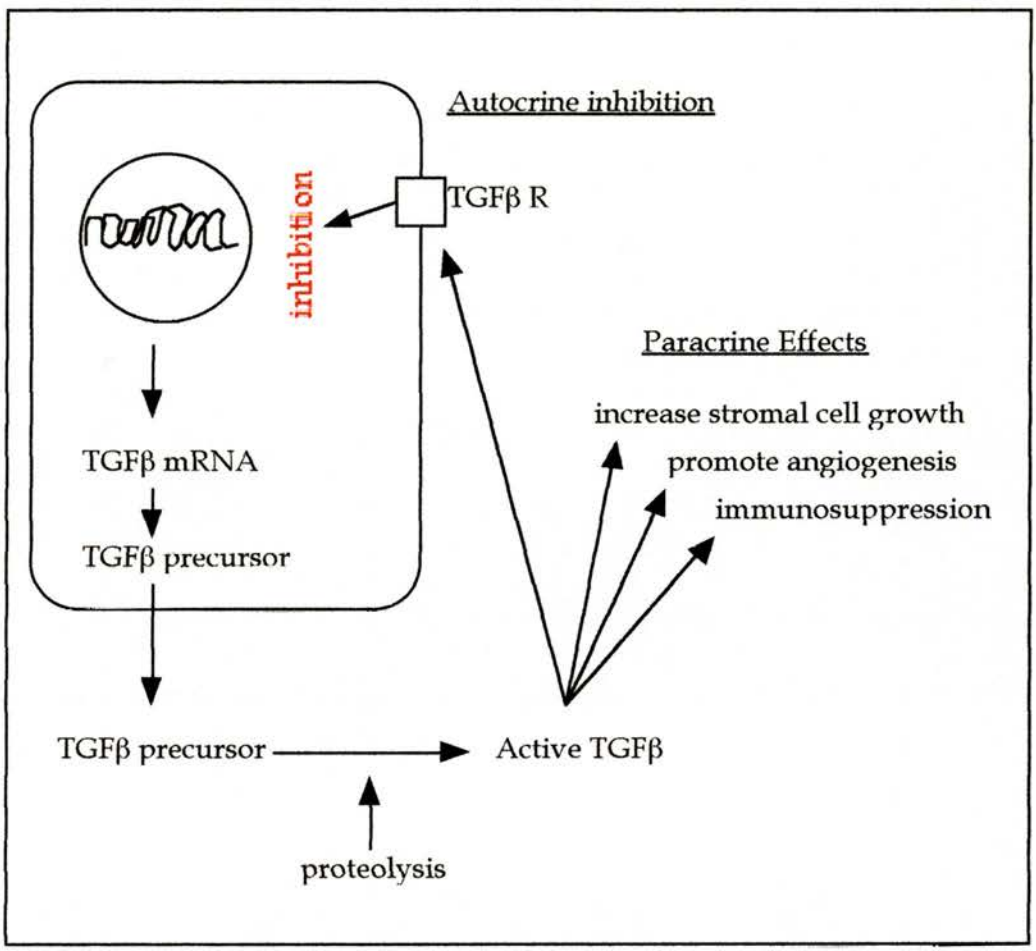


Figure 1.16 Autocrine and Paracrine Actions of TGFβ.

Latent TGFβ is secreted from an epithelial cell. The precursor is proteolytically cleaved, producing active TGFβ. The active TGFβ can exert both autocrine and paracrine effects.

tumour cells but there was no label in tissue where RNA preservation was poor. The level of label varied between individual samples and within the same sample. Stromal cells showed no significant reactivity. Expression of mRNA correlated with positive staining for protein. The levels of RNA found in the study by Walker and Gallacher (1995) are similar to those reported by Barrett-Lee *et al.* (1990) but lower than MacCallum *et al.* (1994) probably due to differences in techniques used.

MacCallum *et al.* (1994) used an RNase protection assay to measure the expression of TGFβ isoforms in 50 breast carcinomas. TGFβ1 mRNA was expressed in 90% of tumours, TGFβ2 was expressed in 78% of tumours and TGFβ3 was expressed in 94%. All 3 isoforms were expressed in 74% of tumours, 20% expressed 2 isoforms, 4% expressed only TGFβ1 and one tumour was negative for all 3 isoforms.

With regard to clinical treatments, there were hopes that TGFβ1 might prove a valuable tool in combating breast cancer. Tamoxifen induces TGFβ mRNA *in vitro* but has no effect on the species of mRNA produced (Arrick *et al.*, 1990). This supports the theory that TGFβ1 stimulation is a mechanism of tamoxifen action on breast cancer cells. Thompson *et al.* (1991) investigated expression of TGFβ1 mRNA in estrogen dependent MCF-7 xenografts and in tumours from patients who had received tamoxifen therapy before surgery. The MCF-7 xenograft tissue responded to tamoxifen treatment with a simultaneous increase in TGFβ1 mRNA levels from low on Day 0 to high from day 7 onwards, demonstrating a significant relationship between TGFβ1 expression and tumour volume reduction. In the human breast tissue, high levels (>2x that of normal tissue) of TGFβ1 mRNA were detected in 80% of tumour specimens. Almost all the tissue from pre-menopausal women had high levels of TGFβ1, which is a significant correlation. No association was found between TGFβ and tumour size, ER status or presence of lymph node metastasis. Six tumours from a total of 11 post-menopausal patients did not respond to tamoxifen therapy even though TGFβ1 mRNA levels were high, showing an association with high TGFβ1 level and tumour progression despite tamoxifen treatment. Thompson *et al.* (1991) suggested that TGFβ1 fails to inhibit breast cancer cell growth either because the latent protein is not properly activated or because the cells have lost the ability to respond to TGFβ1. The TGFβ1 model from which Thompson *et al.* (1991) were working is shown in Figure 1.16. Insensitivity to TGFβ1 in the autocrine pathway would

result in a lack of inhibition of growth. The clinical outcome would be that tamoxifen therapy had failed because a reduction in cell growth was not observed, despite a measurable increase in TGFβ1.

i. Site of TGFβ1 Production in Malignant Breast Tissue

There are 2 theories concerning where TGFβ is produced in the breast. The data described above all point to the epithelial cells being the producers of TGFβ (McCune *et al.*, 1992; Gorsch *et al.*, 1992; Walker and Gallacher, 1995). However, Butta *et al.* (1992) found that mammary stromal cells were the main producers of TGFβ protein, following tamoxifen treatment, which concurs with the findings of Colletta *et al.* (1990) who stimulated the production of TGFβ from ER-negative fetal fibroblasts treated with tamoxifen. Adult breast cancer fibroblasts are known to revert, in certain aspects, to a fetal-like phenotype (Haggie *et al.*, 1987).

Debate also over whether tamoxifen causes an upregulation of TGFβ1 mRNA or a post-transcriptional elevation of the protein. Wakefield *et al.* (1990) found an increase in the TGFβ1 protein but no increase in mRNA by anti-estrogens. They identified a stable stem-loop in the structure of the untranslated TGFβ1 mRNA which could be activated by anti-estrogens. They proposed that, rather than increasing the actual amounts of message, anti-estrogens were acting to increase the 'translatability' of the TGFβ1 mRNA, hence more protein.

1.8.5 Mechanisms of Escape from TGFβ Inhibition

For cells to thrive, they have to overcome the normal inhibitory growth constraints of molecules like TGFβ. This has been demonstrated by Ewton *et al.* (1988) in normal muscle cells when they are forming differentiated myotubes. Although TGFβ is present in the muscle cells, no growth inhibition is observed.

Many malignant cell lines have overcome their sensitivity to TGFβ, which *in vivo* could contribute to disease progression. Various mechanisms have been proposed. Evidence indicates that TGFβ receptors and signalling proteins act as tumour suppressors and it is their loss that causes the insensitivity to TGFβ and a mechanism of escape from growth regulation.

Loss of one or more TGFβ receptors has been identified in some neoplastic tissues, for example, retinoblastoma, breast carcinoma and several leukaemic cell lines (Ravitz and Wenner, 1997). There is evidence for point and frameshift mutations in the type I and II receptors. Markowitz (1995) identified inactivated type II TGFβ receptors in some colon cancers which is associated with microsatellite instability. Mutations in TGFβ receptor types I and II have been reported in prostate cancer (Kim *et al.*, 1996) along with reduced levels of the type II protein (Williams *et al.*, 1996) even though, *in vitro*, most prostate cells are inhibited by TGFβ. Restoration of the type II receptor in receptor negative cells has suppressed their tumorigenicity.

Mutational inactivation of tumour suppressors, for example p53 and Rb1, has also been implicated in the development of TGFβ insensitivity.

Smads and cdk inhibitors function as tumour suppressors so mutations in these proteins are associated with the progression of carcinogenesis. Riggins *et al.* (1997) studied smad gene mutations in human cancers and found that from the 6 known smads, only smad2 and smad4 contained cancer enhancing mutations, which resulted in the interruption of the TGFβ signal. The inactivation of smad4 is implicated in gastric carcinomas (Powell *et al.*, 1997).

Slingerland *et al.* (1994) reported that mutations in cdk inhibitors could also result in a lack of response to TGFβ. They identified the presence of a novel inhibitor in the TGFβ inhibitory pathway, but at the time it remained unidentified. It is now known to be p15. However, the actual role of TGFβ is more mysterious because it is often overexpressed in highly aggressive tumours (Gorsch *et al.*, 1992).

Also, altered expression of cyclin E/A and of cdk2 resulting in aberrant inhibitory responses and overexpression of cyclins E/D causing an acceleration in the transition from G1 to S have been proposed as TGFβ escape mechanisms (reviewed by Slingerland *et al.*, 1994).

However, Kalkhoven *et al.* (1996) demonstrated that, despite TGFβ insensitivity, some breast cancer cell lines were still growth inhibited by anti-estrogens. They concluded that while TGFβ could contribute to growth inhibition in TGFβ-sensitive cells, it could not be the only inhibitory pathway in use, as TGFβ-insensitive T47D breast cancer cells lines were still growth inhibited by anti-estrogens.

1.9 ESTROGENS & THEIR ROLE IN THE BREAST

1.9.1 Estrogen

Estrogen is a steroid hormone and, like the all other steroids (glucocorticoids, mineralocorticoids, androgens, progestogens and estrogens), is a derivative of cholesterol. Because steroids are relatively small at ~300MW and hydrophobic, they diffuse easily across the lipid cell membrane of target cells. They bind to their receptors tightly but reversibly and once the receptor is activated, the complex can bind to DNA and affect transcription. A typical cell has ~10,000 steroid receptors.

Estrogen, the female sex steroid, is vital for the normal formation of the embryonic genital tract and the mammary gland. It elicits many effects on the epithelia of the breast, uterus, prostate and seminal vesicle. However, studies on neonatal mice showed that this effect may be indirect as the epithelial cells display no detectable ER sites; instead, the ER are on the neighbouring mesenchymal cells (Cunha *et al.*, 1983). This indicates that, in hormonally regulated development, mesenchymal-epithelial interactions are essential for correct development. The structure of estradiol is shown in Figure 1.17. Other hormones involved in the development of the mammary gland are prolactin and growth hormone which may act in concert with growth factors such as IGF-1 (Plaut *et al.*, 1993)

Secondary female characteristics are also induced by estrogens. During the menstrual cycles, estradiol, in conjunction with FSH and LH, is responsible for the maturation of the ovarian follicle in the follicular phase of the cycle and the thickening of the endometrium in the luteal phase of the cycle. Another effect of estrogen is bone maintenance, a fact which is particularly evident in post-menopausal women who often suffer osteoporosis due to estrogen depletion (Heerscé *et al.*, 1998).

i. Synthesis of Estrogen

In women of reproductive age, the majority of bioactive estrogen, 17β-estradiol, is produced in the ovaries during the menstrual cycle and also in the placenta during pregnancy. In post-menopausal women, smaller amounts of

estradiol are produced in extra-glandular tissues like the liver, muscle and fatty tissue. They are converted from small amounts of the male hormone, testosterone, by an enzyme called aromatase. Breast cells are capable of aromatising testosterone into 17β-estradiol which emphasises the auto- and paracrine possibilities in the breast (Adams and Li, 1975). New therapies utilising aromatase inhibitors to limit the production of natural estradiol are being tested (refer to 1.11.2vi).

ii. Estrogen Receptor (ER)

Estrogen elicits its transcriptional activity through the ER. The ER was identified by Jensen *et al.* in 1967 (Wolf and Fuqua, 1995) and is part of a large family of related nuclear proteins, which includes the thyroid hormone receptor, vitamin D receptor and receptors for retinoids (Fawell *et al.*, 1990). It is a 65kD protein, 593 amino acids long, which is transcribed from a 6.8kb mRNA with 8 exons (Wolf and Fuqua, 1995). The amino terminus confers constitutive transcription activation, a mid-section binds DNA and the carboxy terminus binds the ligand. The DNA-binding section is highly conserved and has 2 zinc finger motifs which determine the binding specificity to the estrogen responsive element in the DNA (Fawell *et al.*, 1990).

Estrogen binds to its receptor with high affinity. The ligand-bound receptor undergoes conformational change to allow it to bind to the chromatin and to influence the transcription of target genes. Other transcription factors can be recruited to influence transcription of estrogen-regulated genes, for example, the progesterone receptor gene and cathepsin D precursor gene.

ER mutations exist which may contribute to the development of hormone independence. Karnik *et al.* (1994) investigated this theory by testing hormone-sensitive and hormone-insensitive tumours for known ER variants. ER mutations were identified in some tamoxifen-resistant tumours but they did not account for all the hormone insensitivity.

1.9.2 History of Estrogens and Breast Cancer

The notion that the ovaries influence normal breast development (already known, as lactating mothers did not menstruate) was strengthened by the 18th century surgeon, Percival Potts in 1785. He performed an ovariectomy on a 23-year old woman and follow-up examinations showed that, although she was in good health, her breast tissue was gone and she had not menstruated since the operation. The conclusion from this was that substances from the ovaries maintained the structure of the breast and if lost, the breast tissue would diminish.

In 1836, Cooper suggested a link between estrogen and breast cancer after observing that breast tumour growth was affected by the menstrual cycle (McPherson and Doll, 1991). Sixty years later, Sir George Beatson (consultant surgeon in Western Infirmary, Glasgow) noted a direct relationship between the ovaries and progression of breast cancer (Beatson, 1895). A 33-year old woman with breast lesions was referred to him at the Western Infirmary. He suggested that she undergo an ovariectomy because local surgery of the cancer was impossible. Months later, although the woman was by no means cured, the lesions had shrunk in size. Beatson continued this radical surgery by removing the ovaries of other breast cancer patients, some of whom benefitted from the operation with improvements of their symptoms.

More recent clinical associations between estrogen and higher than normal incidences of breast cancer have been observed in women prescribed the non-steroidal estrogen, diethylstilbestrol, as an anti-abortion drug during pregnancy and in transsexual males taking estrogen as a feminising drug.

Less strong relationships with breast cancer exist between oral contraceptives (OCC) and hormone replacement therapy (HRT). The Collaborative Group on Hormonal Factors in Breast Cancer (1996) examined almost all the available data on the relationship between OCC and breast cancer. They found that the risk of breast cancer to women currently taking the contraceptive pill (and to those who had stopped the pill within the last 10 years), was slightly higher than the risk to those who had never taken it. The cancers diagnosed in the 'user' group were of a less advanced stage than those diagnosed in the 'non-user' group. This may have been due to the regular check-ups that users require before being prescribed the medication. The risk of breast cancer and HRT (usually estrogen and/or progesterone replacement) has also been examined. Long-term usage of

HRT is correlated with a small increase in risk of breast cancer and also in endometrial cancer (Burger *et al.*, 1996). The higher than normal exposure to estrogens experienced by OCC and HRT users are often justified by the clinical benefits (McPherson and Doll, 1991).

1.9.3 Effect of Estrogen on Breast Cancer Cells

Most work on estrogens in breast cancer has been done in cell lines. The results are extrapolated to the *in vivo* situation but there are recognised difficulties in doing this.

The 1950s saw the establishment of cell lines for breast cancer but most lacked the estrogen receptor. In the early 1970s, Soule *et al.* established MCF-7, the first ER-positive breast cancer cell line (Levenson and Jordon, 1997). MCF-7 was derived from cells in a pleural effusion, extracted from a 69-year old woman who had undergone mastectomy for malignant adenocarcinoma 3 years prior to the establishment of the cell line. The patient's cancer had been controlled for these 3 years by radiotherapy and hormone therapy so it was assumed that the tumour was hormone dependent. MCF-7 became the standard model for estrogen and anti-estrogen research.

Early research on the growth effects of estrogen on MCF-7 cells was confused by the lack of consistent, reproducible results by different groups. A partial explanation was found, accidentally, by Katzenellenbogen (Levenson and Jordon, 1997) who detected the presence of estrogens in culture media. Subsequent studies revealed that phenol red, a pH indicator in culture media, is a weak estrogen (Berthois *et al.*, 1986). The constant presence of the estrogenic compounds were desensitising the cultured cells to exogenous estrogen during experiments.

Anderson *et al.* (1994) reported that normal and malignant breast epithelial cells metabolise estrogens in different ways, which could contribute to the growth stimulation in breast cancer. They found that, in a normal cell line (MTSV1-7), estradiol and estrone were converted to inactive, water-soluble conjugates while the malignant cell lines (MCF-7, ZR-75-1 and BT-20) converted the steroids to more active derivatives using the enzyme, 17β-hydroxysteroid dehydrogenase. This

inability of malignant cells to de-activate potent mitogenic estrogens may predispose the cells to the further development of the cancer phenotype.

Estrogen may act indirectly to enhance growth of estrogen-responsive cells by inducing intermediary proteins which, in turn, affect growth. For example, Dickson *et al.* (1986) found that estrogen induced the upregulation of TGFα and IGF-1 in MCF-7 cells and another estrogen-responsive cell line, ZR75-1. On estradiol withdrawal, these factors could recapitulate the effects of estrogen by stimulating growth for a short time. Therefore, estrogen may be exerting its mitogenic properties through these other factors. Indeed, TGFα and IGF-1 are often constitutively high in estrogen-independent breast cell lines, indicating that the factors replace the need for estrogen. MCF-7 growth is stimulated by IGF and EGF but not to the same extent as by estrogen. Manni *et al.* (1991) also concluded that, in soft agar, IGF and TGFα were important mediators of estrogen-stimulated MCF-7 cells because their upregulation by estrogen contributed to the overall growth increase of the cells. The addition of neutralising antibodies against IGF and TGFα abolished the growth effects of estrogen in MCF-7.

Clarke *et al.* (1994) reported a stimulatory growth effect of estrogen on normal human breast tissue xenografts. They found an increase in IGF-1 receptor mRNA and a paradoxical increase in TGFβ protein from stromal, not epithelial, cells.

Estrogen upregulates plasminogen activator and other proteases (Dickson *et al.*, 1986). It also increases exposure of laminin receptors on the cell surface of MCF-7 which could increase migratory capability (Dickson and Lippman, 1986). Cathepsin D, an enzyme, and an unknown protein pS2, are also induced by estrogen and the latter is used as a clinical marker of good prognosis (Thompson *et al.*, 1993).

More recently, it has been found that BRCA1 and BRCA2 mRNA levels are elevated in MCF-7 and another breast cell line, BT483, in response to estrogen (Spillman and Bowcock, 1996). The upregulation was abrogated by anti-estrogens which suggests action through the ER.

1.10 ANTI-ESTROGENS AND THEIR ROLE IN BREAST CANCER

Anti-estrogens are so called for their ability to counteract the actions of estrogens. They can be steroidal or non-steroidal, natural or synthetic. Exactly how anti-estrogens work is not fully understood. It is known that they competitively bind to the ER and block the signalling response, often mimicking the structure of estrogen. In this way, they prevent the growth of hormone-dependent tumours by abolishing the normal mitogenic effects of estrogen.

i. Mechanism of Anti-estrogen Action

The way in which anti-estrogens work seems to be a complex mix of direct estrogen antagonism via the ER coupled with a range of indirect effects such as enzyme inhibition and growth factor regulation. Several of the secondary effects are clinically important because of their potential to stem the growth of breast cancer cells. Estrogens have many physiological functions but anti-estrogens do not specifically inhibit any one of these functions. Most anti-estrogens are antagonists of estrogen action but some display agonist activity in certain tissues, for example, in the uterus, and possibly in the liver, where tamoxifen acts as a mitogenic estrogen (Jordon and Morrow, 1994).

There are 2 ways in which anti-estrogens, bound to the ER, can inhibit estrogen. The first appears to be true of pure anti-estrogens which bind to the ER, resulting in a failure of the receptor complex to recognise estrogen response elements in the DNA. Non-steroidal anti-estrogens, such as tamoxifen, allow the ER to bind to the DNA but transcription cannot be initiated due to a change in ligand-receptor conformation, which will not be interactive with other transcription factors. Inhibition of tumour growth is proportional to the strength of binding between anti-estrogen and the ER (Coezy *et al.*, 1982).

It became clear that anti-estrogen binding to ER could not account for all the subsequent actions observed. Sutherland *et al.* (1980) discovered an anti-estrogen binding site, distinct from the ER, after finding that, in the cytosols they tested, the number of ER sites was insufficient for the amount of anti-estrogenic

binding and that estrogen could not reverse the effect. There was no evidence, however, that this novel binding site could mediate anti-estrogenic events, so the authors suggested that the site may simply be regulating the amounts of anti-estrogen presented to the ER. The characterisation of this novel binding site has been abandoned recently due to an inability to purify the protein (Colletta *et al.*, 1994).

Because fibroblasts from breast cancer patients often revert to a fetal phenotype (Haggie *et al.*, 1987), Colletta *et al.* (1990) studied the effect of tamoxifen on ER-negative fetal fibroblasts. Biologically active TGFβ1 was produced by these cells in response to tamoxifen, showing that tamoxifen can elicit anti-estrogenic effects independently of the ER signalling pathway. The existence of a novel binding site and non-ER mediated events may also explain why 10-15% of ER-negative tumours respond favourably to endocrine therapy (Jaiyesimi *et al.*, 1995). This is additional evidence that the non-ER binding site plays an active role in anti-estrogenic signalling.

ii. Anti-estrogen Therapy for Breast Cancer

Anti-estrogen therapies for breast cancer have been alluded to for over a century. Surgical and radiotherapeutic ovariectomy has been used to reduce endogenous estrogen levels. In the 1940s, the synthetic estrogen, diethylstilbestrol (DES) was used as breast cancer treatment, although at the time it was not known why the growth of breast lesions was reduced. It is now known that high doses of DES downregulate the ER and desensitise the cancer cells to the stimulatory effects of endogenous estrogen. In 1958, non-steroidal triphenylethylenes were under investigation for their ability to inhibit the effect of estrogen in the rat uterus. These earlier compounds inhibited breast cancer cell growth but were too toxic for clinical use. Tamoxifen, a later triphenylethylene, is well tolerated in the body and is effective against breast cancer cell growth in approximately 30% of patients (Wolf and Fuqua, 1995).

1.10.1 Tamoxifen

i. History

Tamoxifen, trade name 'Nolvadex', from Zeneca Pharmaceuticals is one such non-steroidal, triphenylethylene anti-estrogen, first synthesised and developed in the United Kingdom in 1966 (Harper, 1966). It was found to be an anti-uterotrophic factor with anti-implantation activity in rats, so was developed as a post-coital contraceptive (Harper and Walpole, 1967). However, further research demonstrated that, in fact, tamoxifen induced ovulation in sub-fertile women (Klopper, 1971). Cole *et al.* (1971) proposed tamoxifen as an efficient treatment for post-menopausal, advanced metastatic breast cancer and further studies established that it did inhibit the growth of rat mammary carcinomas (Nicholson, 1975). Following tests and trials in UK and USA, tamoxifen was approved for administration to post-menopausal patients in 1977 as an adjuvant treatment for metastatic breast cancer.

Tamoxifen has now become the most widely prescribed drug for both metastatic and early stage breast cancers. Studies are underway to determine the clinical usefulness of tamoxifen as a chemo-preventive drug in breast cancer (Harris *et al.*, 1992) and also, with its metabolites, as a useful agent against other types of cancer, such as liver carcinoma (Simonetti *et al.*, 1997) and pancreatic cancer (Mikulski, 1996) and non-malignant diseases such as coronary heart disease, osteoporosis and autoimmune disorders (Grainger and Metcalfe, 1996). The structure of tamoxifen is shown in Figure 1.18. Structurally, tamoxifen bears little resemblance to estrogen.

It is clear that the mechanism of anti-estrogens is a complex balance between direct inhibition at the level of the ER and secondary effects such as enzyme modulation and growth factor regulation (Colletta *et al.*, 1994).

ii. Tamoxifen Research *in vitro*

Coezy *et al.* (1982) demonstrated in MCF-7 cells that the binding of tamoxifen and estrogen to the ER was competitive and mutually exclusive. The ability of tamoxifen to inhibit cell proliferation is correlated with its affinity for the ER. 4-hydroxytamoxifen is a potent metabolite of tamoxifen, detectable in breast

cells *in vivo*. 4-OH-tamoxifen has an ER binding affinity 100x that of tamoxifen and its competitive efficiency is equal to that of estrogen (300x that of tamoxifen). Both compounds are cytotoxic to MCF-7 cells at concentrations of 10μM and above.

The effect of tamoxifen on the cell cycle was investigated by Taylor *et al.* (1983) using synchronous MCF-7 cells. They found a dose-dependent reduction in the number of cells entering S-phase and a decrease of cells in G2, M and S-phases of the cell cycle. A 2-4 hour interval during mid G0 was identified as the tamoxifen-sensitive period. The experiments showed that tamoxifen is a phase specific, growth inhibitory and cytotoxic (>10μM) agent acting at a precise time in G0. Osborne *et al.* (1983) concurred with Taylor *et al.* (1983) with 65% of their MCF-7 cells accumulating in G1. The build-up of cells in G1 took 2-3 doublings to achieve. This led to cytostatic changes, showing that tamoxifen, at pharmacological concentrations, is not fatal to cells in short term culture. Between 5% and 10% of the cells continued to divide, suggesting that they were tamoxifen resistant.

Reddel *et al.* (1985) investigated the sensitivities of ER-positive and ER-negative breast cancer cells to tamoxifen. From 5 ER-positive and 3 ER-negative cell lines, they found that the ER-positive cells had a significantly higher concentration of anti-estrogen binding site and a 4 - 75 fold higher sensitivity to tamoxifen compared to the ER-negative cells. Cell cycle kinetic differences were also found. ER-positive cells accumulated in G1 in a dose-dependent way, concurring with Taylor *et al.* (1983) and Osborne *et al.* (1983). The ER-negative cells showed an increase in the population entering S-phase, M-phase and G2 with a corresponding decrease in G0-G1 cells. ER-positive cells could be rescued by estradiol but this did not occur in ER-negative cells.

Tamoxifen was cytotoxic to MCF-7 cells in a study by Chen *et al.* (1996). Apoptosis, after culture with tamoxifen, was observed both morphologically and biochemically in the cells. TGFβ1 mRNA was elevated in the treated cells, indicating the mediation of cell death by TGFβ.

iii. Other Biological Effects of Tamoxifen

The lack of effective estrogen, or the presence of anti-estrogens, in a system, has many effects. Estrogen cannot reverse some anti-estrogen effects, suggesting that anti-estrogens can exert their cytostatic effects through estrogen-independent mechanisms. In certain cells and tissues, anti-estrogens act as estrogen agonists rather than antagonists.

Guvakova and Surmaez (1997) showed in MCF-7 that the cytostatic effect of tamoxifen is controlled partly by modulation of the IGF-1 receptor and its signalling pathway by reducing the level of both tyrosine phosphorylation and phosphatidylinositol 3'-kinase signalling. Breast cancer cells are normally regulated by activated IGF-1 receptors and the levels of this receptor are higher in tumour tissue than in normal or benign counterparts.

Tamoxifen antagonises the action of calmodulin, an intracellular calcium receptor which activates cAMP-phosphodiesterase during cell proliferation regulation (Rowlands *et al.*, 1990). The action cannot be reversed by estrogen and Gulino *et al.* (1986) suggested that the interaction of tamoxifen with calmodulin may feature in mediating estrogen-independent inhibition of breast cancer growth.

Protein kinase C is a calcium- and phospholipid-dependent protein kinase which binds to tumour-promoting phorbol esters and can be induced by tumour promoters, suggesting that it is important in tumour promotion. Tamoxifen inhibits the activity of protein kinase C, resulting in a reduction of growth promoting signal transduction; another example of ER-independent cytostasis (O'Brian *et al.*, 1985).

Gagliardi and Collins (1993) investigated several anti-estrogens and their effect on angiogenesis using the chick chorioallantoic membrane (CAM) culture system. Tamoxifen exerted the strongest inhibitory effect with a 73% reduction in blood vessel formation. Estrogen had no stimulatory effect on angiogenesis in this system, so tamoxifen is again working through an ER-independent pathway. Protein kinase C has a role in angiogenesis, so its inhibition by tamoxifen may contribute further to tumour growth arrest.

Bracke *et al.* (1994) used MCF-7/6 cells (subclone of MCF-7) to test another activity of tamoxifen. These cells have an invasive phenotype *in vitro* and *in vivo* but display no detectable E-cadherin adhesion sites. Tamoxifen and its metabolites caused a restoration of E-cadherin and increased the aggregation

ability of the cells. As a result, the invasive phenotype was suppressed and the authors suggested a clinical benefit of E-cadherin in breast cancer cells.

Kida *et al.* (1989) identified what they described as an EGF-like protein from MCF-7 cells which was encoded by the pS2 gene. Translation and transcription of the pS2 gene was directly induced by physiological concentrations of estrogen but its synthesis does not affect cell growth. Thompson *et al.* (1993) describe pS2 as a independent marker of good breast cancer prognosis because it reflects tumour differentiation rather than growth.

iv. Effects of Tamoxifen on TGFβ

The effects of estrogens and anti-estrogens can be evaluated *in vitro*. The relationship between tamoxifen and TGFβ was noted in 1986 by Dickson and Lippman. Many researchers have since investigated the pattern of TGFβ expression in response to anti-estrogens. However, studies have resulted in varied findings. The majority of research has been carried out in breast cancer cell lines with a few retrospective studies on tamoxifen-treated breast tissue.

Knabbe *et al.* (1987) demonstrated that tamoxifen induces the expression of biologically active TGFβ from MCF-7 cells *in vitro*. The increase of TGFβ correlated with an decrease in cell growth, leading the authors to conclude that TGFβ is a hormonally regulated negative growth factor in breast cancer cells. MCF-7 cells were responding to tamoxifen through the classic ER pathway because the effect was abrogated by the addition of estradiol.

Colletta *et al.* (1990) achieved the first demonstration of biologically-active TGFβ induction by anti-estrogens in the absence of ER. They showed, by firstly negating the TGFβ present in the medium and serum with anti-TGFβ antibodies, that fetal fibroblasts, which have no detectable ER, were producing TGFβ *de novo*. The fetal fibroblasts were specifically producing TGFβ1 and not TGFβ2. The effect was not completely abolished by the addition of estradiol showing that fetal fibroblasts do not react to tamoxifen in the way that ER-positive cells do. Two mechanisms of action must exist to cause the upregulation of TGFβ. Colletta and co-workers proposed a novel binding site for tamoxifen, perhaps an 'orphan receptor' which has sequence homology to the ER. There is evidence from Haggie *et al.* (1988) that human breast cancer fibroblasts revert to a fetal-like phenotype

which reinforces the concept that stromal cells in breast tumours are capable of producing TGFβ. This does not imply that epithelial cells do not produce TGFβ but that under different circumstances, the different cell types can produce it through different mechanisms, with or without ER. No increase in TGFβ mRNA was detected after tamoxifen treatment in the fetal fibroblasts so the control is likely to be post-transcriptional. Induction in MCF-7 cells is also thought to be post-transcriptional but because that mechanism involves the ER, there must be 2 pathways of control. Wakefield *et al.* (1990) also found that tamoxifen increases the protein rather than increasing TGFβ mRNA. They suggest that tamoxifen exerts its effect post-transcriptionally by facilitating the translation of the message into protein.

Fresh fixed tissue was used by Butta and her colleagues (1992) to establish the pattern of TGFβ staining in breast tumours from patients before and after tamoxifen treatment. Their results were that tamoxifen increased the production of TGFβ1 in both ER-positive and ER-negative tumours, but to a greater degree in the ER-positive tumours. The staining was predominantly in and around the stromal cells of the tumours with a weak and unchanged stain in the epithelial cells before and after treatment. They concluded that TGFβ was influenced by tamoxifen and that, although the ER had an additional effect, it was not necessary for tamoxifen to elicit its effects. That Butta *et al.* (1992) found stromal cells to be the probable site of TGFβ1 production conflicts with the findings of Knabbe *et al.* (1987) but is in agreement with Colletta *et al.* (1990). Butta suggested a negative paracrine effect on the epithelial cells by the stromal cells. More evidence for the production of TGFβ from stromal cells comes from Van Roozendaal *et al.* (1995). They cultured breast cancer fibroblasts and used the resultant conditioned medium to inhibit the growth of a reporter cell line. The fibroblasts produced biologically-latent TGFβ, which became active upon heat treatment. On addition of hydroxy-tamoxifen into the culture, 37.5% of the tumour-derived fibroblast cultures showed a significant increase in latent TGFβ production. The authors conclude their study by saying that the lack of paracrine signals from epithelial cells and/or extracellular matrix may have resulted in the secretion of latent TGFβ rather than the active form.

The plasma levels of TGFβ2 were measured in patients with metastatic breast cancer before and during tamoxifen treatment and correlated them with

clinical outcome (Kopp *et al.*, 1995). In patients who responded to tamoxifen, TGFβ2 levels increased in weeks 2-6 of treatment then decreased towards the end of the trial. Of the 7 patients who did not respond to tamoxifen, only 4 patients showed a late increase in TGFβ2 and the others showed none. The authors suggested that an early increase in TGFβ2 could be used as a predictive marker for positive response to tamoxifen.

Tamoxifen is advantageous for estrogen-responsive and mixed estrogen-response tumours as TGFβ can inhibit the ER-positive cells in an autocrine way and the ER-negative cells in a paracrine way. It has been suggested, therefore, that the tumour-suppressing abilities of this drug are mediated through the TGFβ pathway (Serra and Moses, 1996). This is confirmed by ER-negative tumour patient trials. The patients receive some benefit from this treatment, possibly through a paracrine stimulation of TGFβ from stromal cells, demonstrated *in vitro* by Colletta *et al.* (1990) and Van Roozendaal *et al.* (1995). It has been suggested that tamoxifen induces the stromal cells to produce TGFβ *in vivo* which will act in a negative paracrine fashion on the epithelial tumour cells (Baum *et al.*, 1989).

Thompson *et al.* (1991) studied tamoxifen-induced TGFβ1 production in 2 ways. Estrogen-dependent MCF-7 xenograft tumours in mice were treated with tamoxifen. The tumours regressed under the influence of tamoxifen and this was positively correlated to an increased and sustained production of TGFβ1. The same principle was tested *in vivo*, using biopsy material from 11 post-menopausal patients who had undergone surgery because of tamoxifen failure. Of these 11 biopsies, 7 tumours expressed high TGFβ1 mRNA, were ER-negative (6 of the tumours progressed and 1 remained static). The other 4 biopsies were ER-positive, but expressed low TGFβ1 and remained static. There was a positive correlation between TGFβ1 expression and pre-menopausal status but no relationship between tumour size, ER status or stage of tumour. The authors concluded that, since 6 tumours progressed despite a high expression of TGFβ1, a breakdown in either the TGFβ1 autocrine or paracrine pathways had occurred. If the stimulatory paracrine effects of TGFβ1, for example angiogenesis and immunosuppression, are not balanced by an autocrine decrease in growth, the net result is that tamoxifen produces an environment which is conducive to tumour cell growth (Thompson *et al.*, 1991). Gorsch *et al.* (1992) also warn of the association between TGFβ1 and disease progression.

The straightforward induction of TGFβ1 by tamoxifen, seen so often *in vitro* with cell lines (Knabbe *et al.*, 1987; Colletta *et al.*, 1990), is not likely to be so simple *in vivo*.

v. Clinical Side Effects of Tamoxifen

Advantageous Effects: The most significant extra benefit of tamoxifen for breast cancer patients is the prevention of the disease recurring in the opposite breast (Stewart, 1991).

Women who take tamoxifen share many of the beneficial effects of menopausal estrogen replacement therapy (HRT). For example, post-menopausal women experience a lowering of total plasma cholesterol while taking tamoxifen and a slowing of bone loss (osteoporosis). There are also effects on lipid metabolism in the liver. Tamoxifen increases high-density lipoproteins (HDL) and very low density lipoproteins (VLDL) while decreasing low density lipoproteins (LDP). This is advantageous because an increase in the ratio of HDL to LDL is considered to lower the risk of cardiovascular disease (Wolf and Fuqua, 1995).

Disadvantageous Effects: There is a small increase in the number of blood clots in women taking tamoxifen, particularly in women who are receiving chemotherapy as well. Several clinical trials show that women taking tamoxifen as a treatment for breast cancer face a risk of endometrial cancer about two to three times higher than the risk for women in the general population. The risk of uterine cancer in women taking tamoxifen is in the same range as the risk in post-menopausal women who are taking HRT. Tamoxifen has also been considered as treatment for benign breast disease, for example cyclical mastalgia, but the risk of cataracts and hepatic carcinoma (seen in animal models at high dosages) are considered too high for the treatment of non-malignant disease (Fentiman and Powles, 1987), i.e. the disadvantages outweigh the advantages, which is not the case for patients with more advanced breast cancer.

There is a risk of drug resistance. Most breast tumours are of mixed population with varying sensitivity to anti-estrogens so while ER-positive cells will stop growing, ER-negative cells will thrive and overtake the ER-positive population. It is also possible that the tumour cells have the capability to adapt to an estrogen-depleted environment (Miller, 1991).

1.11 DEFENCES AGAINST CANCER

1.11.1 Natural Responses to Cancer

Before considering the medical therapies for breast cancer, the natural reaction to 'foreign' tumours must be considered. It was surmised by Paul Ehrlich early this century that tumours would overwhelm us were it not for our immune systems keeping aberrant cells in check. This notion was the precursor for Burnet and Thomas' theory of immune surveillance (Underwood, 1996), which stated that the immune system was continually seeking out abnormal cells and destroying them. Tumour cells, therefore, should be recognised as abnormal then destroyed by immune cells.

Evidence to support this theory has been presented and reviewed by Roitt *et al.*, 1996. Most tumours display a lymphoid cell infiltrate, demonstrating that the tumour has elicited an immune response and that the lymphoid cells have made some attempt at destroying the tumour cells. Some established tumours can spontaneously regress, for example, melanomas (Menzies and McCarthy, 1997). Further confirmation came from the observation that tumours are more likely to occur when the immune system is compromised, most often observed in neonatal stages, old age and in immunosuppressed patients. For example, one third of individuals suffering from AIDS develop a rare skin cancer called Kaposi's sarcoma, indicating that tumours are more common under immune deficiency conditions. Most of the cancers initiated under these circumstances are virally-induced, while tumorigenic viruses in breast cancer have only been reported convincingly in animal models.

Tumours can be detected by the immune system as being non-self because they display tumour-associated antigens (TAA). TAAs are proteins which can be found in normal adult cells but under different conditions (time, differentiation state, quantity) to tumour cells. They are also expressed in embryonic development, a period of rapid cell division - a feature common to tumour progression but not generally observed in adult cell proliferation. Examples of these oncofetal antigens are α-fetoprotein (AFP), which is produced by liver cancer cells, and carcinoembryonic antigen (CEA), produced by epithelial and colon cancers. Tumour-specific antigens (TSA) are exclusively expressed preferentially

on tumour cells. Some TSAs are common to most tumours and a few are specific to particular tumours, or even to a particular patient.

For a tumour to overcome the efforts of the immune system, some cells must escape detection. This is achieved either by being poorly immunogenic (not displaying tumour antigens), through loss of MHC molecules which are required for presenting antigens, by secreting factors, e.g. TGFβ, to cause localised immunosuppression or by lack of lymphocytic adhesion molecules.

Immunotherapy as a treatment for cancer has been investigated. For example, the BCG bacterium has been injected into axillary lymph nodes of breast cancer patients to encourage a strong immune response which would non-specifically destroy cancer cells, but this has produced poor results.

Current research includes the development of vaccines against individual tumours and antibodies against tumour-specific antigens.

MUC-1 is an example of a tumour specific antigen. It is a cell surface mucin which is normally so heavily glycosylated that its antigenic sites are hidden in the core of the molecule. In resting cells, it is thought to be involved in cell adhesion. In proliferating cells, for example breast epithelial cancer cells, the glycosylation process is incomplete, allowing the antigenic protein core to be exposed (Houghton and Lloyd, 1998). In mice, antibodies against MUC-1 generate the desired cytotoxic T-cell response but the same antibodies in humans produce an antibody response (Apostolopoulos *et al.*, 1998). It is these species differences and possible cross-reaction of vaccines with other antigens that limit the use and success of tumour-specific antigens at present.

1.11.2 Clinical Therapies against Breast Cancer

Options for breast cancer treatment depend very much on the pathological stage of the cancer. For instance, a localised early stage tumour can be removed surgically without need of further treatment and in most cases there is no recurrence. With larger, less well defined tumours (especially if there are nodal metastases), drug or radiation treatment may be required after surgery to ensure that *all* tumour cells are eradicated.

Adjuvant therapy is 'systemic therapy, given at the time of primary local treatment in the absence of demonstrated metastases' (Harris *et al.*, 1992). Several

forms of adjuvant therapy exist, namely radiotherapy, chemotherapy and hormone therapy.

i. Surgery

Many breast cancer patients will be offered surgery at some point during their treatment, unless the cancer is too widespread. Breast tumours are excised, often with the axillary lymph nodes which are usually the first site of metastatic spread. Mastectomy is the removal of the entire breast and, occasionally, this operation can also include the removal of the chest muscles. Surgery is frequently followed by an adjuvant course of radiotherapy, chemotherapy or hormone therapy. Of the patients offered surgery for breast cancer, around 50% of them will be free of disease. The success of breast cancer surgery depends on many factors - stage and grade of tumour, hormone receptor status and pathological appearance. Early tumours, often those detected during screening programmes, have a better chance of clean excision and of long-term disease-free survival. Surgical cure is not possible if the primary tumour has metastasised to other parts of the body so systemic drug therapy is offered.

ii. Radiotherapy

High energy X-rays or Cobalt 60 gamma rays are the sources of radiation in this treatment and their aim is to kill cancer cells and shrink any tumour tissue. The radiation can be administered externally using a machine to direct the rays medially and laterally at the breast or internally by putting radioisotopes inside the body. Fast dividing cells, like cancer cells, are sensitive to radiation therapy so the treatment is relatively specific. Radiation can also be used prior to surgery with the hope that the tumour will be of operable size afterwards.

iii. Chemotherapy

Chemotherapy is commonly a multi-drug regime of DNA alkylators and DNA synthesis antagonists, used to combat local and systemic disease. These cytotoxic drugs are used to stop the division of cancer cells by disrupting cell cycles. Taken orally or intravenously, the drugs have a non-discriminating,

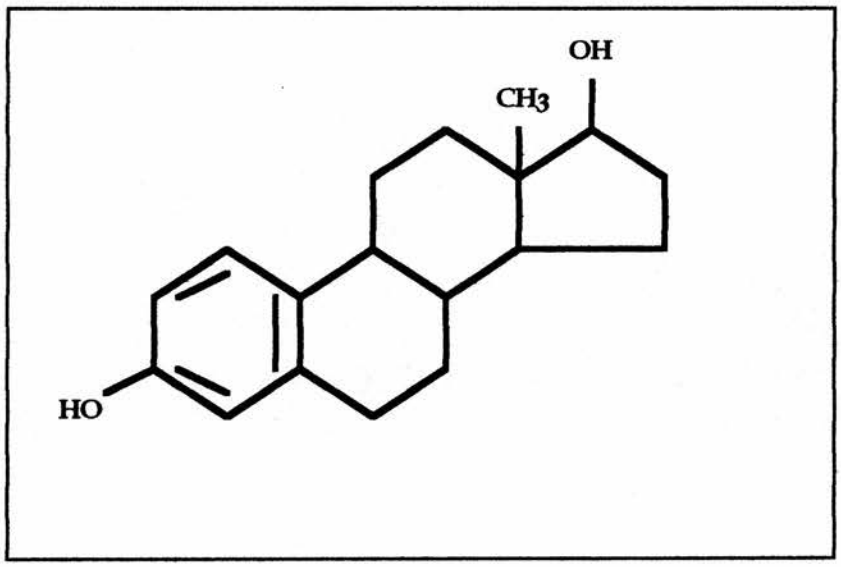


Figure 1.17 Structure of Estradiol

systemic effect on all quickly dividing cells resulting in common side-effects such as bone marrow suppression, hair loss and nausea. In the case of breast cancer, chemotherapy is the preferential adjuvant treatment for poor prognosis cancer in pre-menopausal women and is administered soon after surgery (Stewart, 1991). Drug resistance to chemotherapy can occur with prolonged exposure. Resistant cells display altered membrane permeability, increased efflux (Forrest *et al.*, 1991), decreased drug uptake, increased drug inactivation, increased DNA repair and alteration of expression of drug targets (Kobayashi *et al.*, 1993). However, it is not clear if these *in vitro* observations are as prevalent in tumours *in vivo*.

iv. Hormone Therapy

Hormone therapy is offered, particularly, when the parent cell type of the tumour is hormone responsive, for example breast carcinomas, endometrial cancers and prostate tumours. Specific tissue types can be targeted with their particular hormone/anti-hormone. Breast cancers are commonly treated with an anti-estrogen, tamoxifen, which binds to estrogen receptors, blocking the growth stimulating action of the real hormone.

Patients taking anti-estrogens, with or without prior surgery, experience a longer disease-free survival period before recurrence and a decreased chance of a second primary tumour developing. However, of the 30% of breast tumours which regress under anti-estrogenic therapy, only a temporary regression is experienced. Hormone-independent tumours usually replace the regressed tumour and metastatic disease follows (Darbre, 1990).

v. Chemotherapy versus Hormone Therapy

Deciding on the type of adjuvant treatment requires consideration of menopausal status, nodal status and estrogen receptor status. CMF is the most widely used chemotherapy drug cocktail in breast cancer management. It contains cyclophosphamide, methotrexate and 5- fluorouracil. The choices are by no means clean cut as in several situations, e.g. in patients who are pre-menopausal, node negative and ER negative, it has not been proved conclusive that the treatment, indicated in Table 1.2, is the better option.

vi. Therapies under Trial

Goserelin is an analogue of Luteinising Hormone Releasing Hormone (LHRH) which causes medical castration inhibiting LH and FSH and, in turn, lowering the level of circulating estrogen. This drug provides an alternative to ovarian ablation. Blamey *et al.* (1992) tested goserelin on pre- and peri-menopausal women with confirmed breast cancer. The results showed that over 36% of patients had a favourable response to goserelin which closely compares with surgical ovariectomy and tamoxifen treatments. Taylor *et al.* (1998) confirmed this result, finding that goserelin treatment was as effective as surgical ovariectomy. Jonat *et al.* (1995) and Buzzoni *et al.* (1995) tested goserelin with and without tamoxifen. In the former group, 38% of participants had a positive response after goserelin and tamoxifen treatment compared to 31% in the single treatment group while the second research group reported that 41% of their patients had a beneficial response to the dual treatment. Advantages of goserelin are its easy administration (once monthly injections) and its high tolerability in most patients. However, it is only effective in pre- or peri-menopausal women.

Aromatase inhibitors act to inhibit the conversion of testosterone and estrone to estradiol by aromatase. Clinical trials have concentrated on post-menopausal patients with advanced breast cancer. In volunteer trials, anastrozole (Arimidex), a potent inhibitor of aromatase, serum estradiol levels were reduced by 80% (Yates *et al.*, 1996). Arimidex is well tolerated and in a trial by Jonat *et al.* (1996), 34% of patients benefitted from its effects. Aromatase inhibition is self-limiting, reversible and specific (Miller, 1991).

1.11.3 Advance Knowledge of Breast Cancer

i. Screening

The most consistent way to detect early cancers is by regular mammographic screening. Women are entered into screening programmes at an age when they are statistically most at risk of developing breast cancer. At present, mammography is the most beneficial screening method and in the UK, is offered to women between the ages of 50 and 64, i.e. generally peri- and post-menopausal women. Women in this range are more likely to develop a tumour

and because the breast tissue is radiologically more uniform after menopause, early tumours are more likely to be detected. In an Edinburgh-based trial, the benefit to screened women, after a 10 year follow-up, was a reduction in mortality of 20%, a 3% increase since the 7 year follow-up (Alexander *et al.*, 1994). In a Swedish two-county trial, the mortality of screened patients, compared to non-screened women, started to decrease after 5 years and continued to decrease throughout the 11 years of follow-up (Duffy and Tabar, 1995).

There is no doubt in researchers' minds that screening women, over the age of 50 years, for breast cancer saves lives but great debate exists over the benefit of screening younger women (Taubes, 1997). Mammography is of limited use for younger women as the breast tissue is more dense and very changeable during the menstrual cycle. Tumours are less likely to be detected in this group of women because the natural 'lumpiness' of the pre-menopausal breast tends to mask some abnormalities. Meta-analyses were performed on the results of 7 mammography trials which screened women in their forties. The overall result was a 23% benefit to younger women called for screening compared to the non-screened counterparts (Smart *et al.*, 1995).

Advice to all women is to be 'breast aware' and report any unusual changes as soon as possible because around 90% of lumps are detected by women themselves. The disadvantages of screening are the high rate of false positive results, which is estimated to be 5-11% in younger women (Taubes, 1997), and the anxiety to patients who are recalled for unnecessary testing.

ii. Prophylactic Surgery

Prophylactic bilateral mastectomy is a drastic surgical option, sometimes taken by those with a strong family history of breast cancer and who may even have tested positive for mutations in the BRCA genes as part of research trials. There are several levels of mastectomy, with different amounts of tissue being cleared from the breast and chest wall. A study by Hartmann *et al.* (1999) showed a 90% reduction in breast cancer diagnosis for high risk relatives who had opted for prophylactic mastectomy. The problem with partial mastectomy is that breast tissue is left behind, enough to be subject to carcinogenic effects.

Ovariectomy is sometimes coupled with mastectomy in order to decrease circulating estrogen levels. In the 1930's, Taylor introduced radiation-induced menopause, i.e. ablation of ovarian function, and showed an improvement of conventional local therapy when used in conjunction with loss of ovarian function. Controlled trials of this treatment in 1948, in the Christie Hospital in Manchester, confirmed the results.

iii. Chemoprevention

Drugs are being developed and used in trials to test their efficacy of preventing tumourigenesis. There are 2 possibilities so far - tamoxifen and retinoids.

Tamoxifen: As well as being the adjuvant drug choice for treating breast cancer, tamoxifen is being tested in trials for its ability to prevent breast cancer ever occurring in high risk women. It is proposed that, since proliferation of breast epithelium is driven by sex hormones, anti-estrogenic drugs could reduce the incidence of breast cancer. A trial conducted in the USA has recently been stopped because a clinically significant benefit of tamoxifen was emerging. It was then deemed unethical to subject the control group to a placebo. Trials in the UK and Italy, with different parameters to the American trial, have not revealed any advantage of tamoxifen against the development of breast cancer.

Retinoids: 'Retinoids' is the term used to describe Vitamin A and its isomers, derivatives and analogues. It has been demonstrated that retinoids are potent inhibitors of epithelial carcinogenesis and so are candidates for preventing and treating cancerous epithelia (Kurie *et al.*, 1996). Cell proliferation can be controlled *in vitro* by retinoids and animal models have shown a decrease in tumour incidence if treated with retinoids (Harris *et al.*, 1992). Toma *et al.* (1997) examined the effect of all-trans retinoic acid and 13-trans retinoic acid on MCF-7, ZR-75-1 (ER-positive) and MDA-MB-231 (ER-negative) breast cell lines. Both retinoic acids exerted a dose-dependent anti-proliferative effect, more so in the ER-positive cell lines.

All-trans-retinoic acid induces TGFβ2 production in a wide variety of rat epithelia (Butta *et al.*, 1992). Further studies in rats have demonstrated the value of N-4-hydroxyphenyl-retinamide and tamoxifen interacting synergistically in the

prevention of breast cancer. The main problem with administering retinoids is their long-term effects. They are stored in the liver and eventually cause hepatic damage. Less toxic analogues have been synthesised and are undergoing toxicity tests (Costa *et al.*, 1994).

1.11.4 TGFβ as a Potential Therapy for Cancer

Normal epithelial cells are capable of accurate growth control. The controls must break down, or be by-passed, to allow the progression of cancer. TGFβ is known to be an endogenous inhibitor of growth, and since most epithelial cancers retain their sensitivity for this growth factor until late in cancer progression, it is possible that TGFβ could be used in some therapeutic way (Colletta, 1990).

Systemic use would be impractical because bioactive TGFβ has a half life of only 2.5 minutes (O'Connor-McCourt and Wakefield, 1987) and its many functions could adversely affect other body systems. It would be advantageous to increase local production of the growth factor as it is well known that both epithelial cells and stromal cells are capable of producing it. Because of TGFβ's short half-life, local production is likely to be local action (Wakefield *et al.*, 1990).

The only option would be to increase local production of endogenous TGFβ in an effort to curb cell growth. This is one of the theories behind tamoxifen and other therapies, but as discussed above, it is unclear which cells should be therapeutically targetted.

1.12 AIMS OF RESEARCH

The aim of the present research was to develop a culture system, such that the anatomical and functional relationship between breast cancer epithelial cells and stromal cells would be preserved over several days *in vitro*. The usefulness of that system would be tested by addressing one specific issue, namely, the way in which TGFβ1 affects the growth of human breast cancer tissue under the influence of estrogen and the anti-estrogen, tamoxifen.

There is no doubt that, in ER-positive MCF-7 cells, tamoxifen upregulates the production of TGFβ1 and that, subsequently, TGFβ1 acts as a negative autocrine regulator of MCF-7 cell growth. This has been demonstrated in liquid media (Knabbe *et al.*, 1987) and in soft agar clonogenic assays (Manni *et al.*, 1991). The hormonally regulated growth factor expression in MCF-7 cells works directly through the ER. In ER-negative breast cancer cell lines, tamoxifen can still induce growth inhibition but to a lesser extent than is observed in ER-positive cell lines. The ER-positive cells could be 'rescued' from tamoxifen by the addition of estrogen but the ER-negative cells could not, suggesting that the growth inhibitory effects of tamoxifen in ER-negative cells are not mediated through the ER (Reddel *et al.*, 1985). *in vivo*, where most tumours are of mixed ER status, it is unclear how estrogens and anti-estrogens are working.

It has also been convincingly demonstrated that, in some primary cultures of breast fibroblasts (which are ER-negative), the production of TGFβ can be induced in response to tamoxifen (Van Roozendaal *et al.*, 1995). This has lead to a partial explanation of why a proportion of ER-negative tumours respond to tamoxifen therapy. Tamoxifen must exert its growth inhibiting effects by inducing the production of negative growth factors from the stromal cells, which then acts in a paracrine fashion on the epithelial cells. There is no satisfactory explanation of how the anti-estrogen is acting in the absence of detectable ER. It may be that a low level of ER, undetectable by conventional methods, is sufficient to cause the growth inhibiting effect, or, as Sutherland *et al.* (1980) suggested, the, as yet undefined, non-ER, anti-estrogen binding site could be involved. It is clear that tamoxifen is capable of both the classical estrogen antagonism, displayed by MCF-7 cells, and growth inhibitory actions through novel pathways.

The majority of studies have been carried out using cell lines, such as MCF-7. Speirs *et al.* (1998) reported that, in the last 30 years, 97% of the published literature had used MCF-7 as a breast cancer model. There are recognised problems in using monolayer culture with a single cell type. For example, only one cell type is represented, which reflects neither the histological nor the biochemical situation *in vivo*. The validity of MCF-7 and other cell lines has been brought into question because, in some strains, the cells no longer resemble the original line.

Retrospective *in vivo* trials have also been employed to investigate the TGFβ hypothesis. Gorsch *et al.* (1992) reported the presence of TGFβ1 protein mainly in

the cytoplasm of the epithelial cells of ductal tumours, especially at the cytoplasmic membrane. McCune *et al.* (1992) found that, in all the infiltrating carcinomas they tested, malignant epithelial cells stained positively for TGFβ1, β2,β3, TGFβ1-LAP while stromal fibroblasts and myoepithelial cells were negative or weakly stained.

In contrast, Butta *et al.* (1992) found an increase in TGFβ expression in the stromal cells of breast biopsy tissue taken from patients before and after tamoxifen treatment. They found that tamoxifen had not altered the expression in the epithelial cells. The result of Butta's study adds to the evidence that tamoxifen may be working through an ER-independent pathway and that stromal cells could be pharmacologically targeted for the production of TGFβ1. The authors also mention the need to confirm *in vitro* studies with comparable *in vivo* trials.

As indicated, there is considerable controversy over the TGFβ hypothesis *in vivo*. Breast tumours are usually of epithelial cell origin and the tumour cells are often of mixed ER status. The interaction between the stromal cells and the tumour cells is impossible to establish from the culture of isolated cells or cell lines, and even *in vivo* studies with fresh fixed tissue cannot trace the cellular interaction over time.

This research aimed to assess the feasibility of maintaining breast tissue in as near to an *in vivo* situation as possible, so that the functional organisation of epithelial and stromal cells could be preserved. The intention, then, was to culture the tissue under the influence of estrogen and tamoxifen. At regular time points, the tissue would be fixed, wax-embedded and assessed for the protein expression of the growth regulator, TGFβ1, by immunohistochemistry.

Immunohistochemical techniques do not provide unequivocal information on the site of TGFβ1 mRNA transcription. *In situ* hybridisation would therefore be attempted, with the aim of establishing the site of gene expression and investigating whether tamoxifen upregulated protein expression only, rather than mRNA expression, as reported by Wakefield *et al.* (1990).

CHAPTER 2

GENERAL TECHNIQUES

2.1 Coating Slides

The breast cancer tissue used in this research was sectioned onto glass slides and had to adhere firmly in preparation for rigorous histological procedures. To facilitate this, ordinary glass microscope slides [BDH, Poole, England] were coated with 3-aminopropyltriethoxysilane (APES) [Sigma, St. Louis, USA].

Glass slides were loaded into slide racks and soaked in a solution of Decon 90 detergent [Decon Laboratories Ltd., Hove, England] before being rinsed thoroughly in tap water with final rinses in distilled water and allowed to air dry. The slides were washed twice in distilled water followed by 2 washes in 96% E ethanol and allowed to dry. In a fume hood, 5 troughs were prepared containing the following reagents:- 100% acetone, 2% APES in acetone, 100% acetone and 2 x distilled water. The slides were washed in each solution for 5 minutes then incubated at 37°C overnight. The next day, they were heated at 60°C for 1 hour. The slides were re-packed into their boxes and stored in dust-free conditions.

2.2 Fixation of Tissue

Breast cancer tissue pieces were taken from culture under sterile conditions and put into glass bottles containing a fresh solution of 4% formol saline which is a 1:10 dilution of 40% formaldehyde [BDH] in PBS. The tissue was fixed for 3 hours at room temperature in freshly prepared formol saline.

2.3 Dehydration of Tissue

The formol saline was pipetted from the bottles. The tissue was dehydrated with increasing concentrations of ethanol (75%, 96%, 2 x 100%) every 30 minutes. The tissue was incubated with a 1:1 solution of 100% ethanol and chloroform [BDH] for 10 minutes because ethanol is immiscible with paraffin wax. The tissue was then incubated in 100% chloroform overnight.

2.4 Wax-embedding of Tissue

The tissue was taken from the 100% chloroform and incubated in molten paraffin wax at 60°C for 2 hours. During this time the tissue was permeated by the wax. The tissue was transferred to a fresh solution of wax in metal embedding

moulds, according to the TissueTek 1 protocol. Plastic cassettes were placed on the moulds so that once the wax had hardened, the embedded tissue was supported by the cassette.

2.5 Sectioning of Tissue

Sectioning was performed on a Spencer microtome [American Optical Company, New York, USA]. Wax sections were cut at a width of 4μm. Wax ribbons with 6-8 sections from each tissue block were floated on the surface of distilled or DEPC-treated water in a 50°C waterbath to allow expansion of the wax. Each ribbon was then manoeuvred onto APES-coated, frosted-end slides with a fine-tipped paintbrush and allowed to air-dry. At least 6 slides were prepared from each tissue block. Freshly cut sections were incubated in a 40°C oven overnight to allow thorough drying. The sections were ready for staining at this stage.

2.6 Haematoxylin and Eosin (H&E) Staining

The sections were dewaxed in xylene for 10 minutes. They were rehydrated through a series of decreasing ethanol concentrations (100%, 96% and 75%) to distilled water. The sections were stained in Ehrlich's Haematoxylin (a basic, nuclear stain) for a predetermined time, usually 6 minutes. After a rinse in distilled water, the sections were 'blued' in tap water - the minerals and ions in tap water which are not present in distilled water turn the reddish stain to a permanent blue colour. Eosin (Yellowish) [BDH] is a pinkish orange stain which stains connective tissue. The sections required 2 minutes in eosin followed by a rinse in distilled water. The sections were dehydrated in ethanols, cleared in xylene then mounted using DPX [BDH] and glass coverslips. The mounted slides were dried overnight before viewing on a Zeiss 'Axioskop' light microscope.

2.7 Agar Plates for Bacterial Cultures

The agar was LB Lennox L Agar [Gibco BRL, Paisley, Scotland] which is composed of Select Peptone 140 (10g/L), Yeast Extract (5g/L), Agar (12g/L) and NaCl (5g/L). In a 1L conical flask, 16g of LB agar was dissolved in 500ml

distilled water. The flask neck was covered with foil and taped. When fully dissolved, the agar was autoclaved then allowed to cool to 50°C in a waterbath. Ampicillin [Gibco BRL] was mixed into the agar to give a final concentration of 50mg/ml. The agar was poured into 100mm diameter Petri dishes [Bibby-Sterilin, Stone, England], just enough to cover the bottom of the dish. The agar plates were cooled then stored at 4°C, upside down to avoid condensation.

2.8 Competence Induction in Bacteria, *E.Coli* dh5α

The Rubidium Chloride technique was chosen for its reliability and high efficiency. The protocol was modified from one recommended by Promega Corporation [Southampton, England].

An overnight culture of bacteria was established from 10μl *E. coli* in 2ml LB broth. The bacteria were grown at 37°C in an orbital incubator on 225rpm. The resultant culture was used to inoculate 250ml LB broth, supplemented with 20mM Magnesium Sulphate and grown to a concentration of 4×10^7 cells/ml which took approximately 3.5 hours. The cells were pelleted by 5 minutes centrifugation (4,500*g*) at 4°C. The cells were gently resuspended in 100ml ice-cold solution 1 (30mM Potassium Acetate [BDH], 10mM Calcium Chloride [BDH], 50mM Manganese Chloride [Sigma], 100mM Rubidium Chloride [Sigma] in 15% glycerol [BDH], pH 5.8). On ice, the cells were incubated in Solution 1 for 5 minutes then pelleted at 4,500*g* for 5 minutes at 4°C. They were gently and thoroughly resuspended in 10ml of Solution 2 (10mM PIPES (Piperazine N,N bis-2, hydroxypropanesulphonic acid [Sigma], 75mM Calcium Chloride [BDH], 10mM Rubidium Chloride [BDH] in 15% glycerol [BDH], pH 6.5). The bacteria were incubated in Solution 2 for 30 minutes on ice then aliquoted and frozen to -70°C immediately.

2.9 Tissue Culture

All tissue culture was performed in a Microflow biological safety cabinet [Airflow, Andover, England] using sterile disposable plastic-ware.

CHAPTER 3

A NOVEL IN VITRO CULTURE SYSTEM FOR FRESH BREAST TISSUE

3.1 Breast Cancer Cell Culture Background

Human breast cancers are notoriously more difficult to culture than their normal counterparts (O'Hare, 1991). This is because the epithelial component of breast tissue is embedded in dense stroma, occluding the cells of interest from the nutrients required to maintain the tissue *in vitro*. Tumours are often of a hard, gritty consistency caused by the stromal reaction (desmoplasia). Cellularity can range from as little as 10% to 80% (O'Hare, 1991).

Cell lines are the main research tool. They offer a sustainable population of homogeneous cells maintained in an environment which is easily manipulated. In 1937, Cameron and Chambers succeeded in transplanting primary carcinomas and observed the 'spilling out' of epithelial cells from the stroma (Lasfargues and Ozzello, 1958). Since then, however, only about 50 true breast cancer cell lines have been created with only about 20 of these considered useful in research (O'Hare, 1991). They have proved difficult to establish because of the heterogenous nature of breast tumour tissue and the large amount of stromal tissue surrounding epithelial structures of the breast. Also, on culturing primary breast tumours, investigators have found that the culture conditions favour only the normal cells and not the tumour cells (Schor *et al.*, 1991). Once a primary culture is established, it is even more difficult to maintain the cells past a few, if any, doublings.

Two methods which have been employed to dissociate epithelial cells from their surrounding stroma are mechanical disruption (shearing, mincing, aspiration) and enzymatic disaggregation, using proteases to digest the extracellular matrix (Visscher and Crissman, 1994). Breast cancer cell lines can also be created by cultivation of metastasised tumour cell suspensions e.g. pleural effusions.

MCF-7 was the first hormone responsive breast cancer cell line. It was established by Soule *et al.* in 1973 (Levenson and Jordon, 1997), and has become the most-studied cell line in breast cancer research. Work on MCF-7 has shown that its growth is promoted by estrogen and inhibited by tamoxifen *in vitro* (Lippman *et al.*, 1976; Darbre *et al.*, 1984; Knabbe *et al.*, 1987). These observations confirmed the potential of hormone therapy in breast cancer treatment. MCF-7 cells have been used in many studies and are still instrumental in the development of commercial products. However, it is likely that many MCF-7 lines will be

contaminated with other cell types and may have lost or gained characteristic features, making the cells different to the original line.

One disadvantage of cell lines is their homogeneity. They lack *in vivo* interactions with different cell types. Also, cell lines are not a true representation of the *in vivo* situation. However, they are a simple and adequate model for initial research.

i. Other Culture Techniques

Simple cell cultures have been modified in many ways. Attempts to culture solid breast cancer tissue have also been made, in the hope of achieving a system which would have the manipulability of cell culture but which would maintain tissue architecture and cellular interactions.

Three-dimensional cell cultures include the use of soft agar to support single breast cancer cells so that they can divide and create colonies of cells in the semi-solid medium (Calvo *et al.*, 1984) and the culture of breast tumours as floating aggregates (Poulsen *et al.*, 1982) so that the effect of hormones with regard to uptake of tritiated thymidine (growth rate) could be measured. Both techniques achieved limited success. *Co-cultures* have been used by Armstrong and Rosenau (1978), who found that human breast cancer cells could be maintained more successfully than organ or monolayer cultures if the cells were co-cultured beside murine embryonic mesenchymal cells. The mesenchymal cells nurtured the weaker breast cells so that when the mesenchymal cells died, the breast cells were still thriving. The authors reported ~80% success with this protocol. *Xenografts* are cells from cell lines or tumour, grown in immunodeficient mice to study the behaviour of the cancer cells. Human breast cancers have proved to be one of the most challenging tumours to grow in this way (Hurst *et al.*, 1993). Attempts, previous to Hurst *et al.* managed only 6.5% and 9% success, respectively. Bailey *et al.* (1980) suggested that their poor success rate (8 tumours from 102 different specimens) was due to one of the following - dense stroma of breast tumours preventing proper diffusion of nutrients, slow growth rate of breast tumours leaving them more susceptible to host attack mechanisms or differing hormone receptor status between tumour and host, i.e. mice not supplying enough hormones to sustain the receptor positive tumours. Beeby *et al.* (1975) used *organ culture* to study the effects of hormones on breast cancer. On removal from the

patient, organ cultures were set up in liquid medium and put on to stainless steel grids in Petri dishes. The tissue was in contact with both the medium and the gas phase of the culture set-up. Hormones were added to the medium. After 24 hours in culture, the tissue was fixed and processed for histological examination. The results showed good morphological preservation after culture. Atwood *et al.* (1995) used organ culture to study the effects of lactogenic hormone withdrawal on involution of the mouse mammary gland. They excised whole mammary glands and cultured them in medium for about 20 days in total. Plaut *et al.* (1993) primed mice with hormones then excised the mammary glands and cultured them in medium supplemented with growth factors and hormones.

ii. Present Research

For this research, the enzymatic disaggregation approach was attempted first but superseded by a novel soft agar culture system. This was developed from a culture system used by Macintyre *et al.* (1988) in which chick embryonic structures were successfully cultured for several days. The soft agar culture system was chosen for this research because it offered 3-dimensional support for the tissue and because drugs could filter through the loose agar molecules, allowing the tissue to be non-invasively manipulated *in vitro*. Agar was chosen rather than matrix molecules because it is inert and would not exert any influence on the cultured tissue. Matrices composed from extracellular matrix proteins, especially collagen, are known to encourage epithelial cell attachment and spreading (O'Hare, 1990).

Manni *et al.* (1991) demonstrated the efficacy of a soft agar system for the culture and hormone manipulation of MCF-7 cells. In addition, they showed that estrogen-stimulated growth of MCF-7 cells was mediated by IGF and TGFα while TGFβ-mediated the growth inhibition by anti-estrogens.

It was important to design a system which could maintain the tissue in as near to the *in vivo* situation as possible because the evidence is that the stromal component of the tissue interacts with the cellular areas, forming barriers, adhesive substrates and relaying signals (Ruoslahti, 1992). Epithelial-stromal interactions are essential for the development of the embryonic mammary gland. Epithelial mammary structures only form if mammary stroma is present. Kratochwil (1969) demonstrated this using combinations of salivary and mammary structures.

Salivary stroma encouraged the formation of salivary structures from mammary epithelial cells and vice versa.

The initial study was undertaken to evaluate the viability of tumour tissue in such a culture system and, if promising, to utilise the culture system for the study of hormones on intact breast tumour tissue. Viability was assessed by measuring morphological apoptosis in sectioned tissue.

The agents to be tested were 17β-estradiol, tamoxifen and a combination of both. Coradini *et al.* (1994) examined the effect of tamoxifen on proliferation in breast cancer cell lines. They found that tamoxifen alone did not exert a clear anti-proliferative effect but did antagonise the effect of estrogen when estradiol was present. Darbre *et al.* (1984) reported the abolition of estradiol's proliferative effect by tamoxifen occurred at a ratio of at least 1:100 (estrogen:tamoxifen).

3.2 Methods

i. Selection and Characterisation of Patients

The tissue used throughout this research was taken from patients attending the Breast Cancer Clinic of Consultant Surgeon, Mr. Paul Preece, in Ninewells Teaching Hospital, Dundee. The tissue was taken randomly and with no prior knowledge of patient history. A record was kept of all biopsies, listing initials of patient, date of birth and patient identifier number, left or right breast tumour and date of excision. A duplicate copy was held at the hospital.

The average age of the breast cancer patients from whom the biopsies were taken was in the 55-57 age group with a range from 34 years of age to 96 years of age. A chart in Appendix 4 shows the frequency of patient ages. There was almost an equal number of right versus left breast biopsies (refer to Appendix 5).

Pathological requirements for diagnosis of the tissue were of the utmost importance and it is with sincere gratitude that any surplus tissue was received for this study.

Appendix 6 demonstrates the usage of the donated tissue. Tissue was stored at -80°C if more than 24 hours had elapsed since excision.

ii. Breast Tissue Collection and Preparation

Biopsies of breast cancers were collected no later than 24 hours after excision from Ninewells Teaching Hospital, Dundee, Tayside. They were handled as aseptically as possible in the operating theatre and adjacent pathology prep area. Tumour tissue was divided by the pathologist so that material for diagnosis was taken first and any surplus tissue was provided for this study. The tissue was stored in DMEM [Gibco BRL] supplemented with 10% FCS [Gibco BRL], 50 IU/ml Penicillin and 50μg/ml Streptomycin [Gibco BRL] and 2μM L-Glutamine [Gibco BRL] at 4°C until collection. They were transported in a pre-cooled ice box and stored at 4°C until use. Before culture, the tumour tissue was cleared of attached fat then washed 3 times in sterile PBS [Unipath Ltd.], to remove traces of FCS and phenol red, both of which are reputed to have weak estrogenic properties (Berthois *et al.*, 1986). The tissue was placed into sterile Petri dishes and kept moist with a drop of warmed, sterile PBS.

The amount of tissue varied from tumour to tumour, depending on the size of the original lesion and on how much tissue the pathologists could spare.

iii. Enzymatic Disaggregation of Breast Tissue

In order to test the contribution of stromal cells to breast epithelial cell growth, primary breast epithelial cells (prepared from enzymatic disaggregation) were to be compared to fresh breast tissue grown as explants.

The protocol used was developed from a method described by O'Hare (1991) and employed by Atherton *et al.* (1994). The biopsy material was sliced, in a criss-cross fashion, into very small pieces with large, rounded scalpel blades (No. 24) [Swann Morton]. The resultant tissue pieces were suspended in 10ml DMEM [Gibco BRL], supplemented 5% FCS [Gibco BRL], 50 IU/ml Penicillin and 50μg/ml Streptomycin [Gibco BRL] and 2μM L-Glutamine [Gibco BRL]. The tissue was passed through the opening of a 10ml pipette several times to disrupt the tissue structure further. The cell suspension was centrifuged at 1600rpm (200*g*) for 5 minutes. The supernatant was drawn off and replaced with a solution of crude collagenase type 1A [Sigma] in complete DMEM at a concentration of 200 units/ml. The tissue was incubated with collagenase overnight at 37°C. The suspension was centrifuged at 1600rpm (200*g*) for 5 minutes and the supernatant drawn off. The tissue pellet was washed vigorously 5 times through a 10ml

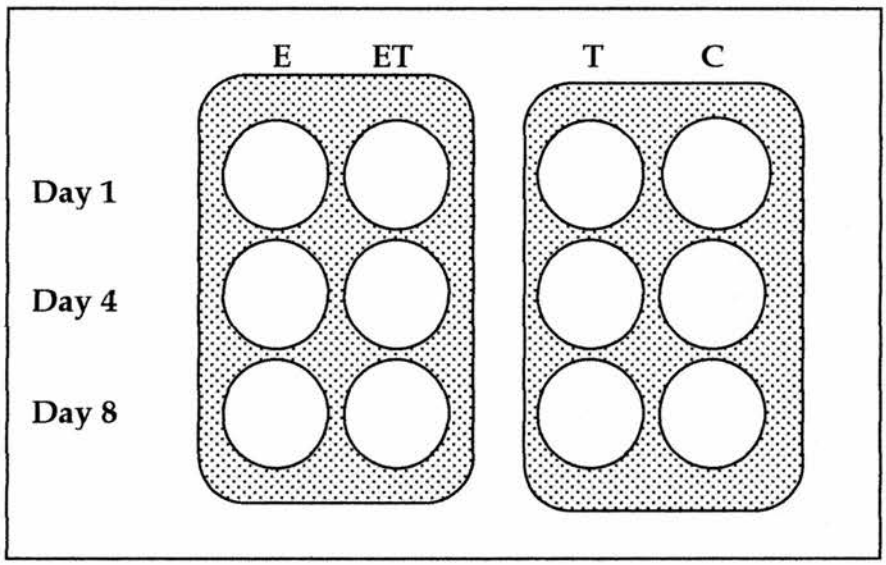


Figure 3.1 Experimental Soft agar Culture System.

96-well microtitre plates were used as culture dishes. Breast tissue was cultured with estrogen (E), estrogen and tamoxifen (ET), tamoxifen (T) and without drugs, as a control (C).

pipette with complete DMEM. After centrifugation, the pellet was dispersed through a stronger solution of collagenase (750 units/ml) for 1 hour at 37°C with frequent agitation. The collagenase medium was drawn off after centrifugation and the pellet washed 5 times in fresh DMEM. The cell suspension was resuspended in 3ml of complete DMEM, seeded in a culture flask and incubated at 37°C in 95% air/5% carbon dioxide.

The cell suspension was examined daily under an inverted light microscope for cell attachment to the culture flask substrate. If, after 7 days, no cell attachment was apparent, the culture was discarded.

iv. Tissue Viability in Soft Agar Culture

Each tumour was sliced with sterile scalpel blades into as many ~2mm^3 cubes as possible. Soft agar was prepared by mixing equal parts of 0.6% Bacto agar [Difco Laboratories, Detroit, MI, USA] and double strength, phenol-red-free Minimum Essential Medium (MEM) [Sigma]. 0.6g of Bacto agar was dissolved in 100ml of distilled water, aliquoted and autoclaved. MEM powder was dissolved in half the recommended amount of distilled water to produce the double-strength culture medium, then filter sterilised. The MEM was supplemented with 20% dextran-charcoal stripped (estrogen-free) Calf Serum (DC-CS) [Sigma], 100 IU/ml Penicillin and 100µg/ml Streptomycin [Gibco BRL] and 4µM L-Glutamine [Gibco BRL] resulting in final concentrations of 10%, 50 IU/ml, 50µg/ml and 2µg/ml respectively.

Before use, the agar was boiled in a domestic microwave on low power then heated thoroughly in a 40°C water-bath for 30 minutes. An equal amount of MEM was warmed to 37°C then added to the agar immediately before use giving final concentrations of 0.3% agar and 1x MEM. The soft agar was added in 200µl aliquots to the wells of a 96-well culture plate [Nunc] (refer to Figure 3.1). Using sterile forceps, the cubes of breast tissue were pushed under the surface of the agar, one cube per well.

The multiwell plates containing the biopsy tissue were incubated at 37°C in a humidified box in an atmosphere of 95% air and 5% carbon dioxide. The tissue explants were taken out of culture under sterile conditions and then fixed, dehydrated, wax-embedded and sectioned as described in 2.2-2.5.

The effects of culture in terms of histological survival in the agar was subsequently analysed by assessing percentages of apoptotic cells at each time point.

Apoptosis, also known as programmed cell death (PCD), is a specific type of cell death involving single cells or small groups in a tissue where other cells are functioning properly. Apoptosis is distinct from necrosis because it has a physiological or genetic involvement whereas tissue necrosis is due to injury. Apoptotic cells quickly lose their contacts with other cells. Nuclear material condenses within 1-2 hours and the chromatin is packaged into nuclear fragments by the nuclear membrane. The cytoplasm shrinks and organelles are packaged into vesicles (apoptotic bodies). The membrane binding of all particles is essential so that the digestive enzymes do not spill out into the environment.

Each section was scored for apoptotic cells in 5 fields of view under x63 magnification. The first field was taken as the 'top' of the section, the second field was 'one field down and one to the right', the third was 'one field down and one to the left' and so on. Apoptotic cells were counted as those with dark, condensed nuclei, shrunken cytoplasm and few, if any, cell contacts. Healthy cells had large, paler staining nuclei and retained their position and contacts in the architecture of the tissue.

v. Tissue Viability in the Presence of Conventional Experimental Drugs and Hormones

Tamoxifen [Sigma] and 17β-Estradiol [Sigma] were prepared from powder into 10^{-4} M stock solutions by diluting them as appropriate in ethanol or methanol. These drugs are light sensitive so they were stored in the dark at 4°C. For working stocks of the drugs, the 10^{-4}M stock solutions were diluted in MEM to give concentrations of 10^{-5}M for Tamoxifen and 10^{-7}M for Estradiol. The working stocks of drugs were x10 stronger than required so that when 20µl were added to each well of 180µl medium, the final concentrations were 10^{-6}M for Tamoxifen and 10^{-8}M for Estradiol. All precautions for using sex steroids were observed i.e. handling in a fume hood, avoiding inhalation and ensuring minimal exposure during use. The drugs were added as shown in Figure 3.1 with estradiol and combinations in one plate and single drugs and control in another plate. Blank rows and columns separating the experimental wells were filled with sterile PBS to

humidify the atmosphere. Estrogen-containing wells were all in the same plate to avoid cross-contamination with the single drug treatments.

Because the agar is made of loose sugar molecules diluted further by the medium, it was assumed that the drugs filtered through the soft agar and into the tissue. This was demonstrated using histological stains in place of the experimental drugs. Haematoxylin, eosin and trypan blue were pipetted onto the top of the soft agar wells, as described for the drugs. The diffusion of the stains was observed. Eosin diffused evenly throughout the 200μl of agar within 1.5 hours while the other 2 stains took 3 hours. When the tissue was removed from culture and sliced, it was noted that the colour had permeated to the core of the tissue cube. The tissue cubes were fixed, wax-embedded and sectioned. Without dewaxing and rehydrating, the stains could be seen in the sections of tissue.

3.3 Results of Research

i. Enzymatic Disaggregation of Breast Tissue

Enzymatic digestion of breast cancer tissue was attempted in order to produce single cell suspension of breast epithelial cells. Collagenase was used to loosen the fibrous breast tissue and free the epithelial cells. It was hoped that any epithelial cells in the suspension would adhere to the culture flask and continue to grow, producing a primary cell line.

In the present research, enzymatic disaggregation was performed on 17 biopsies. Early in culture, 6 of the cell suspensions became irreversibly infected. The remaining 11 biopsies were examined daily for cell attachment. It was observed that the cells remained as floating aggregates rather than single cells and that none of the cells settled onto the substrate.

It became clear that adequate numbers of fresh epithelial cells would not be established using this technique and that valid direct comparisons with explant tissue (see below) would not be possible.

Furthermore, the disaggregation process was wasteful of tissue and the extensive manipulation of the tissue clearly carried a high risk of infection that could threaten the success of the whole study, which was based on large numbers of cultures.

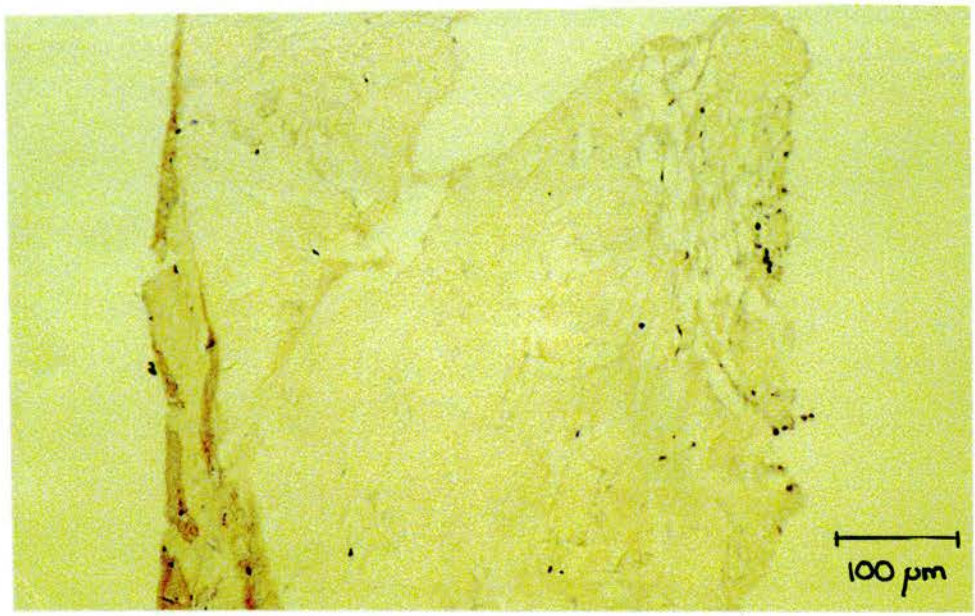


Figure 3.2 Section of Acellular Breast Tumour.

The section of tissue is very fibrous with few nuclei.

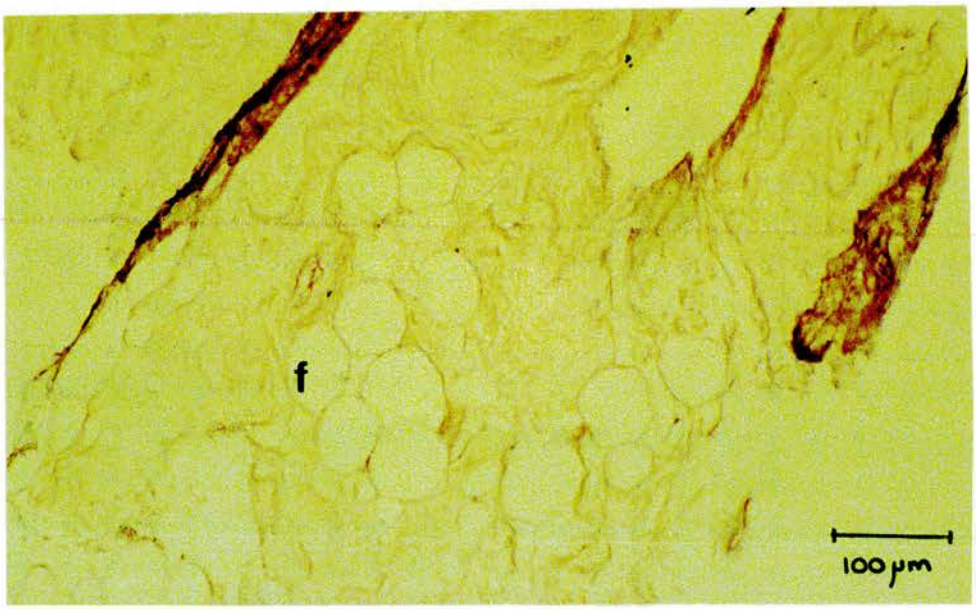


Figure 3.3 Section of Fatty Breast Tumour.

The section is fibrous and completely acellular. The spaces were fat cells (f).

Time Point→ Tumour↓	0	1	2	3	4	5	6	7	8	9	10	12	14	16	18	21	22	28	29	31
025	0	10		31.2	26.2		34.2													
036	1.9	7.0	9.6	5.8	6.5	Ac	Ac	4.4	96.5	44.9	Ac									
037	4.1	7.3	7.5	19.5	Ac	9.5	36.0	13.9	Ac	64.3	32.7									
038	6.7	Ac	Ac	Ac																
039	1.5	10.1	6.7		19.8	8.9	9.8		33.9											
040	4.6	5.9			Ac	66.0	2.4													
041	9.9	20.5			21.5	Ac	27.2													
103	0.1		12.8								18.9		77.9	95.7		68.3				
122	7.6	13.2	11.1	22.2	53.5	19.0	24.8													
128	4.5		23.9		52.0		49.0		18.0		28.9	14.1	33.1	28.6	26.0				63.4	38.8
130	7.3	25.0	9.4		5.6							Ac	95.9	Ac		33.3		33.0		
132	0	3.9	3.2		4.6		19.6		0.8			7.0		20.3			6.5		12.6	
134	5.6								26.8				Ac							

Table 3.1. Percentage of apoptotic cells in time course breast tissue, averaged from at least 5 fields of view at x96 magnification. The acellular sections in a tumour series are denoted by 'Ac'.

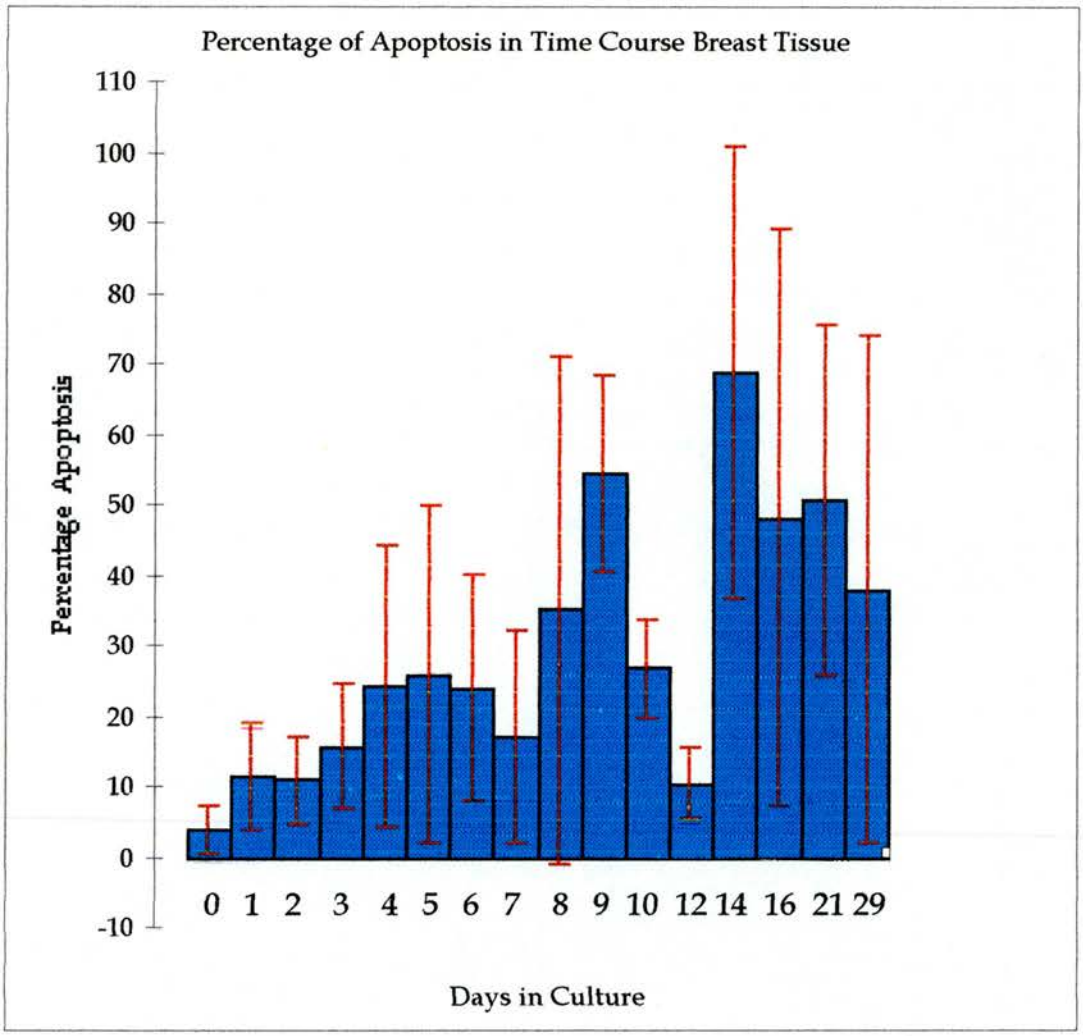


Figure 3.4 Chart of Mean % Apoptosis in Time Course Breast Cancer Tissue.

ii. Success of the Soft Agar Culture System

Because of the lack of success with enzymatic disaggregation, the focus of the study became the development of a novel culture system, designed to sustain human breast tissue in a sterile environment, with minimal disruption to its architecture.

Bearing in mind that the tissue had been handled in a non-sterile environment in the pathology labs (clinical considerations were paramount), the culture system had to be low maintenance so that the risk of infection was minimised. The soft agar culture system was a simple set-up which withstood the necessary handling during the culture time. Due to extreme care and precise sterile technique, every culture performed during the present study remained infection-free throughout the culture period. The tissue explants were easily manipulated by the addition of drugs and hormones into the agar.

It was rare, however, for a tumour to yield a complete set of sections from the cultured pieces. This highlights the difficulties in using a culture system which is ultimately retrospective. Not until the whole process of culture, fixation, sectioning and staining was complete could one assess the histology of the tissue. Cultured tissue was often acellular or fatty as demonstrated in Figures 3.2 and 3.3. Every effort was made to identify the 'gritty' texture in the biopsy (a characteristic of some tumours) and to take the tissue cubes from the same area but there was no conclusive way to predict the nature of each tissue cube before culture.

iii. Cell Viability in Cultured Breast Cancer Tissue

Seventeen biopsies, yielding a total of 135 individual cultures, were used in the cell viability experiments. However, only 82 points could be included in the study - the others could not be counted due either to acellularity of the tissue explant or to fragmentation of sections during processing. The duration of the culture period ranged from 0 to 31 days, depending on the availability of tissue. The soft agar culture system proved to be very successful in maintaining breast biopsy tissue for many days. The results for percentages of apoptosis in cultured fresh breast tissue are shown in Table 3.1 and Figure 3.4.

Statistical Analysis of Cellular Viability in Cultured Breast Cancer Tissue: A statistics programme, DataDesk®, was used to analyse the data collated from

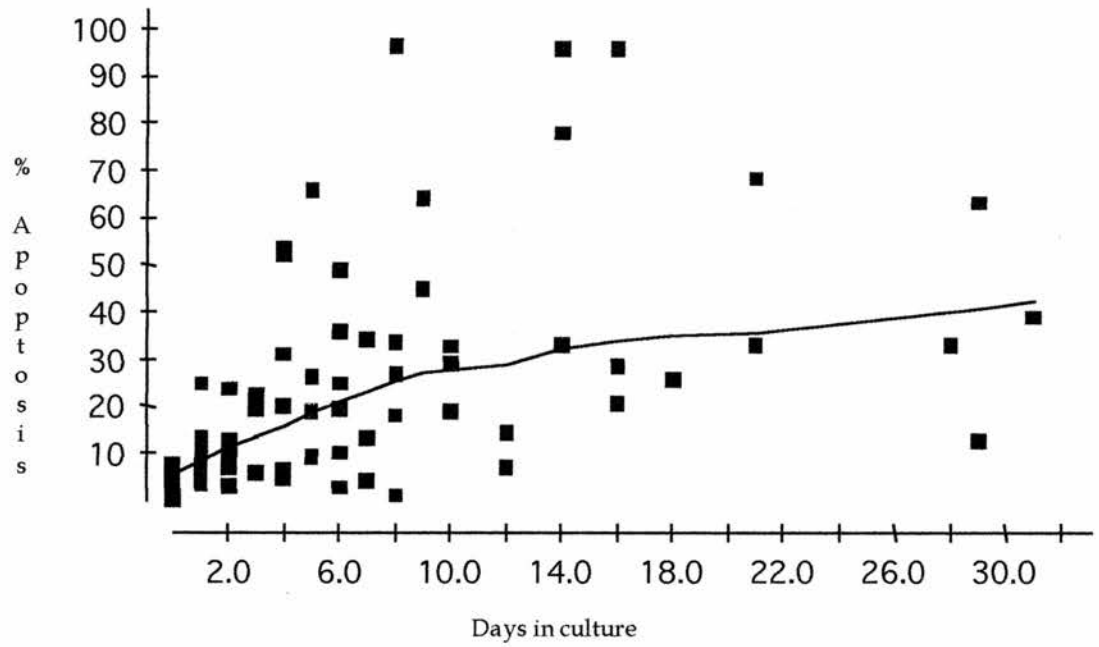


Figure 3.5 Scatterplot of Percentage Apoptosis in Cultured Breast Tissue.
The level of apoptosis rises in the first week of culture, and continues to do so throughout the culture period, though at a slower rate.

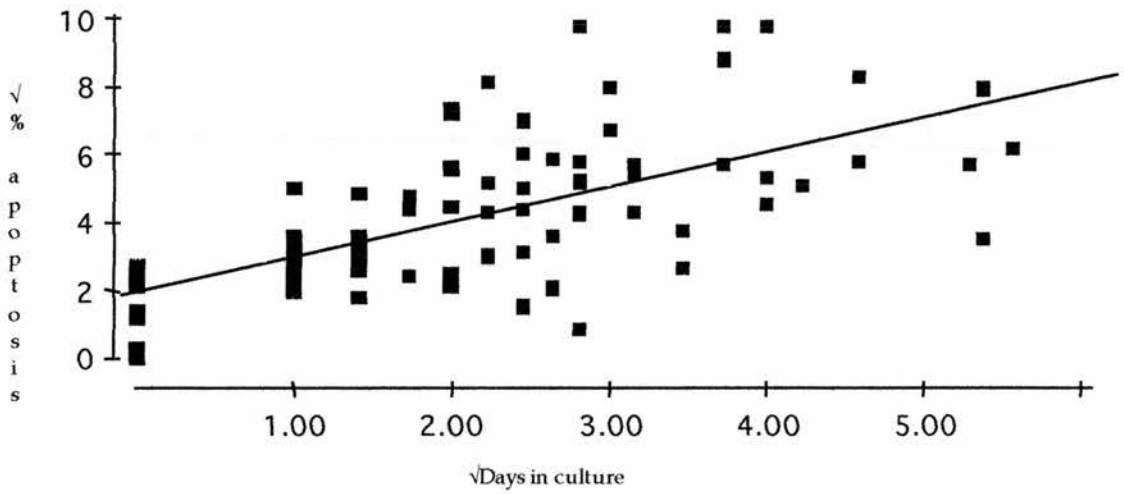


Figure 3.6 Transformed Data of the Percentage Apoptosis in Cultured Breast Tissue.
The linear relationship between the percentage of apoptosis and time in culture is shown.

measuring apoptosis in the time course tumours. A scatterplot of the data is shown in Figure 3.5. The trend line on this figure was obtained by LOWESS smoothing (Locally Weighted Regression Scatterplot Smoothing), an integral tool in the DataDesk® programme. There is considerable variation in the data, but the smoothing line indicates that the percentage of apoptosis increases rapidly from Day 0 to Day 8 and thereafter rises more slowly over the remaining culture period. The non-linear nature of the smoothing line demonstrates that transformation of the data is required before correlation coefficients can be calculated. DataDesk® offers customised transformations which can incur the risk of data trawling, therefore it was decided that only standard types of transformation would be used. Of the standard transformations offered by DataDesk® ($\sqrt{y}$, log y, $1/y$, $1/\sqrt{y}$), taking the square root of each variable generated the best result. The Pearson product moment correlation was used to establish the correlation coefficient between the length of time in culture and percentage of apoptosis. The coefficient was 0.627. Figure 3.6 shows the transformed data and the linear relationship of the data.

A regression equation was calculated to determine the slope of the data line. Any unknown point could be calculated from the regression equation. A data print-out from DataDesk® is provided in Appendix 3.

$$\sqrt{\%}\text{ apoptosis} = 1.02 \times \sqrt{\text{days in culture}} + 1.98$$

Equation 3.1 Regression Equation for Cultured Breast Cancer Tissue Viability Data.

The trend-line, based on the actual data, shows that apoptosis increases at a faster rate during the first 8 days of culture than at any other time. The trend-line suggests that the cumulative frequency of apoptosis at this time is around 20%, a figure deemed acceptable for further study. After 8 days of culture, apoptosis increased more slowly reaching over 40% after 4 weeks in culture.

Two of the time course series are shown in Figure 3.7a-h and Figure 3.8a-f. Figure 3.7 depicts an ER-positive, ductal carcinoma from a post-menopausal patient. The time points shown are days 0, 4, 8, 12, 16, 29 and 31. There was a substantial, but variable, degree of apoptosis in this tumour. However, as the micrographs of day 31 show, many of the cells are still viable. Figure 3.8 shows an

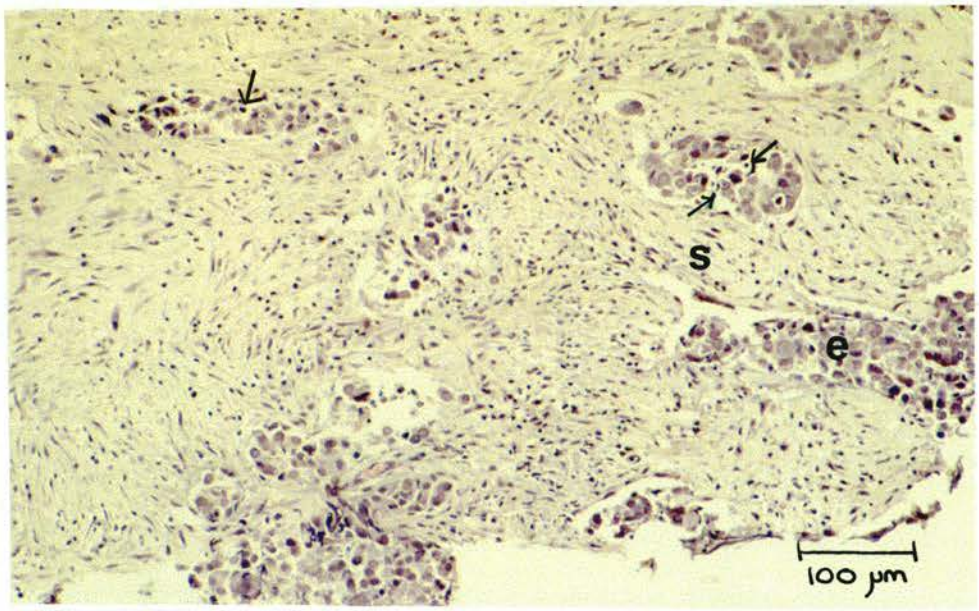


Figure 3.7a Tumour 128, Day 0 (H&E; x10).
Note the good histology of the epithelial islands (e) and the stromal cells (s). The morphological apoptosis score for this section was 4.5 %. Examples of apoptotic cells are denoted by ➔.

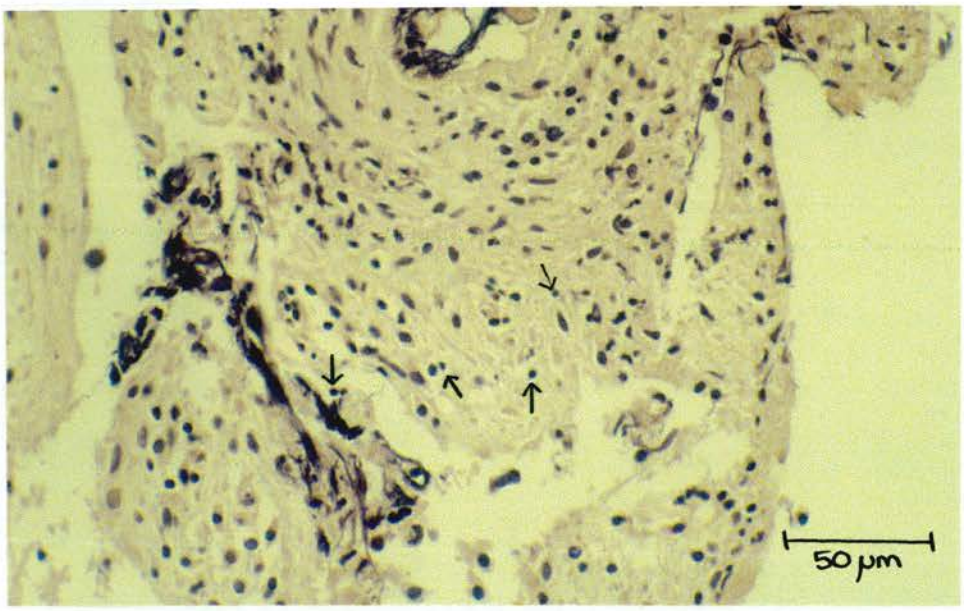


Figure 3.7b Tumour 128, Day 4 (H&E; x25).
The overall score for day 4 is 52% but this particular section of the tumour shows reasonable cell viability. Examples of apoptotic cells are denoted by ➔.

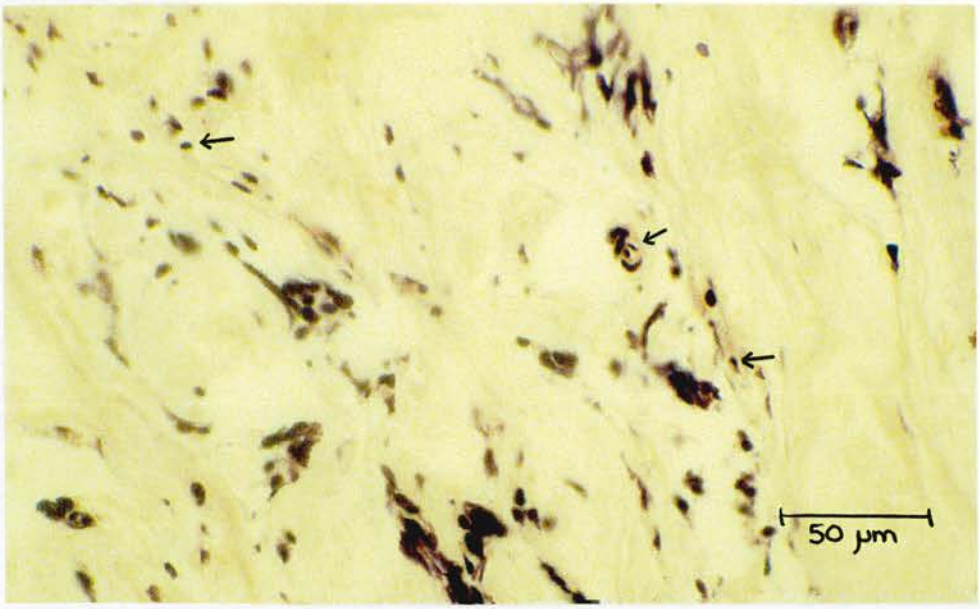


Figure 3.7c Tumour 128, Day 8 (H&E; x25).
The apoptosis score for this section was 18%. Examples of apoptotic cells are denoted by ➔.

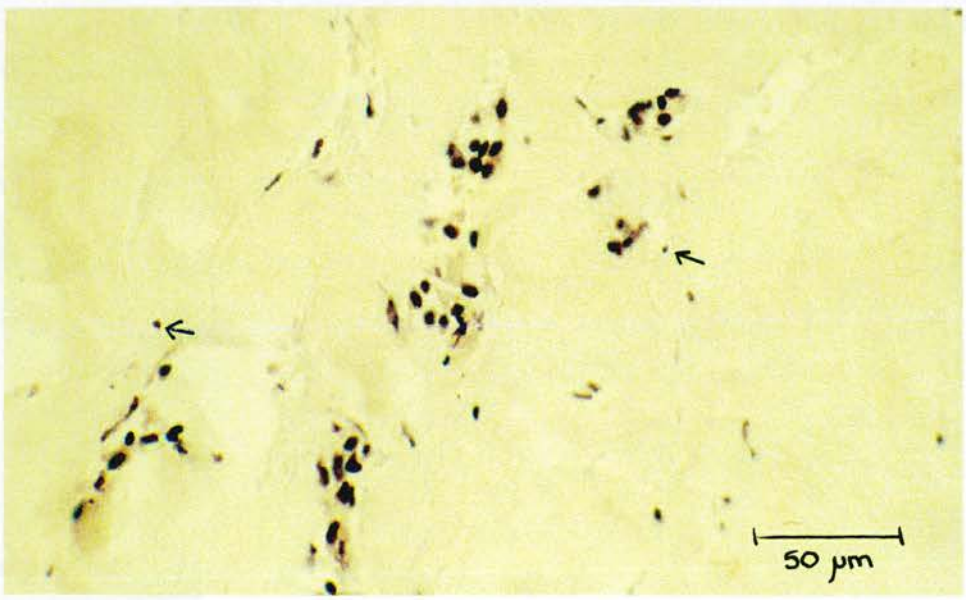


Figure 3.7d Tumour 128, Day 12 (H&E; x25).
The apoptotic score for day 12 is 14.1%. Examples of apoptotic cells are denoted by ➔.

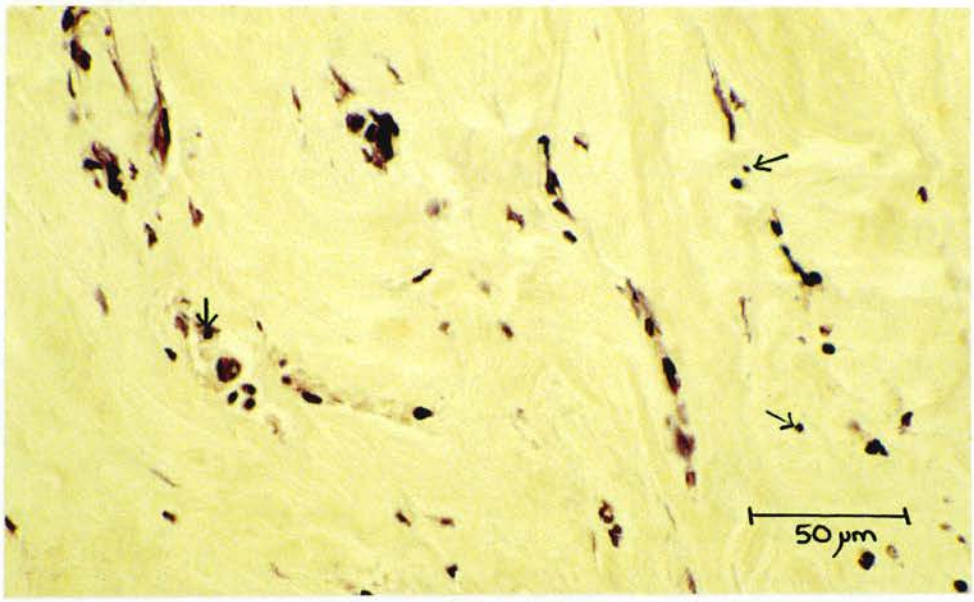


Figure 3.7e Tumour 128, Day 16 (H&E; x25).
The apoptosis score for this section was 28.6%. Examples of apoptotic cells are denoted by ➔.

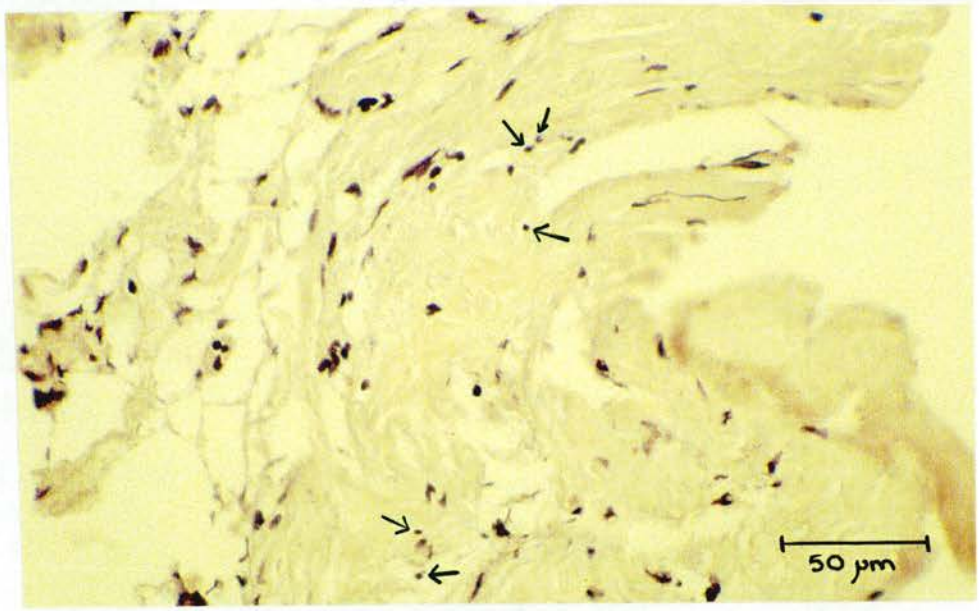


Figure 3.7f Tumour 128, Day 29 (H&E; x25).
The apoptotic score for for day 29 was 63.4%. Examples of apoptotic cells are denoted by ➔.

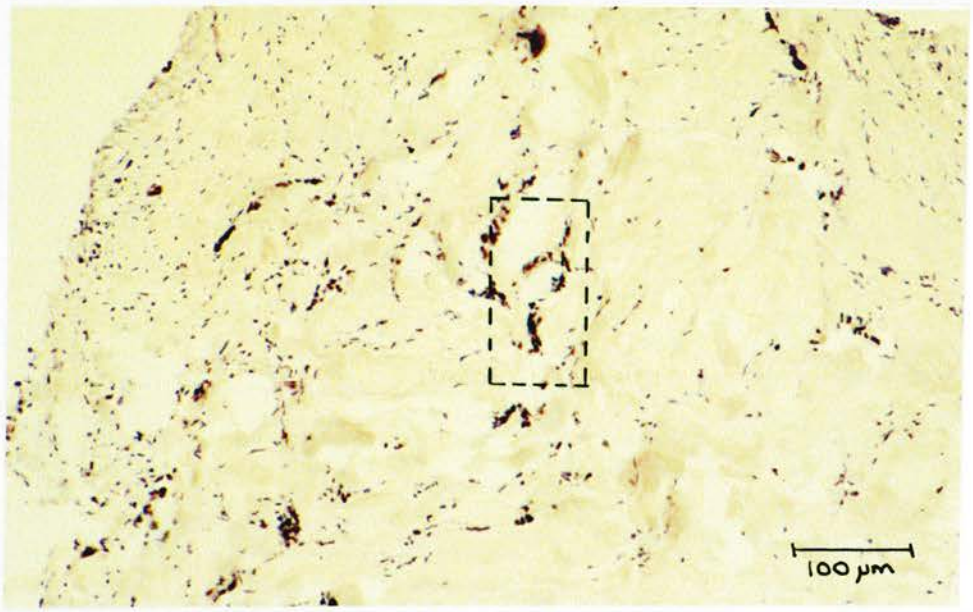


Figure 3.7g Tumour 128, Day 31 (H&E; x10).
The apoptosis score for this section was 38.8%.

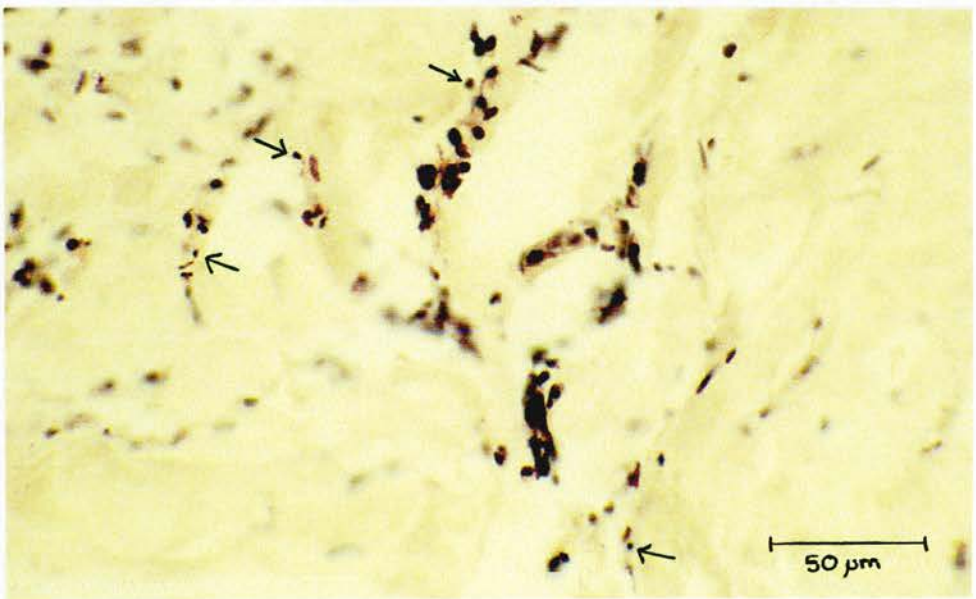


Figure 3.7h Tumour 128, Day 31 (H&E; x25).
This figure is a higher power micrograph of the boxed area in the above figure and shows epithelial cells. Examples of apoptotic cells are denoted by ➔.

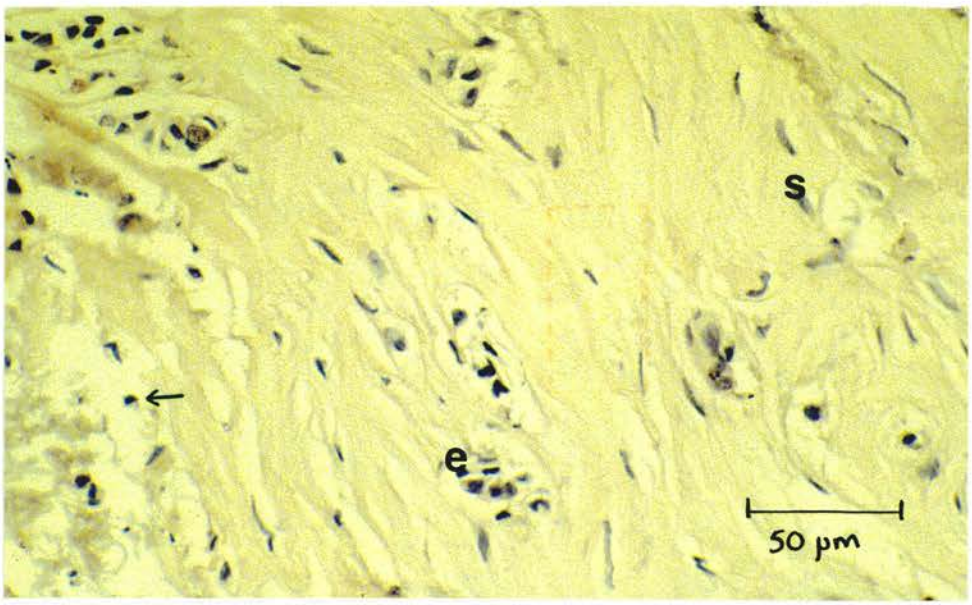


Figure 3.8a Tumour 132, Day 1 (H&E; x25).
Epithelial islands (e) and the stromal cells (s) are evident. The morphological apoptosis score for this section was 3.9%. Examples of apoptotic cells are denoted by ➔.

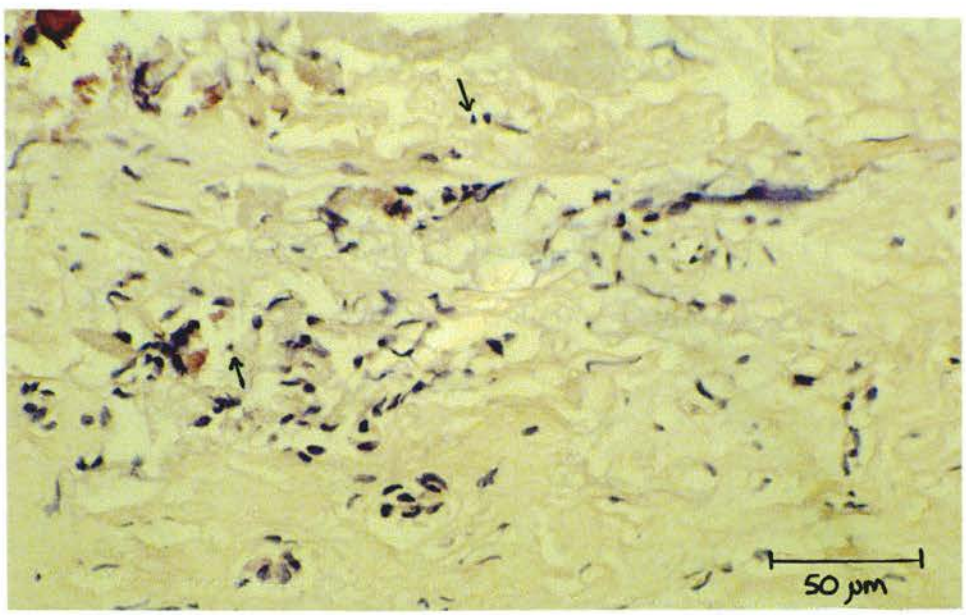


Figure 3.8b Tumour 132, Day 2 (H&E; x25).
The apoptotic score in this section was 3.2%. There is little difference between the day 1 and day 2 sections. Examples of apoptotic cells are denoted by ➔.

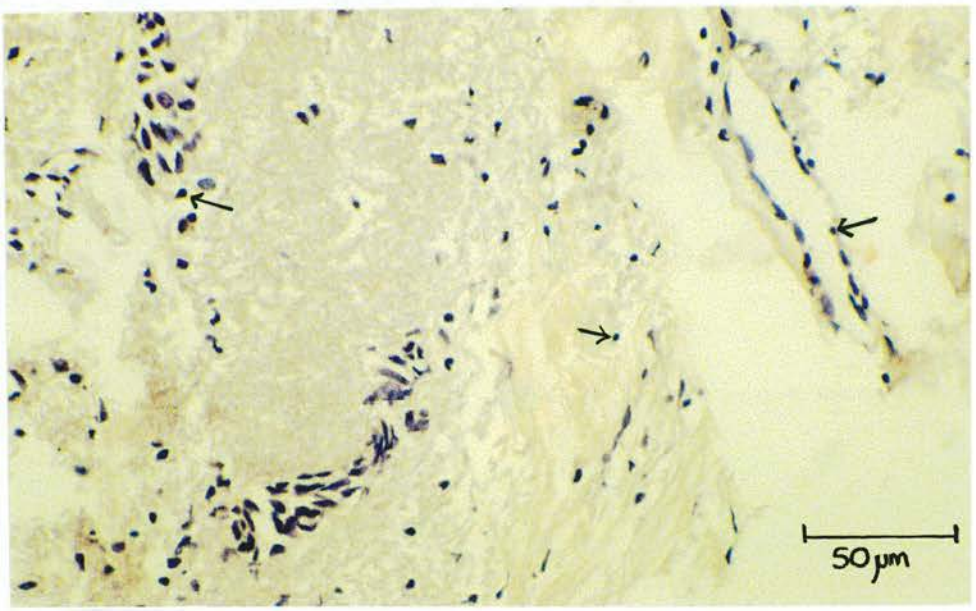


Figure 3.8c Tumour 132, Day 4 (H&E; x25).
The apoptosis score for this section was 4.6%. Examples of apoptotic cells are denoted by ➔.

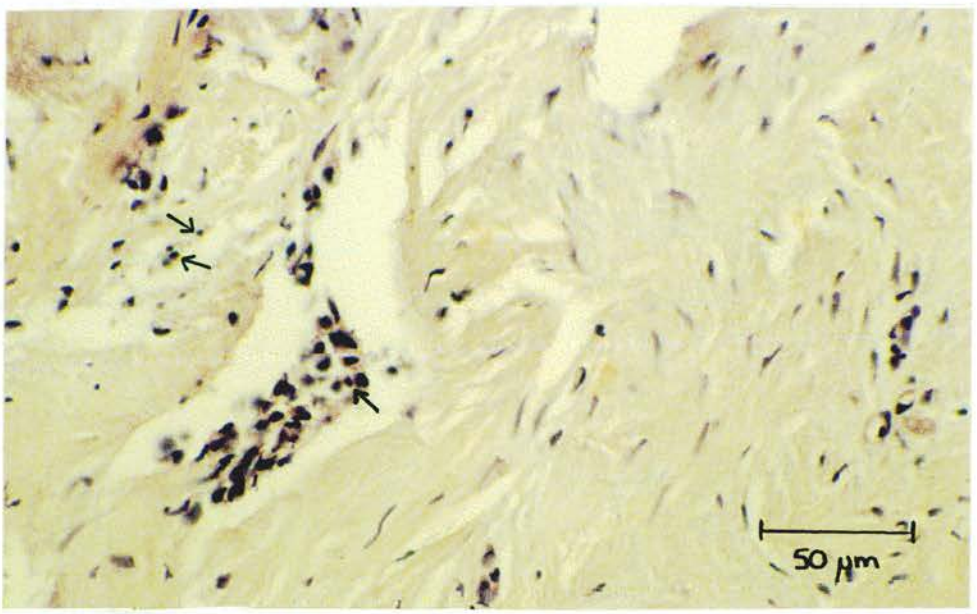


Figure 3.8d Tumour 132, Day 6 (H&E; x25).
The apoptotic score in this section was 19.6%. Examples of apoptotic cells are denoted by ➔.

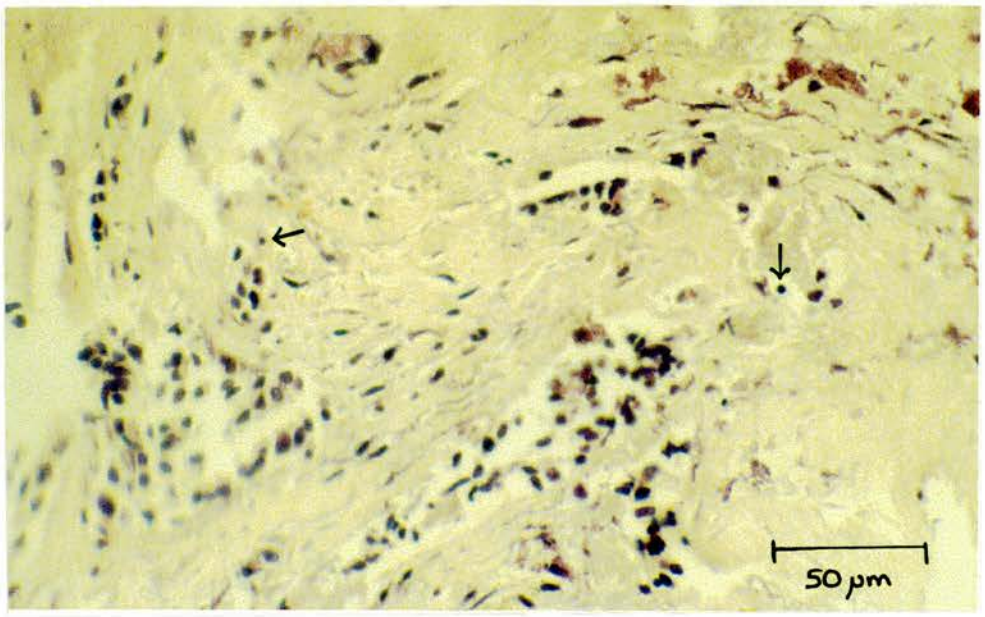


Figure 3.8e Tumour 132, Day 22 (H&E; x25).

The apoptosis score for this section was low at 6.5%. The epithelial cells remain in reasonable health. Examples of apoptotic cells are denoted by ➔.

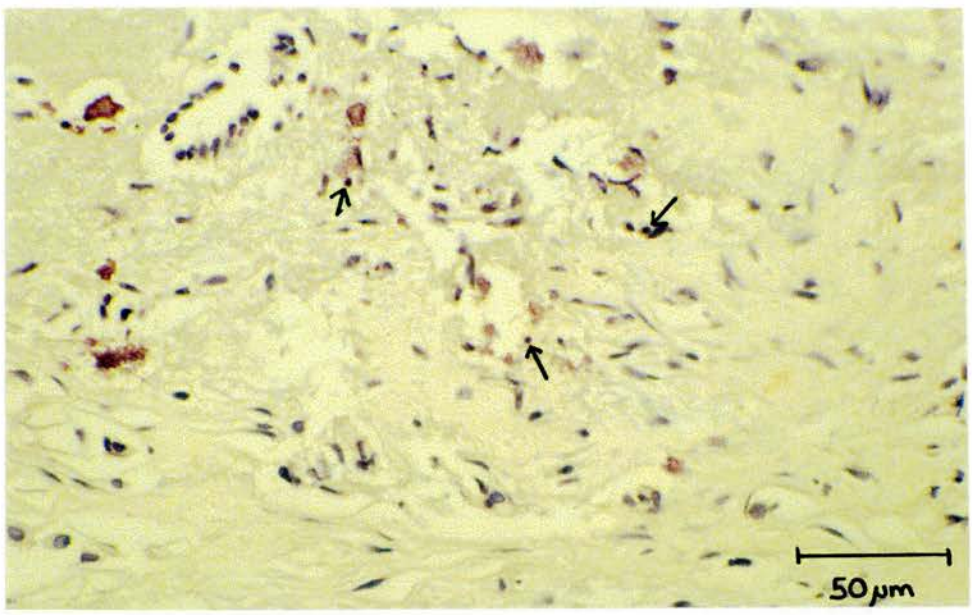


Figure 3.8f Tumour 132, Day 28 (H&E; x25).

The apoptotic score in this section was 12.6%. Examples of apoptotic cells are denoted by ➔.

Drug Treatments	E1	E4	E8	ET1	ET4	ET8	T1	T4	T8	C1	C4	C8
Mean % of Apoptosis	11.2	4.4	12.2	10.1	4.7	3.5	9.0	6.0	7.8	12.5	9.9	4.5
Standard Deviation	15.7	2.3	17.8	9.5	1.9	2.3	2.6	2.6	9.7	9.7	2.5	2.2

Table 3.2 Mean Percentages of Apoptosis in Drug Treated Breast Tissue.
E = estrogen, T = tamoxifen and C = control (no drugs), cultured for 1, 4 and 8 days.

ER-positive, ductal carcinoma from a pre-menopausal patient. The time points are days 1, 2, 4, 6, 22 and 28. There was a low degree of apoptosis in this tumour, indicated by the micrographs, which show a high proportion of viable epithelial and stromal cells.

The micrographs add to the evidence that cell viability at day 8 in culture is satisfactory for subsequent experiments.

iv. Cell Viability in Drug-treated Breast Cancer Tissue

There was no significant difference between the experimental and control tissues during the culture period. Percentages of apoptosis were measured in the same way as in the time course studies, by counting apoptotic cells as a percentage of the total number of cells in 5 fields of view at a magnification of x63 on a light microscope. The effect of drugs on the levels of apoptosis over an 8-day culture period are tabulated in Table 3.2.

No overall effect on apoptosis was observed with any of the drug treatments. A chart of the data, including error bars based on standard deviation, is shown in Figure 3.9. The control tissue, i.e. no drug treatment, showed higher variability than had been recorded in the comparable time course studies, described in 3.3iii.

For differences between control and experimental tissue to be established, the effect of the drug on apoptosis needed to be greater than the magnitude of the control tissue viability. On analysing the control tissue, it became clear that the margin of error was so great that if the drugs were exerting an effect on the rate of apoptosis, it would be masked. Fewer data points were available for the experimental controls than in the previous time course experiments, which explains the larger error bars. However, comparison of apoptosis rates in drug-treated explants with data from the time course experiments still gives no suggestion of any drug-induced effect.

v. Retrospective Analyses

For a subset of tumours, the pathology reports were made available, along with the ER status of the tumours and the menopausal status of the patients. It was of interest to investigate whether or not there was a difference in viability

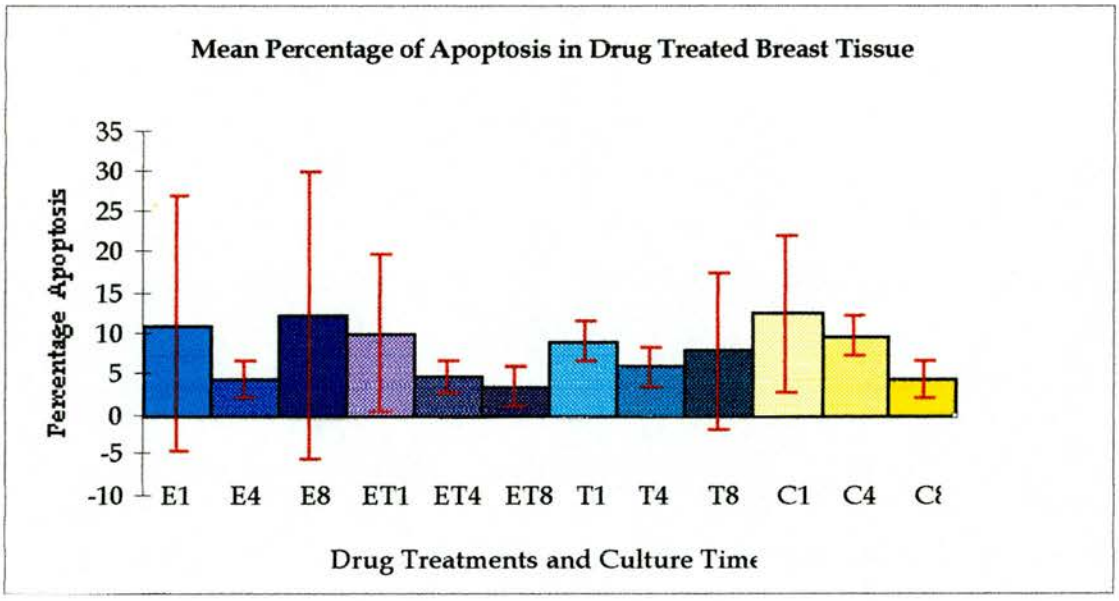


Figure 3.9 Mean Percentage of Apoptosis in Drug-treated Breast Tumour Tissue.

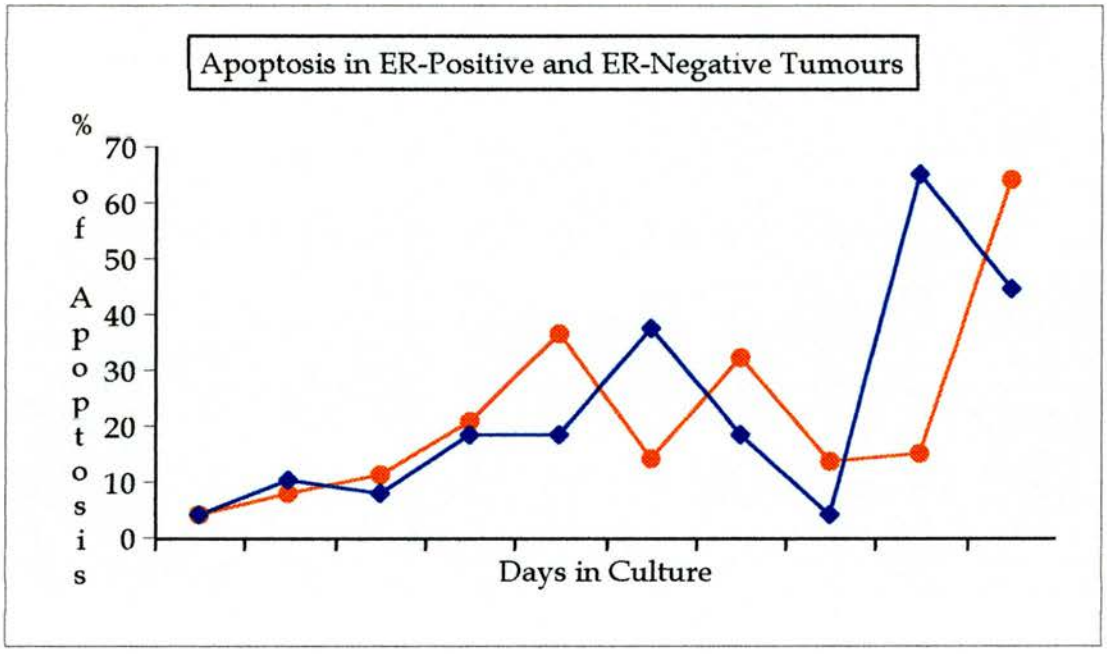


Figure 3.10 Apoptosis in ER-positive (●) and ER-negative (■) Tumours.
There is no significant difference between the average apoptosis rates over 9 days of culture in ER-positive and ER-negative tumours.

between ER-negative and ER-positive tumours during the culture period. The culture system was estrogen-depleted, so there was some question of whether the ER-positive tumours would survive as well as the estrogen-independent tumours.

The apoptosis data were grouped according to ER status and the 2 groups were compared. The result is shown in Figure 3.10. No significant difference in apoptosis was observed between the 2 groups of tumours over a 9 day culture period.

3.4 Discussion of Results

i. Enzymatic Disaggregation of Breast Tissue

Despite much effort, establishing cell lines from primary breast tumour tissue has never been particularly successful. This is often due to the large amount of fibrous stroma present in the breast and the faster growth of fibroblasts and myoepithelial cells over the cancerous epithelial cells (O'Hare, 1991).

Past attempts have utilised hormones and growth factors, for example hydrocortisone, insulin, EGF and many others, in the final growth medium after collagenase treatment (Pandis *et al.*, 1992). In the present research, the chosen technique was very simple with a basic growth medium, so it is perhaps not surprising that no cell lines were established. However, the technique was worth trying, if only for the fact that, had it worked, one would have succeeded in establishing a breast cancer cell line which required no exogenous growth stimulants - an ideal study material for subsequently examining the effect of hormones and growth factors and for comparing growth effects in the presence and absence of stromal support.

The problem of infection in the cultures also decreased the chances for success. Sterility was not a priority for the pathologists involved in handling the tissue.

For the present study it was more important that the breast tissue architecture should be maintained, in order to assess the 'natural' interactions of stromal and epithelial cells.

If enzymatic disaggregation was to be attempted again, certain improvements could be made. The region of tumour in the biopsy could be better identified by the pathologist to maximise the number of epithelial cells and limit

the amount of fibrous tissue. The collagenase treatment could be optimised and, perhaps, combined with other proteolytic enzymes. It might be advantageous to add growth-stimulating factors into the medium and, once a cell line was established, the cells could be weaned off the factors in order then to examine the effects of experimental drugs.

ii. Soft Agar Culture System

The novel tissue culture system devised for the culture of breast tissue proved successful on several counts. First, being a low maintenance system, the risk of infection was kept to a minimum which limited wastage of the precious breast tissue. Second, although apoptosis clearly increased over the culture period, the tissue remained in a satisfactory state of health, with adequate cell viability, for the desired experiments. This was fundamentally important because the tissue was cultured in a minimal medium and hormone-depleted serum. A previously reported explant study, which utilised mouse mammary tissue, required estrogen, progesterone, aldosterone, hydrocortisone and EGF in the growth medium to sustain the growth of the explants (Plaut *et al.*, 1993).

The use of fresh tissue explants could be superior to cell lines, which do not properly reflect the *in vivo* situation, and might be applied for the culture of other tissues. It may be, however, that the large stromal component of most breast tissue contributes to the survival of the explants *in vitro*, and that tissue with less stroma may not survive so well. It would be of interest to culture other tissue types, in order to assess the benefit of stromal tissue for cell viability. The problem of unpredictability of tissue content for each cultured fragment remains and there are no easy answers.

iii. Cell Viability in Cultured Breast Cancer Tissue

The results showed that breast biopsy material could be maintained with a satisfactory degree of health for up to 31 days in some cases. Apoptosis increased most steeply in the 0-8 day part of the time course, but based on previous studies, an 8-day tissue culture period with time points at one and 4 days was deemed sufficient for experimental drugs to exert any effect.

Apoptosis proved to be a practicable counting system for assessing cellular viability. It was expected that a degree of apoptosis would be observed in the tissue sections because of the simple culture conditions. However, the point of the exercise was to assess whether or not the health of the tissue was adequate for experiments on growth regulation, which it proved to be.

Further development of the system should concentrate on the later range of time points, extending the culture period, so that more complex experiments can be undertaken, perhaps reflecting cancer therapy *in vivo* where responses are commonly assessed over periods of weeks or even months rather than days..

iv. Cell Viability in Drug-treated Breast Cancer Tissue

The findings exclude any dramatic effect of estrogen, tamoxifen or a combination of both on apoptosis of breast cancer cells in this culture system.

In order to observe an effect - if, indeed, an effect exists - improvements to the system could be made. The standard deviation could be reduced mainly by sampling more tissue, for example multiple replicates for each time point. A wider range of drug concentrations might also be tried or addition of an effector which would increase the efficacy of the experimental drugs. An example of an effector is TGFβ, which, when added to EGF in an NRK-49F clonogenic assay, produces more colonies than EGF alone (Macintyre *et al.*, 1988).

In retrospect, apoptosis was not the optimal measurement for evaluating the effect of drugs on cell growth. A more precise and quantitative method could have been used, for example thymidine uptake or Proliferating Cell Nuclear Antigen (PCNA) staining, which measure cell growth rather than cell death.

It was assumed that 1, 4 and 8 days would be long enough for an effect to be seen, based on previous studies - both cell lines and fresh tissue culture. Arrick *et al.* (1990) cultured MCF-7 cells with estradiol for 40 hours and within that time the hormone had induced the production of 3 TGFβ isoforms. Guvakova and Suramaez (1997) tested the effect of tamoxifen on cell growth and IGF-IR expression in MCF-7 cells. An inhibitory effect on cell growth was observed within the 4-day culture period. Karey and Sirbasku (1988) studied the effect of estradiol on MCF-7 cells. Their culture time was 8 days, during which they observed a dose-dependent increase in cell growth in response to estradiol. The production of active TGFβ from MCF-7 cells in response to tamoxifen was recorded by Knabbe *et al.*

(1987) after only 24-48 hours in culture. The subsequent conditioned medium (which contained TGFβ) was used to inhibit the growth of the same cells which produced the protein. Poulsen *et al.* (1982) cultured breast biopsy tissue as explants then tested the effect of estradiol on the growth rate of the tissue. Using thymidine uptake, they found that a significant hormonal effect could be recorded at 18-24 hours of culture. From these reports, it is more than probable that the drugs used in the present study would have had time to affect the growth of the breast cells.

Another reason why no significant effect of tamoxifen on cell death was observed may be that tamoxifen could arrest growth within the culture period but not cause morphological apoptosis. There is debate over whether tamoxifen is a cytostatic or a cytotoxic drug. Osborne *et al.* (1983) support the theory that tamoxifen is cytostatic, causing an accumulation of breast cells in early G1 of the cell cycle rather than directly killing the cells. Reddel *et al.* (1987) examined several breast cancer cell lines for their sensitivity to tamoxifen. They found a cytostatic effect at low concentrations of tamoxifen (0.1 to 1.0 μM) but at higher concentrations (5 to 25 μM), the drug had a cytocidal effect on the MCF-7 cells. The present study used 10μM tamoxifen which is in the range for cytocidal effects on monolayer cell culture. It is possible that the concentration of tamoxifen was not strong enough to exert cytocidal effects on cultured explant tissue. The culture system could be adapted to cover these possibilities.

v. Future Improvements

With regard to many pieces of tissue being misinformative at the end of the culture period, a better understanding of the macropathology of the tissue would have helped in the selection of pieces for culture. However, it must be remembered that the pathological requirements were of the utmost importance and that any tissue donated for this study was gratefully received.

The main problem of the culture system was the inconsistent cellularity in any series of tumour explants. In the time course experiments, 135 explants were set up but only 82 were of sufficient cellularity to be included in the study. This equated to 60% success rate of having cellular sections. Had 2 independent explants been set up for each time point, the chance of there being no cells between the 2 tissue fragments would be 15% i.e. 85% chance of success. For 3 pieces, a

94% success rate could be achieved; for 4 explants, the chance is 97% and for 5 explants there is less than 1% chance that the time point will have no cells.

However, it must be remembered that each explant requires lengthy histological processing - fixation, wax-embedding, sectioning and staining - which may be prohibitory. Producing many replicates from a small amount of tissue could restrict the time range of a time course. An acceptable compromise would be 3 or 4 replicates, so that there is a balance between a reduced chance of acellularity and a longer range of time points.

If the study were to be repeated, the measurement of cell proliferation and of growth arrest, rather than cell death, would be more suitable for the assessment of drug effect on cultured tissue. Methods for cell proliferation assays include thymidine uptake where only proliferating cells use the radioactively-labelled thymidine. Counts of how many cells were proliferating before and after drug treatment could be assessed. 5-bromodeoxyuridine (BrdU) incorporation is another possible method. BrdU is a thymidine analogue which can be incorporated into replicating DNA and be stained with anti-BrdU antibodies. Non-proliferating cells would not stain. PCNA is a similar method. The protein is only found in proliferating cells so anti-PCNA antibodies can be used to determine which cells are proliferating.

vi. Future Developments

Future developments for the culture system could include the use of reporter cells in the agar. Substances secreted by the cultured tissue could be quantitatively measured by reporter cells whose growth would be affected by the secretion. An example of this is the secretion of TGFβ by chick embryos in soft agar culture (Macintyre *et al.*, 1988). TGFβ stimulates the growth of the reporter cell line, NRK-49F, which forms colonies in response to the growth factor.

Similarly, the culture system could be used as a clinical assay to investigate the efficacy of therapeutic drugs on patient biopsies. Changes in growth rate could be assessed for several drug treatments. The optimal treatment, regarded as that which would kill tumour cells and not normal cells, could be established within the cultured biopsy tissue in a very short time. This would be preferable to administering a drug regime to a patient, merely in the hope that it might be successful. By eliminating ineffectual regimes, therapy could be tailored to any

individual, thereby increasing the chance of overcoming the disease quickly. As an aside, identifying the correct therapy first time would also save on health service resources in the long-term.

CHAPTER 4

IMMUNOHISTOCHEMICAL LOCALISATION OF TGFβ1 PROTEIN IN CULTURED BREAST CANCER TISSUE

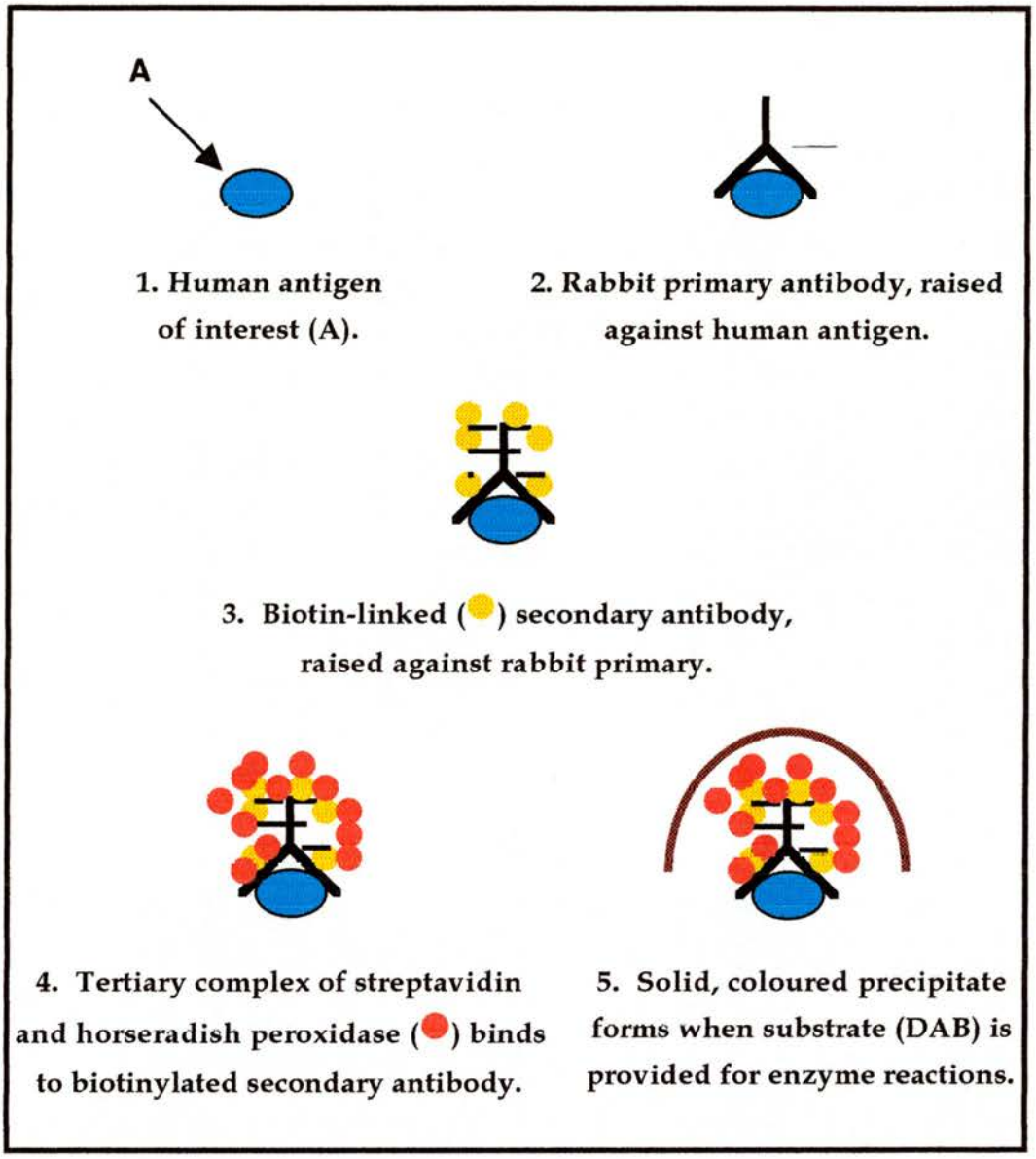


Figure 4.1 Schematic Representation of the Streptavidin-Biotin Horseradish Peroxidase Immunohistochemical Process.

4.1 Immunohistochemistry Background

Non-fluorescent immunohistochemistry has evolved from the early works of Nakane and Pierce (1966) on antibody-enzyme conjugation techniques. Sternberger *et al.* (1970) developed the unlabelled Peroxidase Anti-Peroxidase (PAP) system, improving the sensitivity of previous methods. Heitzmann and Richards (1974) were the first to introduce the Avidin-Biotin method. This system relies on the extraordinarily high affinity that avidin has for biotin, which is approximately one million times higher than the affinity of most antibodies for their corresponding antigens. Biotin, also known as Vitamin H, is a water-soluble vitamin of the Vitamin B complex family. Avidin is a 68kD glycoprotein, extracted from egg-white, and has a binding constant of $10^{-15}M^{-1}$ at 25°C. Avidin can often cause non-specific staining through its carbohydrate component so bacterial streptavidin is often used in its place as it is a neutral, non-glycosylated protein. The attachment of biotin to proteins, particularly antibodies, allows the detection of those labelled proteins once they are bound to avidin (which is normally conjugated to an enzyme, for example horseradish peroxidase or a fluorochrome, for example fluorecein). Due to the flexibility of this system, it has been utilised for developing a variety of valuable immunohistochemistry applications. Immunohistochemical techniques are becoming a major tool for surgical pathologists and immunopathologists in the detection of specific antigens in tissue sections.

For research, an antibody is raised against a protein of interest from the same or similar species. Fluorescent or enzyme conjugated antibodies allow immediate detection of the desired protein. Enzymatic techniques are more permanent; fluorescent labels degrade under visible or ultra-violet light. To amplify the signal as much as possible, indirect techniques of labelling are employed. As shown in Figure 4.1, a primary antibody will bind directly to the protein of interest. A secondary antibody is raised against species specific epitopes in the animal in which the primary was raised, in this example, rabbit. The secondary antibodies are conjugated to enzymes, for example biotin or alkaline phosphatase, or to fluorescent molecules, for example fluorescein or Texas red. Fluorescently-labelled protein can be viewed under ultraviolet light at this stage. A tertiary complex binds an enzyme to the secondary antibody and a fourth layer provides the substrate on which the enzyme works, the product of which is a solid, coloured precipitate which can be viewed under a light microscope.

Immunohistochemistry is a powerful diagnostic technique in pathology. Antibodies are available against specific biological markers which are particular to certain types of breast tumour. For example, Millis *et al.* (1996) report that poorly differentiated DCIS is very often ER-negative, PGR-negative but positive for c-erb-B2 and p53.

i. Present Research

The aim of using immunohistochemistry in the present research was to reveal the location of TGFβ1 protein. Controversy exists over the site of production. Colletta *et al.* (1992) showed that, in response to tamoxifen, stromal fibroblasts would produce TGFβ1 while other researchers (Gorsch *et al.*, 1992; Walker and Gallacher, 1995) found that the epithelial cells were the major producers of TGFβ1.

The Streptavidin-Biotin Horseradish Peroxidase (SAB-HRP) method was chosen for its sensitivity and ease of use. Because so many tumours were to be processed, it was decided to invest in a piece of equipment that would guarantee technical constancy. The Shandon 'Sequenza' Immunohistochemical Staining Station [Life Sciences International] was chosen for its ease and economy of use and the way in which slides could be stained without dehydrating. The experimental set-up of the equipment allowed a fixed volume of liquid to be in contact with the sections at any given time. The stained tissue sections were assessed for the intensity of the stain and for any changes under the influence of the experimental drugs.

In a selection of tumours, it was difficult to determine exactly which cells were epithelial and which were stromal. Anti-cytokeratin antibodies were used to highlight only the epithelial cells so that the pattern of TGFβ1 expression could be fully explored.

4.2 Methods

i. TGFβ1 Immunohistochemical Staining

Tissue Preparation: By the time that tissue was ready for immunohistochemistry, it was already sectioned and dried onto APES coated slides (as described in Section 2.3-2.6).

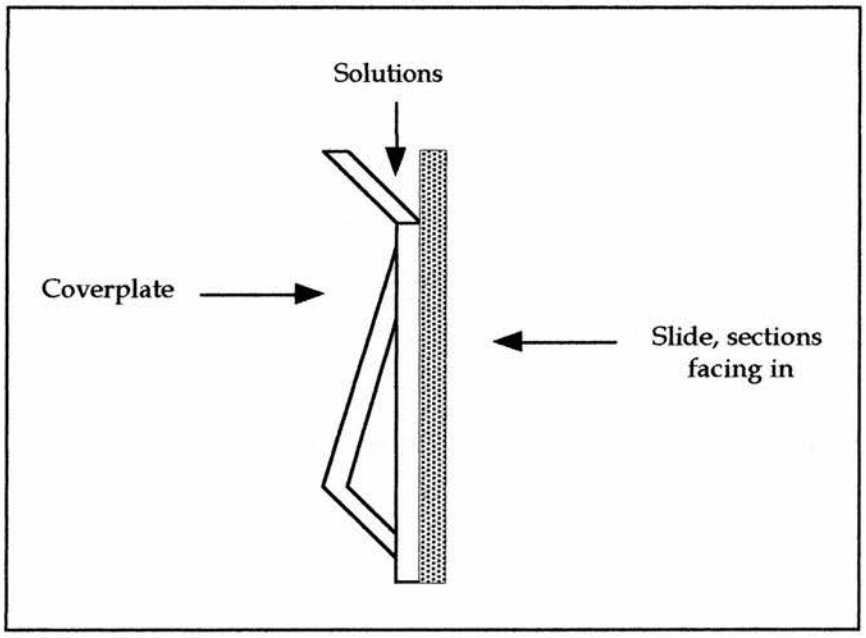


Figure 4.2 Diagram of Shandon 'Sequenza' Coverplate and Slide.

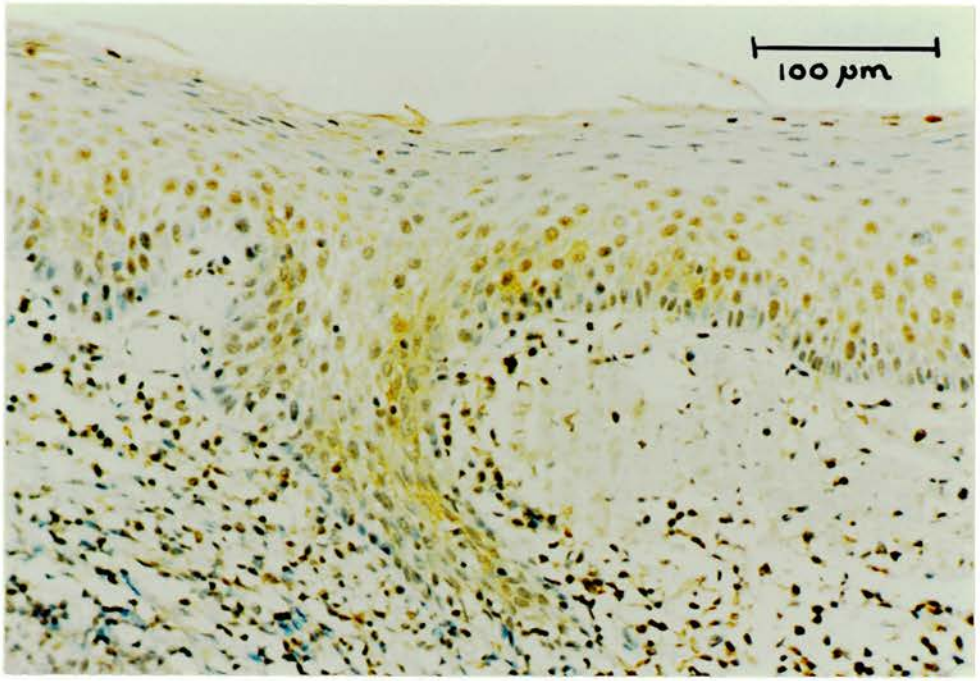


Figure 4.3 Tonsil Tissue as a Positive Anti-TGFβ1 Antibody Control (x16). Positive staining is clearly evident in the outer cortex of the tonsil tissue, showing that the TGFβ1 production is not particular to breast tissue.

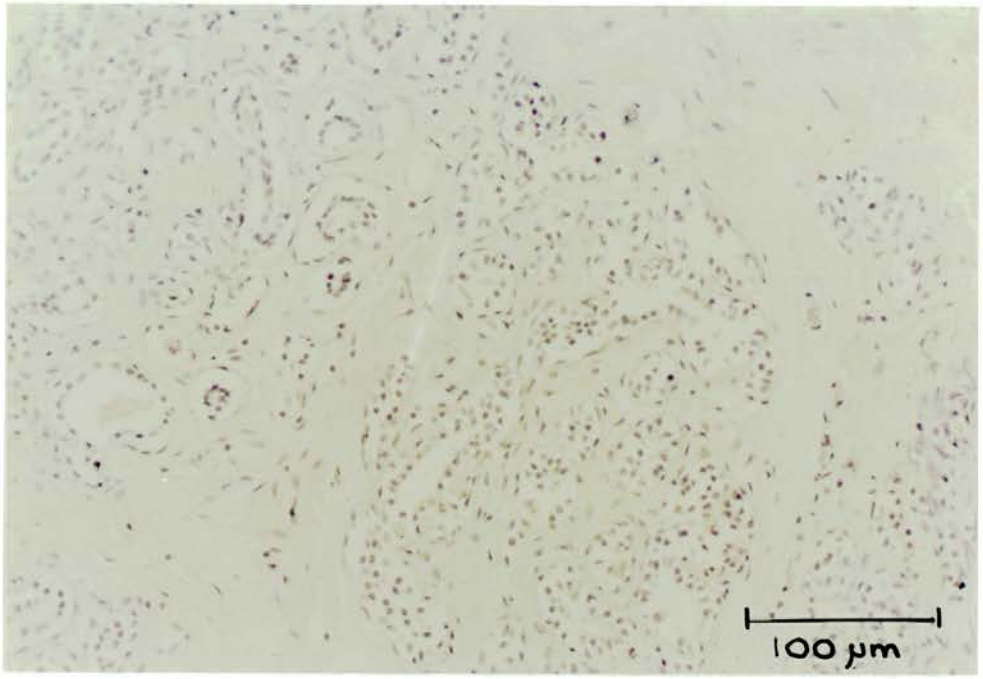


Figure 4.4 Breast Tissue as a Negative Anti-TGFβ1 Antibody Control (x16). This section of breast tissue was incubated without primary antibody. There is no antibody staining, showing that other reagents in the protocol do not binding non-specifically to other antigens in the tissue.

The sections were dewaxed through xylene (2 x 5 minutes), rehydrated through 100% and 96% ethanol (1 x 2 minutes each) then to methanol (2 minutes) and methanol peroxide (10 minutes). A solution of 30% Hydrogen Peroxide [BDH] was added to methanol [BDH] at a ratio of 1:10, giving a final concentration of 3% peroxide in methanol. This solution acted as a block to endogenous peroxidase activity by providing the substrate (peroxide) on which peroxidase works.

Antigen Unmasking: The slides were washed in running tap water then transferred to citrate buffer (10mM Citric Acid Monohydrate [BDH], pH 6.0) and microwaved on full power in a domestic microwave for 2 bursts of 3 minutes, a variation of the technique employed by Cattoretti *et al.* (1993). The high temperature attained in the microwave unmasks any antigenic sites that may have been encrypted by protein crosslinking during fixation. The slides were allowed to cool naturally to room temperature, with the addition of distilled water to ensure the sections did not dry out. They were washed in a Tris Buffered Saline (TBS - 200mM Tris, 1.54M NaCl, 20mM $MgCl_2$) then transferred to a Shandon 'Sequenza' (refer to Figure 4.2) and washed again with TBS, ready for immunohistochemistry.

Blocking: The slides were incubated with normal rabbit serum (NRS) [Scottish Antibody Production Unit, Carluke, Scotland], diluted 1:10 in TBS for 30 minutes at room temperature, to block any non-specific binding sites. The serum was not washed off the slides before application of the primary antibody.

Primary TGFβ1 Antibody: The antibody used in this research was a commercially supplied polyclonal chicken anti-human TGFβ1 immunoglobulin [R&D Systems, Abingdon, England; Catalogue no. AB-101-NA]. It had less than 2% cross-reactivity with human TGFβ3. It did not cross react with other isoforms of human TGFβ or any other cytokine. The stock concentration was 1mg/ml. Evaluation of optimal antibody concentration was carried out with various antibody dilutions (1:500, 1:250, 1:100, 1:50 and 1:10) on control tissues (tonsil for the positive control and breast tissue for the negative control - refer to Figures 4.3 and 4.4). The optimal dilution for stain intensity was 1:100, a concentration of 1μg/100μl. The anti-TGFβ1 antibody was made up in TBS at the appropriate dilution. The incubation time for anti-TGFβ1 was 18 hours (overnight) at 4°C. The primary antibody was washed off with 2 x 5 minute washes with TBS.

Secondary Antibody: The secondary antibody was a biotinylated rabbit anti-chicken immunoglobulin, raised against chicken-specific epitopes in the primary antibody, and conjugated to biotin [Jackson ImmunoResearch Laboratories, Inc.,

West Grove, PA, USA]. The stock concentration was 1.2mg/ml. The optimal concentration was evaluated by testing various dilutions of secondary antibody (1:1500, 1:1000, 1:500 and 1:200) on tissue which had been primed with a primary antibody dilution of 1:100. The optimal dilution was 1:1000, a concentration of 1.2μg/ml. The incubation time was 30 minutes at room temperature. The secondary antibody was washed off with 2 x 5 minute washes of TBS. During this incubation, the tertiary complex was made up and allowed to stand at room temperature so that the component solutions could mix.

Tertiary Complex: The tertiary solutions were Streptavidin in 0.01M Phosphate buffer, 0.15M NaCl, 15mM NaN_3 at pH 7.2 and Biotinylated Horseradish Peroxidase in 0.01M Phosphate buffer, 0.15M NaCl, 15mM NaN_3 [Dako A/S, Glostrup, Denmark]. The streptavidin binds to the biotin in the tertiary complex and in the secondary antibody so forms the link between the protein of interest and the detection system. The sections were incubated with the tertiary complex for 30 minutes at room temperature. The tertiary complex was washed off with 3 x 5 minute washes of TBS.

Colour Developer: The colour developer was Diaminobenzidine (DAB) [Dako]. DAB provided the substrate on which horseradish peroxidase worked. The reaction product was a solid, brown-coloured precipitate which formed in the target tissue. One DAB tablet was thawed to room temperature then dissolved in 20ml TBS with 20μl 30% Hydrogen Peroxide (final concentration = 0.00033M). DAB is a suspected carcinogen so was handled with all necessary precautions. The DAB was applied to the experimental tissue for 10 minutes, during which time the brown colour could be seen developing on the positive slides. The DAB was washed off with tap water and the slides were returned to a slide carrier and washed again in water to remove all traces of DAB. Waste DAB was de-activated with a solution of Presept [Johnson & Johnson, Ascot, England] and washed down the sink with excess water.

Counterstaining: The sections were counterstained in Ehrlich's Haematoxylin for 30 seconds, rinsed in distilled water, 'blued' in tap water and dehydrated through 96% and 100% ethanol to xylene. They were mounted in DPX mountant [BDH] with glass coverslips [BDH] and dried overnight.

All sections from each biopsy were treated in exactly the same way to eliminate technical variability.

ii. Assessment of Staining Intensity

The slides were viewed and scored for staining intensity on a Zeiss 'Axioskop' light microscope. Stain intensity was graded on a scale of 1 to 5, where 1 was no stain, 2 was pale stain, 3 was low level stain, 4 was medium level stain and 5 high level stain. Epithelial cells, stromal cells and the general background were scored separately. All sections were scored blind.

iii. Anti-cytokeratin Immunohistochemical Staining

Intermediate filaments, or cytokeratins, are abundant cytoplasmic structural proteins which form fibres of about 8-10nm in diameter. They are common in cells which are prone to mechanical stress, for example, epithelial cells.

About 29 cytokeratins have been identified and the different types can be used as markers for cellular differentiation and to identify tumour tissue.

Tissue Preparation: The tissue was prepared as before. Briefly, the sections were dewaxed, rehydrated and incubated with methanol peroxide.

Antigen Unmasking: The manufacturer of the anti-cytokeratin antibody [Sigma] recommended protease digestion to unmask hidden antigens. The sections were incubated with a solution of 0.1% trypsin [Gibco BRL] in 0.1% calcium chloride [BDH] (pH 7.8) for 10 minutes at 37°C. After TBS wash, the slides were transferred to the Sequenza.

Blocking: The blocking step was as before with 10% rabbit serum for 30 minutes.

Primary Anti-Pan Cytokeratin Antibody: The primary was a monoclonal mixture of antibodies [Sigma; Catalogue no. C-2931; Clone C-11]which recognised cytokeratin epitopes in most human epithelial cells. The dilution was 1:100 in TBS and the incubation time was 1 hour at room temperature.

Secondary Antibody was a biotinylated rabbit anti-mouse antibody [Sigma], diluted 1:300 in TBS and incubated for 30 minutes at room temperature.

The tertiary step, colour developing and counterstaining were as described for the TGFβ1 immunohistochemistry.

4.3 Results of Research

i. Pattern of TGFβ1 Protein Expression

Almost all sections stained positively with the anti-TGFβ1 antibody. From 97 measured data points, 3 (3.1%) showed no TGFβ1 expression, 7 (7.2%) were 'debatable' (+/-), 14 (14.4%) showed low TGFβ1 expression, 48 (49.5%) showed moderate expression and 25 (25.8%) showed high expression.

Figures 4.5 (a-d) show a tissue explant of 'debatable' TGFβ1 staining. This tumour was ER-negative and ductal in origin (histological grade 2). It was taken from a pre-menopausal patient. The explant, from which the micrographs were taken, had been cultured for one day in estrogen and tamoxifen. Its epithelial viability was good and these cells showed a very low reactivity with the anti-TGFβ1 antibody.

Figures 4.6 (a-d) show a tissue explant which expressed low amounts of TGFβ1 protein. The pathology report was not available for this patient. The explant was fresh-fixed, i.e. not cultured, so the preservation of the duct structures was excellent. One or two of the ducts are normal (Figure 4.6b), with a typical circular form and a single layer of epithelial cells. The tumour ducts are filled with cells and have an irregular appearance. At higher powers, one can see that not all the cells are positive for TGFβ1 protein and that the overall stain is patchy (Figure 4.6c).

Figures 4.7 (a-d) show a tumour which is producing moderate amounts of TGFβ1 protein. The patient was pre-menopausal and the tumour was ER-negative and thought to be of a medullary type carcinoma. This particular explant was cultured with tamoxifen for one day. The ducts are reasonably well preserved, with some normal ducts amongst the tumour (Figure 4.7b). In this tumour, the stromal cells are staining as strongly as the epithelial cells, as shown in Figure 4.7c.

Figures 4.8 (a-d) show a high TGFβ1 producer. The tumour was an ER-positive, ductal carcinoma (grade 2) from a pre-menopausal patient. The explant was control tissue from a day 8 culture. What is striking about the section, is the excellent cell viability and tissue architecture, despite the 8 day culture period. All the cells are staining strongly for TGFβ1 protein. In the low power (x16, Figure 4.8b), the few stromal cells present, appear negative but at higher power (Figure 4.8d), there is moderate staining in some interlobular stroma.

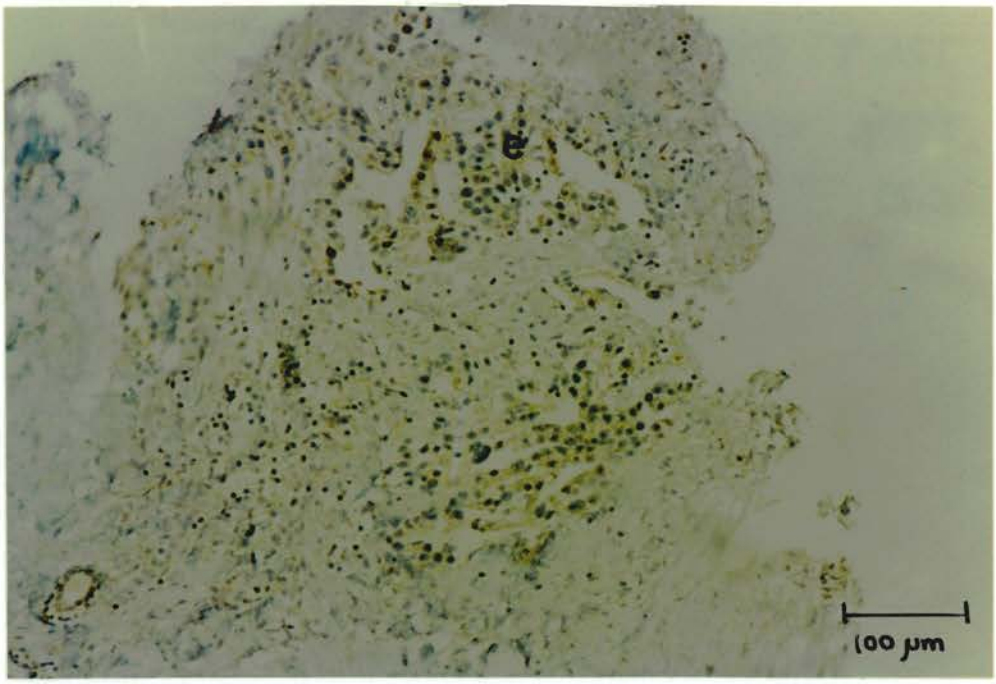


Figure 4.5a Tumour 126, 'Debatable' TGFβ1 Production (x10).
This section of tumour 126 had been treated with the estrogen/tamoxifen combination for one day (ET1). The large epithelial cells (e) are faintly positive for TGFβ1; the stromal cells are negative.

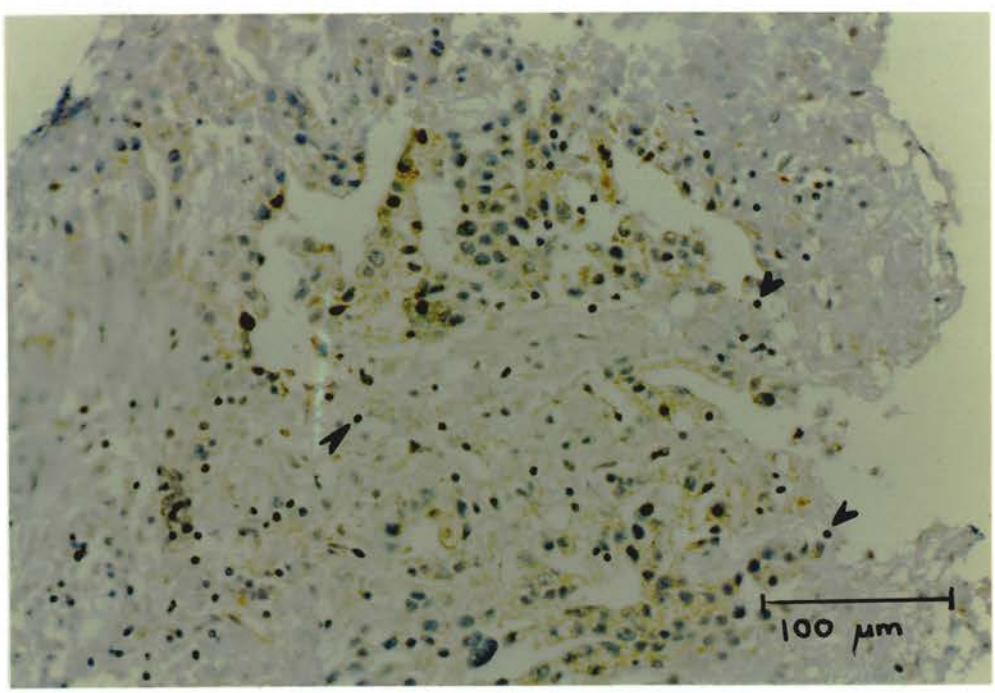


Figure 4.5b Tumour 126, 'Debatable' TGFβ1 Production (x16).
At slightly higher power, the faint stain in the epithelial cell can be seen more clearly. The dark, round nuclei (➢) are probably evidence of a lymphocytic infiltrate.

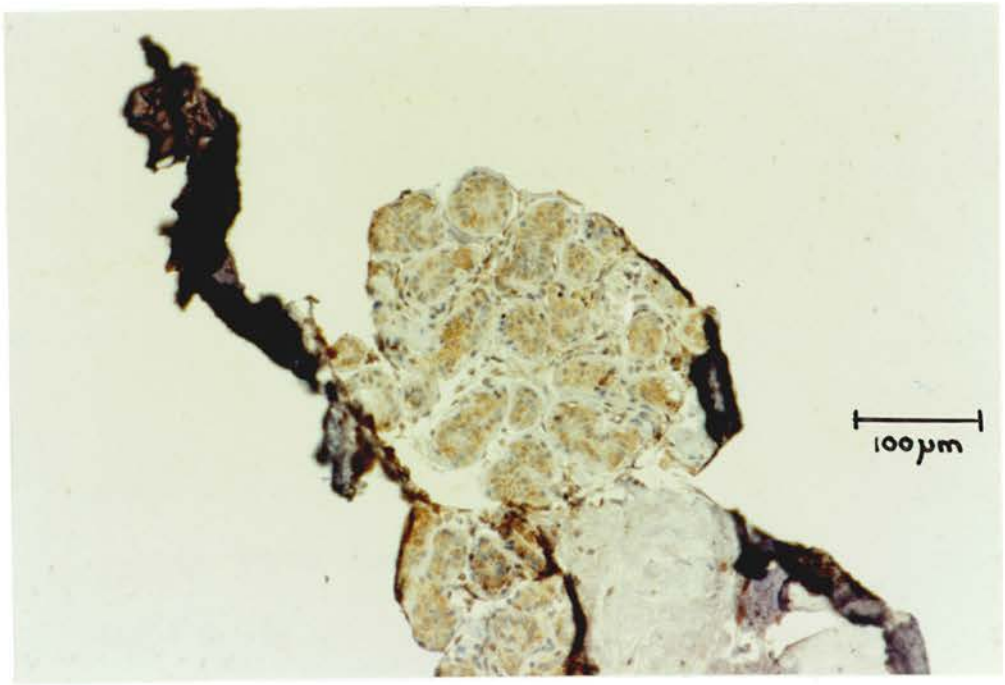


Figure 4.6a Tumour 052, 'Low' TGFβ1 Production (x10).
This section of tumour 052 was fresh-fixed tissue. There are several duct structures present and the epithelial cells show modest TGFβ1 production.

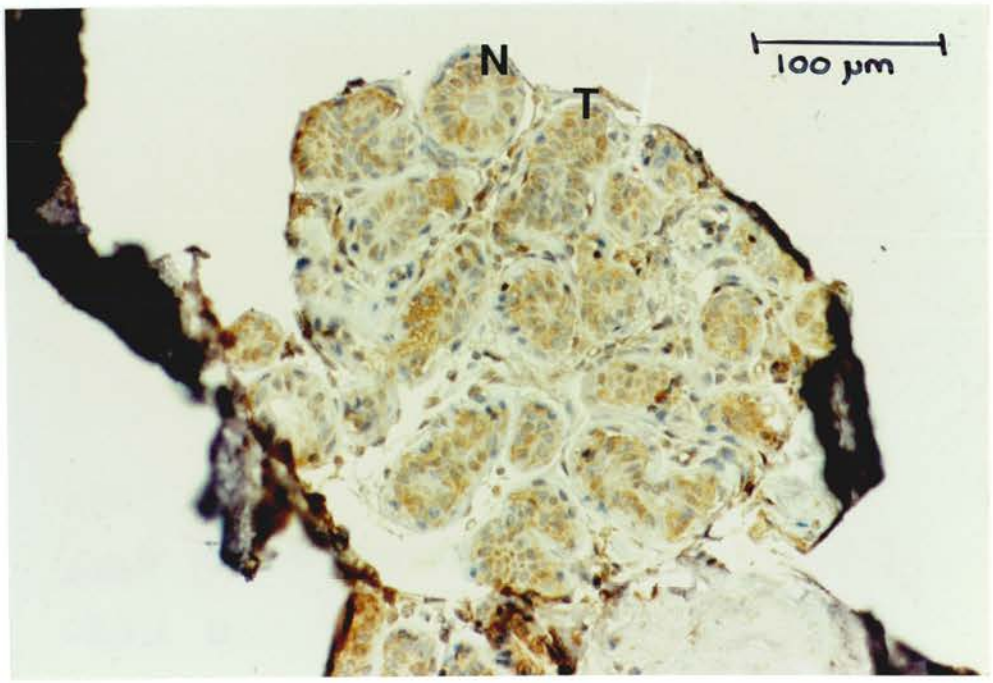


Figure 4.6b Tumour 052, 'Low' TGFβ1 Production (x16).
At slightly higher power, normal ducts (N), with a single layer of cells, and tumour ducts (T), with multiple layers of cells, can be seen.

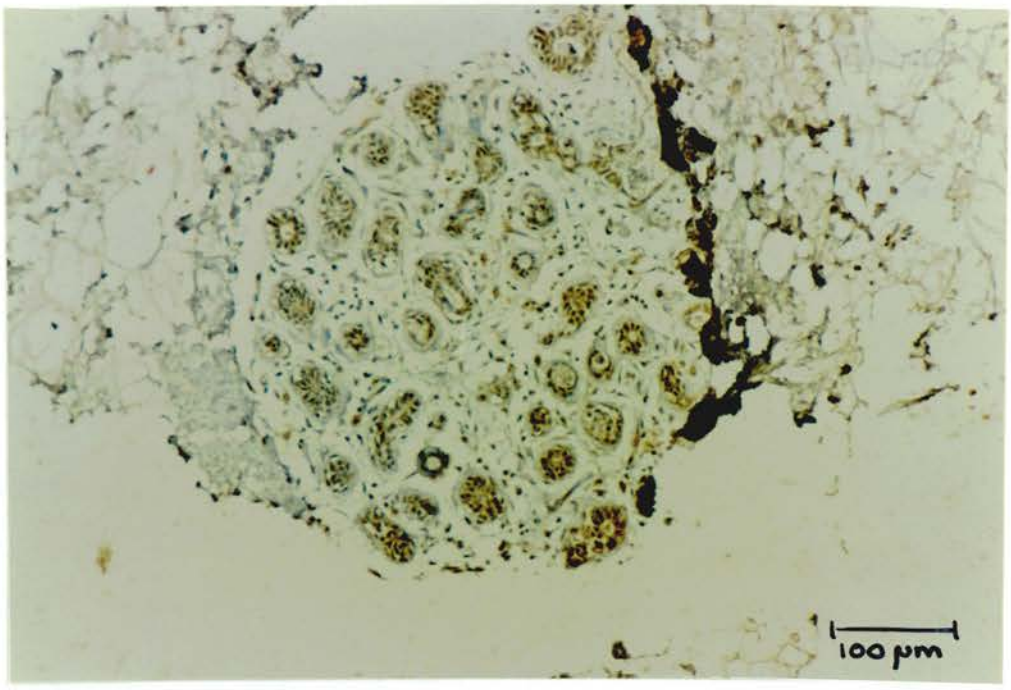


Figure 4.7a Tumour 069, 'Moderate' TGFβ1 Production (x10).
This section of tumour 069 was tamoxifen-treated for one day in culture (T1). There are several duct structures present and the epithelial cells show moderate TGFβ1 production.

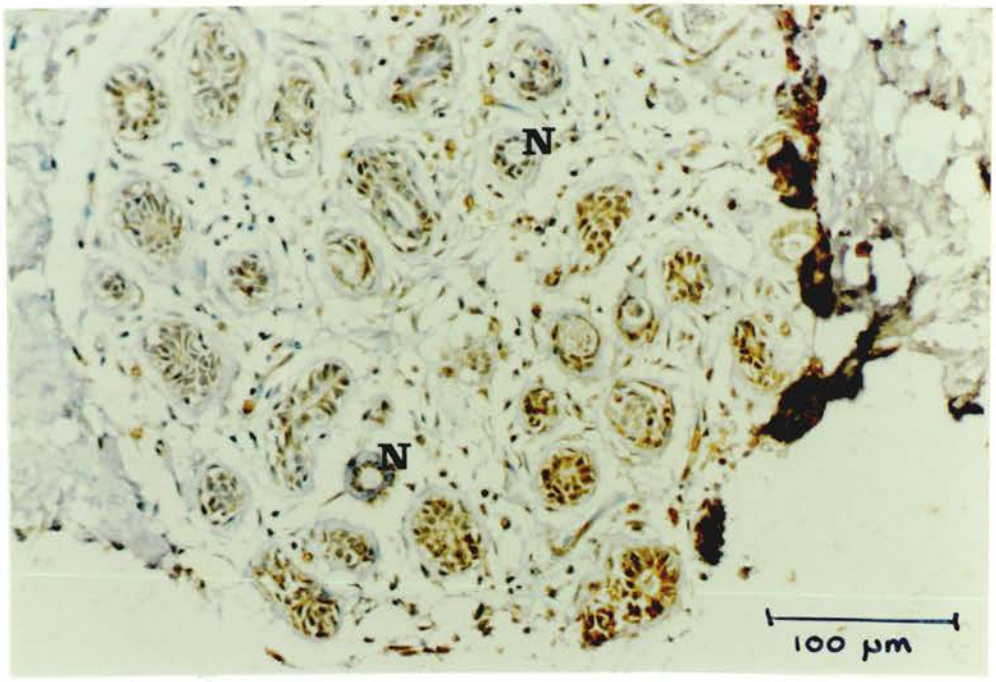


Figure 4.7b Tumour 069, 'Moderate' TGFβ1 Production (x16).
Most ducts are cancerous. The normal ducts are indicated (N).

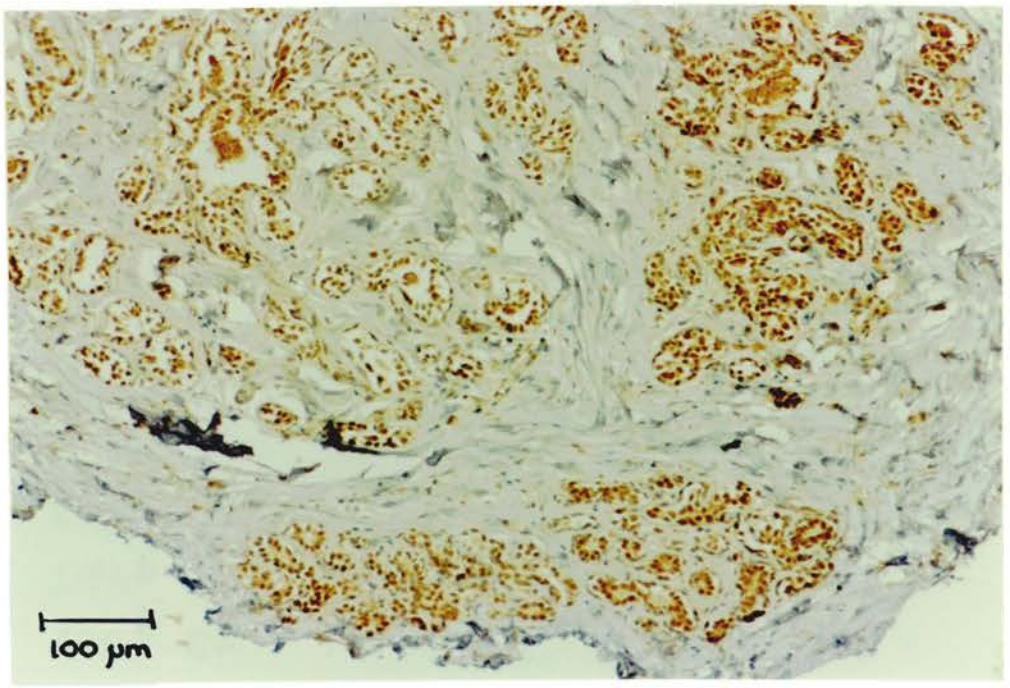


Figure 4.8a Tumour 132, 'High' TGFβ1 Production (x10).
This section of tumour 132 was a control explant, cultured for 8 days (C8). The micrograph shows an extensive duct system of good viability. The epithelial cells are all strongly stained for TGFβ1 protein

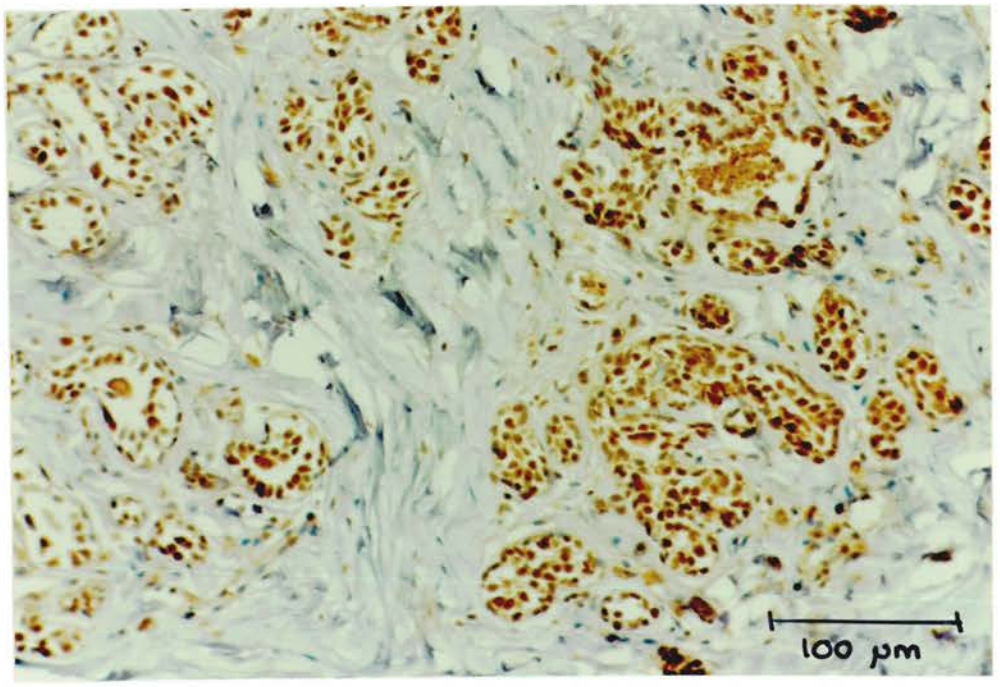


Figure 4.8b Tumour 132, 'High' TGFβ1 Production (x16).
At slightly higher power, the multi-cellular nature of the ductal cancer is apparent.

Some tumours were highly cellular, showing signs of a strong cellular stromal reaction. In these cases, it was difficult to differentiate between cell types. It was possible that the fibrous tissue could be made up of invading epithelial cells as well as stromal cells. Anti-cytokeratin antibodies were used to identify the epithelial cells. The pattern of expression was then compared to that of the TGFβ1 pattern in the same section so that the identity of TGFβ1 producers became clear.

A TGFβ1 section is shown with a comparable cytokeratin section in Figures 4.9 and 4.10.

ii. TGFβ1 Production in Response to Experimental Drugs

Twenty-four biopsies yielded enough cancerous tissue to culture Days 1, 4 and 8 time points. Tumours which yielded only 1 or 2 time points were also included in the study - there were 6 of these. They were immunohistochemically stained for TGFβ1 protein. Due to the inability to predict histological quality before culture, several of the sectioned explants (sometimes entire tumour series) had to be disqualified from the study, due to acellularity and/or poor sectioning.

Positivity for TGFβ1 stain was observed in almost all cellular sections. It was clear, however, that the levels of TGFβ1 stain were variable.

Statistical analyses were performed to assess whether or not the differences in TGFβ1 levels were significantly related to the experimental conditions. The ranked data for the TGFβ1 experiments were categorised in 3 ways. First, the data were assessed by drug treatment; second, by length of drug exposure time and third, by initial level of TGFβ1 staining in fresh fixed tissue. Subsequent statistical analyses were non-parametric, using the Mann-Whitney U statistic for comparisons between 2 samples and the Kruskall-Wallis statistic for testing multiple conditions.

The data collected from the effect of drug treatment experiment showed no obvious pattern when ranked. Statistical testing confirmed that there was no significant difference between the levels of TGFβ1 in one drug group compared any of the other drug groups. The same result was derived in the category of length of drug exposure time.

Significant differences were observed in one group in the category of initial TGFβ1 production using a one-tailed Kruskall-Wallis statistical test. Tumours expressing maximal amounts of TGFβ1 (rank 5) in the fresh tissue showed no significant response to estrogen, tamoxifen or to the combination treatment

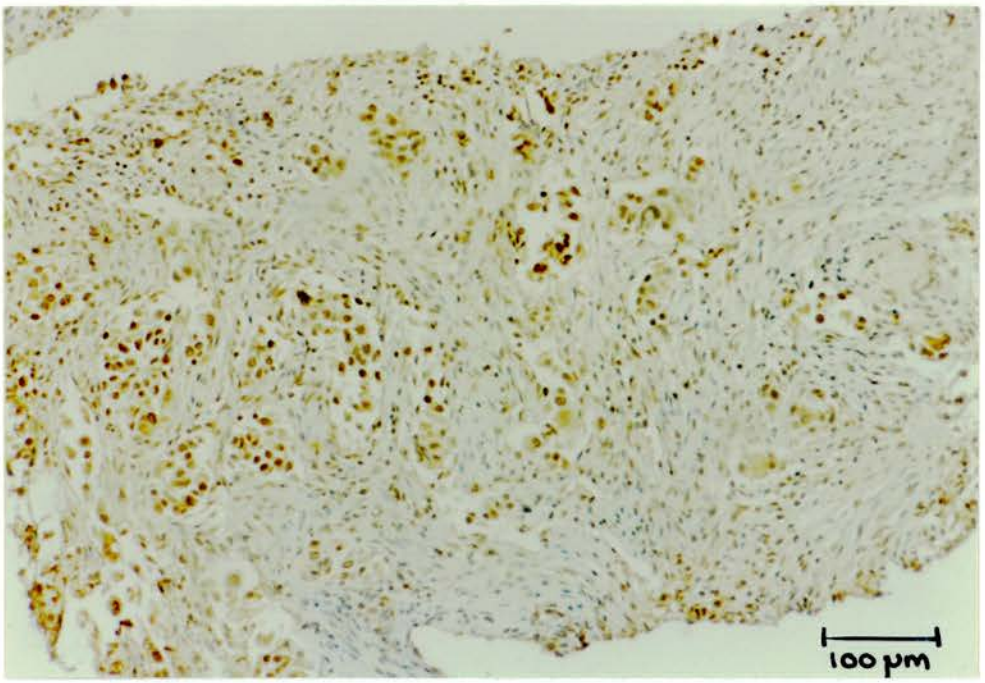


Figure 4.9 Tumour 124, 'Moderate' TGFβ1 (x10).

This section of tumour 124 is highly cellular. The epithelial islands (e) are distinct and are producing moderate amounts of TGFβ1. The other cells are 'debatable' to low producers.

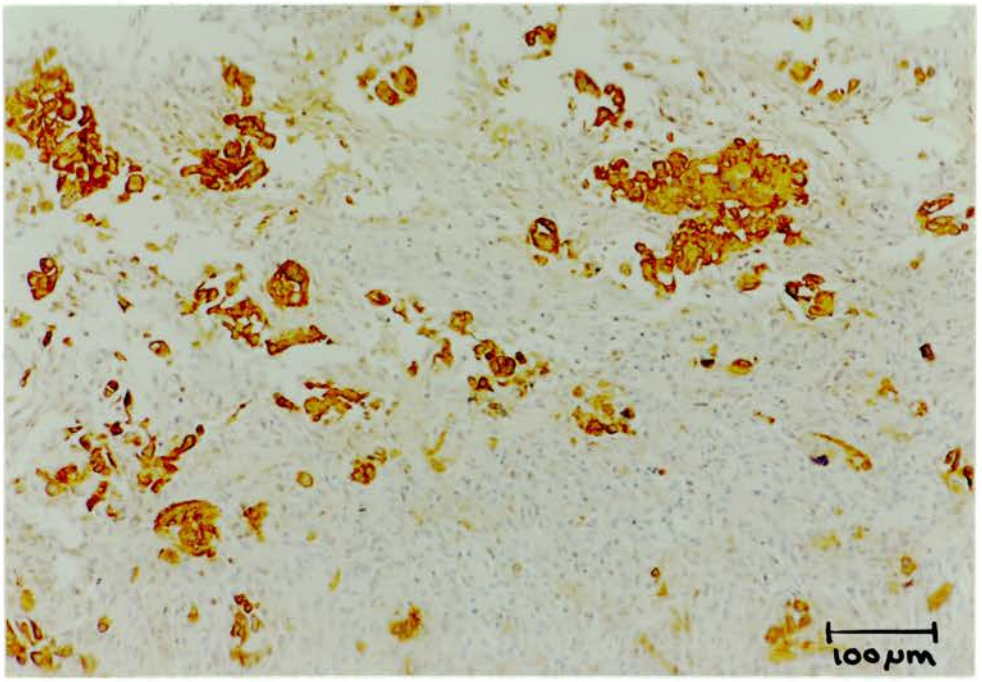


Figure 4.10 Tumour 124, Cytokeratin Production (x10).

The epithelial cells are clearly marked by the anti-cytokeratin antibody, showing that the other cells are, indeed, stromal, and not invaded epithelial cells.

($p>0.05$). Tumours expressing low initial levels of TGFβ1 protein (rank 3) did not respond significantly ($p>0.05$) to drug treatments either.

Tumours expressing medium amounts of TGFβ1 protein (rank 4) did respond significantly to one of the drug treatments ($p=0.027$). Post-hoc testing using a Mann-Whitney U test (ties included), showed that TGFβ1 production was significantly increased in the estrogen-tamoxifen treated tissue compared to the control ($p=0.0147$) and estrogen alone ($p=0.0322$). There was no significant increase in TGFβ1 production of estrogen-tamoxifen treatment compared to tamoxifen alone.

iii. Comparison of TGFβ1 Production with Viability Studies

Viability counts were made on the drug treated tissue in the same way as on the time course tissue i.e. for every drug condition, the percentage of apoptosis was assessed and graphed with error bars (the results of the time course cell viability are discussed in Chapter 3).

In short, no significant effect of the drugs on apoptosis could be statistically proven. Because of this, no comparison with TGFβ1 staining intensity could be drawn, even in the rank 4 group where a significant increase in TGFβ protein was observed.

4.4 Discussion of Results

i. Pattern of TGFβ1 Protein Expression

The experiments show that fresh breast tissue, cultured over a period of 8 days, can be successfully analysed by immunohistochemistry. The technique allowed the staining of distinct sites of TGFβ1 antibody positivity in the cultured breast tissue.

Almost all the tumours sections and all the control sections treated with the anti-TGFβ1 antibody stained positively to some degree, which demonstrates that TGFβ1 is a ubiquitous protein, expressed in most breast cancer cells as shown by McCune *et al.* (1992) and Walker and Dearing (1992).

The staining intensities were variable because each tumour came from a different person. Even tumours of the same pathological type will have different

physical and biochemical properties. It was hoped that pooling the data would reveal a trend between TGFβ1 and drug treatment. A similar study by Gorsch *et al.* (1992) gave results of moderate TGFβ1 expression in 30% of their paraffin-embedded breast tumours and high TGFβ1 expression in 70% of tumours. The results of the present study differ from the previous study in that the commonest staining was moderate in this case as compared with high expression by Gorsch *et al.* This is probably due to technical variation. However, both studies concur that the majority of breast tissue expresses TGFβ1 and that few tumour sections (10.3% in the present study) are negative for TGFβ1 stain.

The immunohistochemistry in the present study revealed that, with the exception of a few TGFβ1-negative cases, the epithelial cells showed stronger antibody positivity than the stromal cells.

There are 2 theories concerning the site of TGFβ production in the breast. The following 3 studies were immunohistochemical in nature and performed on paraffin wax-embedded breast tissue. They all showed that the most prominent TGFβ1 staining was confined to the cytoplasm of the ductal epithelium.

McCune *et al.* (1992) localised the 3 TGFβ isoforms in different breast lesions. They found that almost all active breast epithelia contain TGFβs 1, 2 and 3, indicating the potential for TGFβ to act as a paracrine and autocrine factor in the regulation of breast cell growth. Gorsch *et al.* (1992) undertook a retrospective study on breast cancer tissue, using anti-TGFβ1 antibodies. Positive TGFβ1 stain was most prominent in the cytoplasm of the ductal epithelium. In addition, they found that intense TGFβ1 staining was associated with disease progression and aggressiveness. The same relationship did not exist for TGFβ2 or 3, suggesting that only TGFβ1 is involved in the progression of disease. Walker and Dearing (1992) found a range of staining patterns. Of the invasive tumours they tested, 33% of them showed low to moderate epithelial staining and 11% showed prominent staining. This study also revealed an association between TGFβ1 staining and lymph node metastasis.

It is known that stromal cells can respond to TGFβ (Chiquet-Ehrismann *et al.*, 1989) and, in contrast to the above studies, several workers have shown that TGFβ is detected in the stromal cells of the breast. Butta *et al.* (1992) carried out a retrospective *in vivo* study of breast cancer patients before and after tamoxifen treatment and found that tamoxifen upregulated TGFβ production from mammary stromal cells and that the staining intensity of the epithelial cells was unchanged

after the administration of tamoxifen. These findings concur with an *in vitro* study by Colletta *et al.* (1990) whereby the production of TGFβ was stimulated by treating ER-negative fetal fibroblasts with tamoxifen. Van Roozendaal *et al.* (1995) cultured breast cancer fibroblasts and used their conditioned medium to inhibit the growth of a reporter cell line. The fibroblasts produced biologically latent TGFβ, which became active upon heat treatment. On addition of hydroxy-tamoxifen into the culture, only 37.5% of the tumour-derived fibroblast cultures showed a significant increase in TGFβ production - again, the protein was latent which is in contrast to the situation in MCF-7 epithelial cells (Knabbe *et al.*, 1987) and in fetal fibroblasts (Colletta *et al.*, 1990) where active TGFβ is secreted. The authors conclude their study by saying that the lack of paracrine signals from epithelial cells and/or extracellular matrix may have resulted in the secretion of latent TGFβ rather than the active form.

The results of the present study correspond with the former group of studies i.e. that epithelial cells are the main sites of TGFβ1 production. This conclusion implies that TGFβ1 is acting as a negative autocrine factor, inducing cell cycle arrest in the same cells from which it is produced, and that upregulation of TGFβ1 as therapy for breast cancer can be a worthwhile practice, as long as the signalling pathway is intact.

As part of the bigger picture, these results highlight the autocrine hypothesis (reviewed by Sporn and Roberts, 1985) which states that malignant transformation and cancer cell growth progression may be the result of a failure to respond to negative autocrine growth factors, such as TGFβ1, as well as a response to stimulatory autocrine factors.

ii. Cytokeratin Production in Tumour Sections

The anti-cytokeratin antibody clearly stained the epithelial cells. In some sections, stromal cells stained positively for TGFβ1 protein as well as the epithelial cells, as demonstrated in Figures 4.8d and 4.9.

It can be stated then, that, *in vivo*, the TGFβ pathway is not, as a rule, stimulated directly by tamoxifen either in the epithelial cells or in the stromal cells. Perhaps, *in vivo*, where cellular interaction is normal, the two cell types co-operate in regulating the production of growth factors.

iii. TGFβ1 Production in Response to Experimental Drugs

The TGFβ1 immunohistochemical data were grouped in different ways then subjected to statistical testing.

Grouped by Drug Treatment: No significant differences in TGFβ1 levels were observed between the different drug groups, suggesting that estrogen, tamoxifen or a combination of both do not affect the amount of TGFβ1 being produced. The expected results, extrapolated from previous studies, would suggest that tamoxifen upregulates the synthesis of TGFβ protein and estradiol downregulates its production (Knabbe *et al.*, 1987). However, when Arrick *et al.* (1990) examined the response of ER-positive breast cancer cell lines to estradiol with respect to TGFβ production, they found that the 3 isoforms were affected in different ways. The level of TGFβ2 and β3 mRNA was decreased by the addition of estradiol into the culture system while the level of TGFβ1 mRNA remained unchanged.

Grouped by Time in Culture: No significant statistical difference in TGFβ staining intensity was observed between the culture periods of one day, 4 days and 8 days. No conclusion could be drawn as to the time at which the drugs may affect TGFβ protein synthesis. It is possible, however, that treating the different tumours as a single group may have concealed some subtle effect of the experimental conditions.

Grouped by Staining Intensity in Fresh Tissue: The data were grouped by initial staining intensity in their fresh tissue counterparts. 'No stain' was matched with no TGFβ1 expression (rank 1), rank 2 was the category for patchy or 'debatable' stain, pale stain equated to low TGFβ1 expression (rank 3), medium stain was matched with moderate TGFβ1 expression (rank 4) and intense stain equated to high TGFβ1 expression (rank 5). A significant statistical difference of the effect of the drugs was observed only in the rank 4 group.

The rank 5 group showed no effect of drugs on TGFβ levels, perhaps because the cells could not be induced to produce any more TGFβ than they had already been doing.

Similarly, no differences were observed in the rank 3 group. This could have been due to the fact that the cells were incapable of producing more protein - only a residual amount was detected by the antibody. A breakdown in the signalling mechanism which would normally have relayed the effects of the drugs could explain this inability to increase or decrease the production of TGFβ1.

The rank 4 group showed a significant increase in the level of TGFβ1 protein in the Estrogen-Tamoxifen group compared to the control and the estrogen group but not compared to the tamoxifen group. The presence of estrogen *and* tamoxifen caused a stimulatory effect on TGFβ protein synthesis, indicating that the anti-estrogenic signalling pathway was intact but that the fresh tissue was not producing protein to its maximal capacity. This result may also point towards an effector role of estrogen on tamoxifen. Darbre *et al.* (1984) found that in serum-free cell line culture, a combination of estrogen and tamoxifen was a more effective growth inhibitor than tamoxifen alone.

What is clear from the literature is that there is not, and never has been, a unanimous consensus on what is happening in breast cancer cells in response to estrogens and anti-estrogens. The evidence from cell lines is often contradictory and with very few *in vivo* trials undertaken, it is difficult to say how the present results might have been expected to correlate with published findings. Nevertheless, my evidence does not support a consistent, simple or direct relationship between estrogen antagonism and TGFβ1 upregulation in human breast cancer.

iv. Comparison of TGFβ1 Production with Viability Studies

Because the control tissue in the present research showed wide variability with respect to apoptosis, the effects of the experimental drugs on apoptosis could be neither confirmed nor denied.

In part, this reflects the need for multiple replicates of explant tissue at each time point so that the end-point data is derived from adequate numbers of cells. However, a further useful conclusion from this work is that apoptosis may not be a reliable indicator of tissue response to experimental drugs. A more productive method of measuring cell survival could be thymidine uptake or MTT proliferation assay.

TGFβ1 normally upregulates p15 expression (Hannon and Beach, 1994) at both the mRNA and protein levels (Sandhu *et al.*, 1997), which suggests that p15 is a effector of TGFβ1-mediated cell cycle arrest. p15 is located on chromosome 9, at a site which is frequently abnormal in human tumours. The malfunction of p15 has been suggested as a factor in the breakdown of the TGFβ1 pathway (Sandhu *et al.*, 1997). It is possible that there has been a breakdown in the inhibitory pathway of TGFβ1 in the rank 4 group of tumours, where a significant increase in TGFβ1 under

the influence of estrogen and tamoxifen did not correlate with a detectable increase in cell death.

The actual site of TGFβ1 gene expression cannot be established conclusively by immunohistochemistry. It may be that the protein is transported to the epithelial cells by some other mechanism, rather than actually produced in these cells. An aim of this project was to establish the site of TGFβ1 production using *in situ* hybridisation.

v. Future Improvements

The immunohistochemical study highlighted the problem of retrospective research because many data points were lost or unreliable due to poor tissue sections. Again, this highlights the need for multiple explant replicates in culture.

CHAPTER 5

CLONING TO GENERATE RIBOPROBES

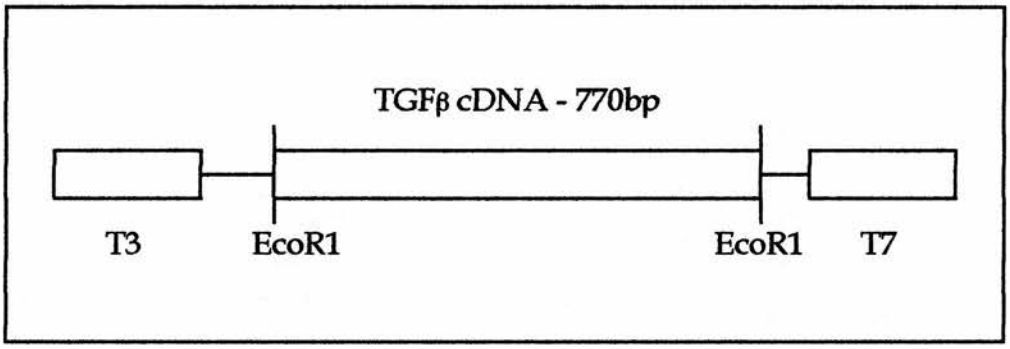


Figure 5.1 Illustration of the TGFβ Insert in pBluescript.

5.1 Background

Riboprobes are short, single-stranded nucleotide chains of RNA. They are generated by *in vitro* transcription from a linearised template. RNA polymerase promoters are required in the vector DNA which the contains the template. Their advantage over DNA probes is that RNA-RNA hybridisation bonds are stronger than RNA-DNA which in turn are stronger than DNA-DNA (Wetmur *et al.*, 1981). The shortness of riboprobes means that they have high penetrance into target tissue but they can only cover a small area. Being single-stranded means that the probe strands are unlikely to renature with one another.

Having found low hybridisation with DNA probes, riboprobes were made for the detection of TGFβ1 mRNA. TGFβ1 cDNA was used as the template from which the riboprobes were to be transcribed.

5.2 Methods

i. TGFβ Riboprobes

Transforming Growth Factor β1 cDNA: The cDNA used was a gift from Dr. Janet Smith, Department of Anatomy, Cambridge University and was used with permission from the original owner, Dr. Rosemary Akhurst, formerly of the Department of Genetics, Glasgow University.

The cDNA was a full length TGFβ coding sequence (i.e. no introns) inserted into the EcoR1 site of the multiple cloning site in plasmid pBluescript (refer to Figure 5.1). The insert length of the cDNA was 770 base pairs.

Transfection: The bacteria used in this transfection were ampicillin-sensitive dh5α *E. coli*, made competent by the Rubidium Chloride method (refer to section 2.8). Required for transfection were 100ml of competent bacteria, 100ng DNA (1ml of plasmid), S.O.C. medium [Gibco BRL] which is complex cell growth medium used to ensure maximal transfection efficiency and ampicillin LB Agar [Gibco BRL].

One microlitre of plasmid was added to 100μl of competent bacteria in 5ml polypropylene tube [Sarstedt, Numbrecht, Germany]. They were left on ice for 20 minutes while the orbital and the bench incubators were set to 37°C. The bacteria were incubated at 42°C in a waterbath for 1 minute to heat-shock the cells into taking up the plasmids. S.O.C. medium was added (900ml) then the tubes were

incubated in the orbital incubator [Gallenkamp] at 37°C and 225rpm for 45 minutes. The bacteria were then plated out onto the agar plates at high (400ml/plate) and low (100ml/plate) concentrations. The plates were incubated for 16-18 hours at 37°C in bench incubators [Merck].

Inoculation: The bacteria that grew on the plates were all successfully transformed as the plasmid confers ampicillin resistance, so any untransformed bacteria would die in the Ampicillin+ agar. One large colony from each plate was picked with a sterile Gilson pipette tip and added to 2ml of Ampicillin+ LB Agar. The colonies were 'shaken' overnight in the orbital incubator at 37°C and 225rpm. The next day, the cultures were cloudy with bacterial growth.

Miniprep DNA Purification: Miniprep techniques allow small-scale purifications of plasmid DNA from bacterial cells. The system used here was the Wizard® Plus SV Miniprep DNA Purification System [Promega]. This kit eliminates the problems of variable DNA yield and time-consumption as it is a reliable and simple method. When high copy number plasmids are used, the resultant DNA can be used in restriction enzyme digestion reactions without further manipulation.

The general principles of the kit were to lyse the bacterial cells and free the plasmids then wash the plasmids of any cell debris so that the final product was clean plasmid DNA. The result of the purification step is circular plasmid DNA containing the insert of interest. The method was as follows:-

The overnight bacterial culture of transfected *E. coli* cells was centrifuged at 10, 000*g* for 10 minutes then resuspended in Resuspension Buffer (50mM Tris-HCl pH 7.5, 10mM EDTA and 100mg/ml RNase A). The resuspended cells were incubated with 250ml Lysis Buffer (0.2M NaOH, 1% SDS) for 1-5 minutes and then a further 5 minutes with 10µl Alkaline Protease. Alkali treatment breaks open the bacterial cell membrane and releases the plasmids into the buffer. Neutralisation causes the cell debris and proteins to precipitate so the lysate was incubated for 10 minutes at 10,000g in 350ml of Neutralisation Buffer. The pellet was discarded and the lysate was poured into a Miniprep Spin Column and centrifuged at 14, 000g for 1 minute. During this spin, the plasmid DNA stuck to the membrane in the spin column and any debris flowed through and was discarded. The DNA was washed by spinning the column with Wash Solution twice then spun into a sterile Eppendorf tube with nuclease-free water. The DNA content was quantified on a spectrophotometer and by running an agarose gel.

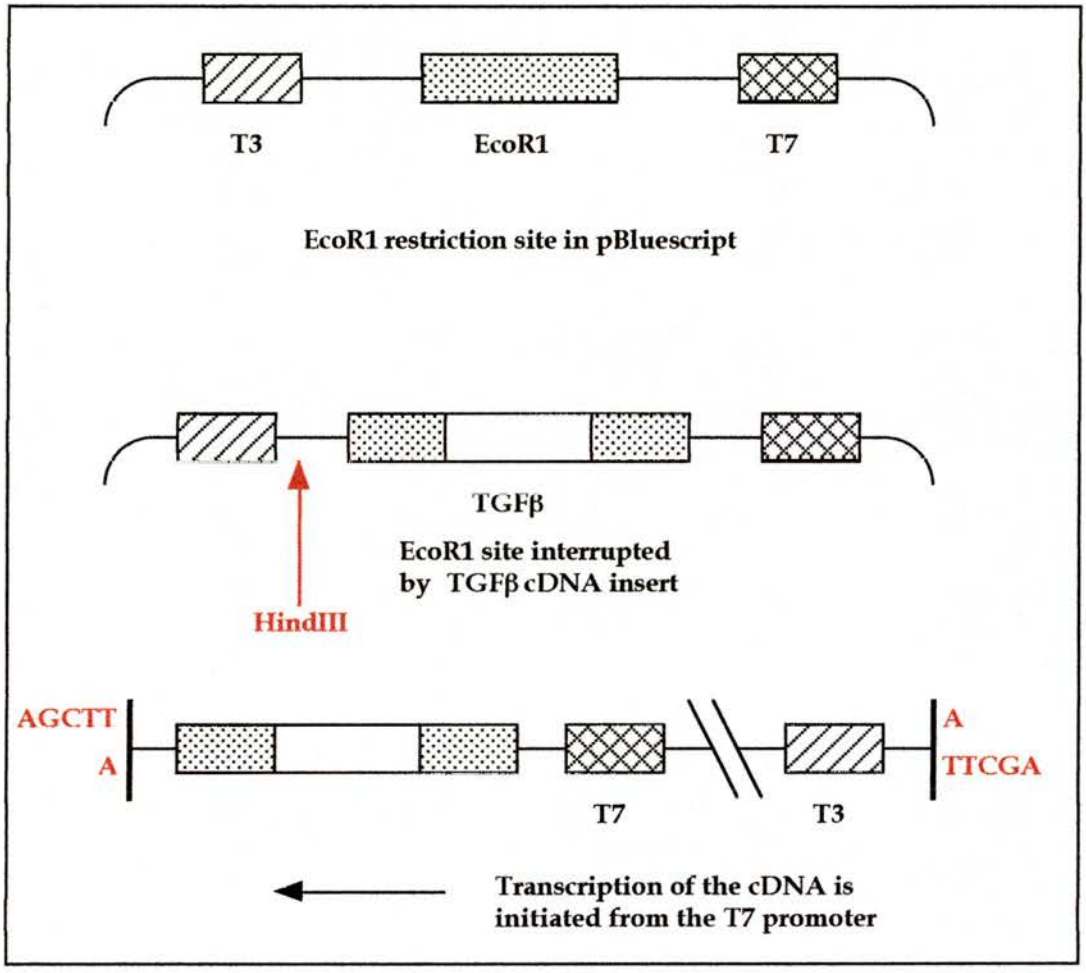


Figure 5.2 Restriction Enzyme Digest of TGFβ cDNA in pBluescript.

The circular DNA of pBluescript was cut with EcoR1 and the TGFβ insert ligated into the site. HindIII was used to cut at A / AGCTT, leaving the T7 promoter to initiate transcription of the TGFβ cDNA.

The result of purifying dh5α *E. coli* with TGFβ cDNA insert was 14.5mg DNA/100ml water.

Restriction Enzyme Digest: The plasmid DNA was circular and had to be linearised before transcription of the insert could occur. Linearisation was done with a restriction enzyme which has a single cut site in the plasmid downstream of the insert. The enzyme used in this case was Hind III [Gibco BRL] and was supplied with its appropriate restriction buffer. The digest is represented by Figure 5.2.

The digest mix contained 45ml plasmid (6.53mg DNA), 1μl enzyme (10 units of activity), 5.5μl 10x buffer (final volume was 55μl) and 2.5μl dH_2O to make up to 55μl. The mixture was incubated at 37°C in a waterbath for 2.5 hours.

The result of the restriction enzyme digest was linearised plasmid DNA with the T7 promoter upstream of the insert. The DNA required further purification to get rid of all the enzyme and buffer (as they are not guaranteed DNase-free).

Post-Digest DNA Purification: This was done using QIAquick™ DNA Purification Kit [Qiagen Ltd, Crawley, West Sussex, England]. The recovery efficiency of DNA was 90-95%. The principle of the procedure was to wash all the enzyme and buffer from the DNA. The method was as follows:-

To 1 volume of DNA, 5 volumes of PB Buffer were added then pipetted into a Qiaquick Spin Column. The column was centrifuged for 60 seconds at 10,000g and the flowthrough discarded. The DNA was then washed in PE buffer and centrifuged again. The column was assembled into a sterile Eppendorf tube and the DNA was eluted with 50μl of distilled water. The result of this procedure was pure plasmid DNA with the TGFβ insert, ready for *in vitro* transcription.

Evaluation of Probe Concentration: Labelling of the probe by *in vitro* transcription resulted in quantities of only 20μl, too small to quantify accurately. Instead, a dot blot was performed with the newly synthesised probes against a standard DIG-labelled RNA probe. Standard and experimental probes were diluted 1:10 initially, then dotted onto nitrocellulose paper in doubling dilutions. The spots were air dried then blocked with modified TBS for 30 minutes as in the *in situ* hybridisation protocol. Anti-DIG antibody was incubated with the paper for 2 hours then washed off with TBS modified and water. 'Fast Red' substrate was used to develop the colour reaction. The spots of probe were compared by eye against the known standard and approximately quantified.

ii. β-Actin Control Probes

β-Actin cDNA: This cellular protein is one of the most abundant to be found in mammalian and avian non-muscle tissue. It is a cytoskeletal protein expressed uniformly in cells. It is commonly used as a control probe. The β-actin cDNA from which riboprobes were to be transcribed was commercially supplied by Oncor. It was a 770 base pair fragment of chicken β-actin which, from tests by the supplier, was known to cross-hybridise with human β-actin message.

Creating Restriction Sites on cDNA: The ends of the cDNA were not compatible with the plasmid, pGEM-T-Easy [Promega] so suitable restriction fragments had to be added to allow ligation of the cDNA into the plasmid. Small 8-mer fragments of Nde1 restriction sites were chosen as pGEM-T-Easy has only one Nde1 site in the multiple cloning site and nowhere else in the plasmid. The Nde1 phosphorylated linkers were supplied by Stratagene. The method was as follows:-

The reaction mix contained a 100-fold molar excess of linkers to cDNA which equated to 100ng of cDNA and 104ng of linkers. The reaction mix also required 1μl of 10x buffer, 3 units (1μl) of T4 DNA ligase [Promega], made up to 10μl with nuclease-free water. The mix was incubated at 4°C overnight then heated to 70°C and quenched on ice to stop the reaction.

To cut the linkers down to one each side of the cDNA, it was cut with Nde1 restriction enzyme. The plasmid was also cut to linearise it. The cDNA and plasmid now had compatible sticky ends.

After the digest, the cDNA was purified free from enzyme and excess linkers using a Microcon Purification kit [Amicon Inc., Beverly, MA, USA]. The principle of the procedure is to capture larger fragments of DNA (cDNA) on a membrane and wash it free of smaller pieces (linker). The result is cDNA in 100μl of nuclease-free water.

Preparation of Plasmid: The plasmid, pGEM-T-Easy, was supplied as part of a kit from Promega (pGEM-T-Easy Vector System I). The plasmid was linearised when supplied with Thymidine overhangs at the cut site. The overhanging 3'-T ends had to be removed and the plasmid circularised. The following mix was used to chew-off the 3' ends - 150ng plasmid, 10x T4 DNA polymerase Buffer, 2.5mM of 4 dNTPs and 3 units of T4 DNA polymerase [Gibco BRL]. The mix was incubated at 37°C for 5 minutes to allow the reaction to continue then heated to 75°C for 10 minutes to destroy the enzyme and stop the

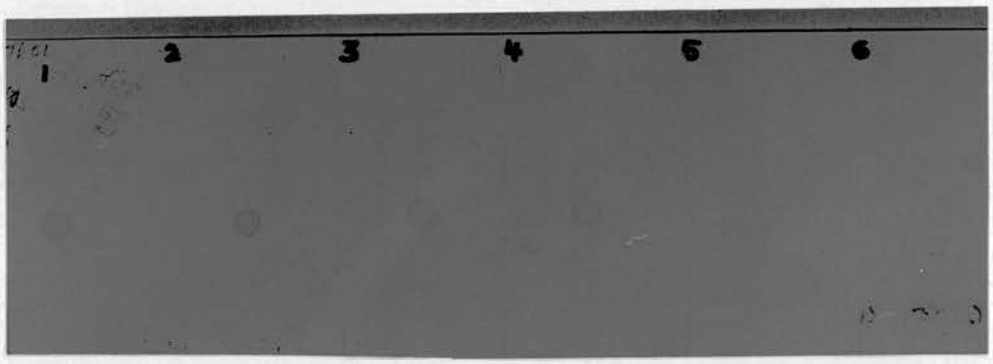
reaction. To purify the plasmid free from excess dNTPs and enzyme, the reaction mix was subjected to Microcon-50 spin columns as before . The plasmid was still linear at this stage so the blunt-ends had to be re-ligated. This was done with T4 DNA Ligase..

For successful ligation of insert with plasmid, one set of ends should be phosphorylated, the other, dephosphorylated. The linkers on the cDNA were phosphorylated so the ends of the cut plasmid were treated with Calf Intestinal Alkaline Phosphatase (CIAP) [Promega], an enzyme which digests off the 5′ phosphate groups preventing self-ligation of the plasmid. CIAP was made to a concentration that would provide 0.01 units of enzyme for each picomole of plasmid ends. The concentration of plasmid DNA was 155µg/ml and the amount required for the ligation reaction was 115ng total. One µl of plasmid (155ng) was used in the CIAP reaction as some DNA would be lost to the subsequent purification procedures. The amount of picomoles of ends in 155ng of plasmid DNA was 0.156 pmol so 0.00156 units of CIAP were required. The reaction mix was 155ng plasmid DNA, 0.00156 units of CIAP, CIAP 10x buffer and nuclease-free water. The mix was incubated at 37°C for 15 minutes then 56°C for 15 minutes. Another 0.00156 units of CIAP were added then the incubations were repeated. The reaction was stopped with the addition of 2µl of 0.5M EDTA and heating to 65°C for 10 minutes. The plasmid DNA and the cDNA were purified free from excess reagents in Microcon spin columns (refer to 6.2ii).

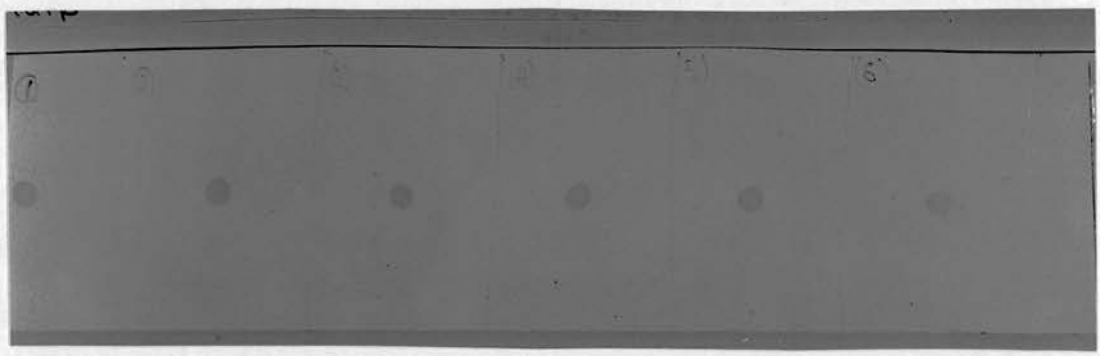
Ligation of cDNA into Plasmid: cDNA was ligated into the plasmid at a molar ratio of 3:1. Plasmid was heated to 90°C to open the cut ends. The reaction mix contained 20µl of cDNA.

Transfection of Plasmid into E. coli dh5α: The transfection, inoculation and DNA purification procedures were carried out as described in Section 5.2i.

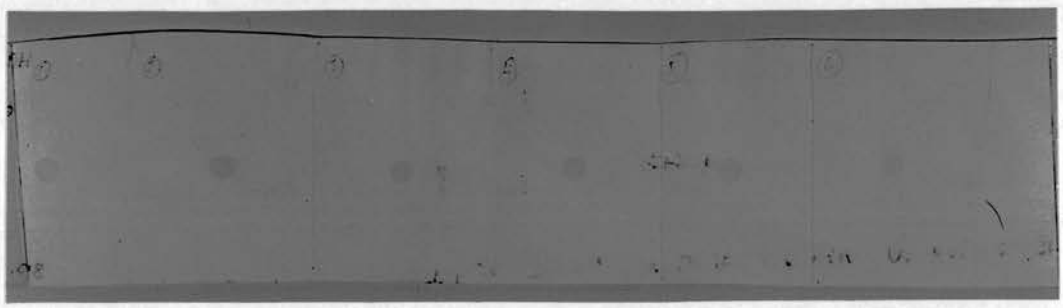
Verification of cDNA Ligation: Because cDNA amounts were so small throughout the previous procedures, it was not possible to test the ligation success before the plasmid had been grown up into sufficient quantities. After DNA purification from the bacterial cells, the plasmid was cut with Nde1 to release the insert. The fragments were gel electrophoresed on a 1% agarose gel with 2µg/ml Ethidium Bromide at 100 volts for 20 minutes. Lambda DNA cut with HindIII [Gibco BRL] markers were run along side the samples to give approximate weights. The expected result was 2 bands on the gel - one about 3kb (plasmid) and the other about 770bp (insert).



(a)



(b)



(c)

Figure 5.3 Nitrocellulose Dot Blots for Riboprobe Quantification.

The control labelled probe (a) was blotted onto the nitrocellulose at concentrations of 100ng/spot, 50ng/spot, 25ng/spot, 12.5ng/spot, 6.25ng/spot and 3.125ng/spot (from left to right). The unknown probe concentrations of TGFβ1 (b) and GAP-DH (c) were measured relative to the known concentration of the control spots. Control spot 1 matched to the 6th spot of TGFβ1 and the 5th spot of GAP-DH which equated to 3.2μg/ml TGFβ1 riboprobe and 1.6μg/ml of GAP-DH riboprobe.

iii. GAP-DH Control Probes

GAP-DH cDNA: Anti-sense control template of human glyceraldehyde-3-phosphate dehydrogenase (GAP-DH) cDNA was obtained from Ambion Incorporated. Probes against GAP-DH are commonly used as controls for *in situ* hybridisation as the gene is expressed in most cells. It was supplied as a 316 base pair insert in Ambion's TRIPLEscript vector which has SP6, T7 and T3 promoter sites. *in vitro* transcription of the fragment from the SP6 promoter resulted in riboprobes of 413 bases in length.

Evaluation of Probe Concentration: RNA *in situ* hybridisation was carried out as in Section 6.2iv with varied concentrations of probe - 100ng/slide, 10ng/slide and 1ng/slide, equating to 0.5ng/μl, 0.05ng/μl and 0.005ng/μl, respectively.

The sections incubated with 0.5ng/μl showed the highest intensity of specific stain. The 0.05ng/μl displayed non-specific staining and the others were negative for stain.

5.3 Results of Research

i. TGFβ Riboprobes Result

TGFβ1 riboprobes were successfully generated by the process described above from a complementary commercial cDNA. The RNA transcripts were dot blotted onto nitrocellulose paper and semi-quantified against a control labelled probe of known concentration. The dot blot (shown in Figure 5.3) indicates that the approximate concentration of the TGFβ probe is 3.2μg/ml.

ii. Actin Riboprobes Result

To clone actin cDNA into *E. coli* and transcribe riboprobes from it, standard protocols for the techniques used were followed.

Cloning the β-actin gene was attempted 7 times but the electrophoretic separation of the cloned plasmid failed to reveal the presence of the cDNA insert. It was decided to choose a housekeeping gene cDNA from which riboprobes could be made.

iii. GAP-DH Riboprobes Result

GAP-DH riboprobes were generated by the process described in 5.2iii from a commercial cDNA. The amount of riboprobe produced was semi-quantified by dot-blot which is shown in Figure 5.3. The approximate concentration of GAP-DH RNA was 1.6μg/ml.

5.4 Discussion of Results

i. TGFβ1 Riboprobes

TGFβ1 riboprobes were used to hybridise with and visualise TGFβ1 mRNA in sectioned breast tissue as a comparison with immunohistochemically stained breast sections.

Because the TGFβ1 cDNA was already inserted into a vector, the generation of riboprobes was straightforward and efficient.

ii. β-Actin Riboprobes

Cloning was necessary because the actin housekeeping gene from which riboprobes were to be made had to be inserted into a vector before the riboprobes could be transcribed.

Attempting to clone cDNA into a vector was time-consuming and ultimately unsuccessful. The reasons for the lack of success in cloning were not obvious but it was assumed that the insert DNA had not ligated into the plasmid in the first place.

iii. GAP-DH Riboprobes

Again, GAP-DH cDNA supplied in a vector was successfully transcribed into labelled riboprobes as with the TGFβ cDNA. The dot blotting of labelled probe onto nitrocellulose shows quickly that the probe is indeed labelled and gives an approximate concentration when compared to control labelled RNA of known quantity.

For quick generation of common riboprobes, it is more efficient to transcribe from a cDNA already inserted into a compatible vector.

CHAPTER 6

IN SITU HYBRIDISATION LOCALISATION OF TGFβ1 mRNA

6.1 Background

In situ hybridisation is used to detect specific nucleic acids sequences in tissue sections, cells or chromosomes. Detection can be related to gene activity at DNA, mRNA and protein levels. The technique was developed independently by Pardue and Gall and by John *et al.* in 1969. Their techniques relied solely on radioisotopes for detection and, since molecular cloning was not established at this time, they could only use sequences that could be purified and isolated by conventional biochemical techniques e.g. mouse satellite DNA, viral DNA, ribosomal RNAs. By the 1980s, molecular cloning was available and radio-labelling methods had improved. DNA sequences of a few hundred base pairs could be detected in metaphase chromosomes. At this time, methods were being developed for the production of chemically synthesised, radio-labelled oligonucleotides which could be used as probes.

Oligonucleotide probes and *riboprobes* are used to detect genes, repeat sequences and RNA. They are made of DNA and RNA, respectively, are single-stranded and of short length (10-50 bases). The advantages of short, single-stranded probes are that first, they can be specifically designed (by automated sequencing for DNA probes and from cDNA templates for RNA probes); second, their short length enables high penetration into target tissue and lastly, single-strandedness means that there is no renaturation of the probe strands. The disadvantages to such small probes is that they cannot cover as much target as conventional cDNA probes and any mismatched nucleotides in the synthetic sequence will reduce stability of the hybrid. In a similar principle to antibody labelling, oligonucleotides and riboprobes can be directly labelled with a fluorochrome or enzyme, or with a hapten such as digoxigenin or biotin which requires antibody detection after hybridisation. An advantage of riboprobes over DNA oligonucleotides is that, for detection of mRNA, RNA-RNA hybrids form stronger bonds than RNA-DNA which in turn form stronger bonds than DNA-DNA.

Radioactive labelling has restricted the application of *in situ* hybridisation because of the safety aspects, the short shelf-life of the probes and subsequent hybridisation pattern and the extensive time required for the radiographic development. The development of *non-radioactive labelling* has overcome the problems experienced with radioactivity and increased the applicability of the

technique. There are two types of non-radioactive labelling. *Direct labelling* requires that the detectable molecule (reporter) is bound directly to the nucleic acid sequence of interest allowing it to be visualised immediately after hybridisation. Direct labelling molecules are usually fluorescent. *Indirect labelling* methods require a probe which contains a reporter (introduced chemically or enzymatically) which will, in turn, become detected by a second reporter, hence the term, 'indirect'. Several detection systems exist, for example, the streptavidin-biotin-horseradish peroxidase complex or the digoxigenin-antidigoxigenin-alkaline phosphatase reaction, both of which are highly sensitive, increasing the flexibility of *in situ* hybridisation. Because indirect methods use at least two layers of reporter molecule, the signal can be amplified in comparison to the direct method.

Probes can be created by a variety of procedures. *In vitro transcription* employs labelled ribonucleotides and RNA polymerases to create 'run-off' RNA transcripts (riboprobes) from cDNA which contains the sequence of interest. The probes can be synthesised complementary to the coding sequence (sense) or complementary to the non-coding sequence (anti-sense). *Nick translation* uses two enzymes, DNase1 and *E. coli* DNA polymerase, as they can add nucleotides to both ends of the DNA duplex. DNase1 creates nicks in double stranded DNA, resulting in an exposed 3' OH group. 5'→3' exonuclease activity of *E. coli* DNA polymerase type 1 removes the nucleotides at the 5' side of the nick and then catalyses the incorporation of new dNTPs from solution at the 3' OH. A new strand is created in a 5'→3' direction of about 200-400 bases with labelled nucleotides. *Random primed labelling* uses the *E. coli* Klenow fragment as a polymerase and a mixture of hexanucleotides to give every possible combination of sequence. The hexamers act as primers and hybridise with the template DNA every 80-100 bases and a new strand is made using the Klenow fragment and labelled/unlabelled nucleotides. *Polymerase chain reaction (PCR)* utilises primers and a DNA template to create large numbers of DNA strands. Taq polymerase accepts modified nucleotides as a substrate so the new strands can be labelled. *End labelling* uses terminal deoxynucleotidyl transferase (TdT) as the enzyme in this technique because it adds nucleotides to the 3' OH end of double or single stranded DNA without the need for a template. It accepts modified nucleotides and if only labelled nucleotides are in the reaction mixture, a fully labelled tail can be created. This method is considered useful for the labelling of short oligonucleotides.

i. Present Research

Immunohistochemistry was ideal for the detection of TGFβ protein location but could not establish whether the protein was manufactured in that place or if it had been transported there. *In situ* hybridisation was used in an attempt to determine where TGFβ mRNA was being transcribed and if transcription of the mRNA was affected by the experimental drug treatments. Non-radioactive *in situ* hybridisation was chosen because it is safer then radioactive labelling and yields results more quickly. *Digoxigenin* was chosen as the colorimetric detection system. Digoxigenin is a steroid isolated from the plants *Digitalis purpurea* and *Digitalis lanata*. The flowers and the leaves of these plants are the only organisms in nature to express digoxigenin so no cross-reactivity of the anti-DIG antibody occurs in other biological tissues. Both DNA and RNA probes were used to determine the pattern of TGFβ expression. The alkaline phosphatase method of detection was chosen for its reliability.

6.2 Method

i. Equipment and Reagent Preparation

All glassware (slide carriers, measuring cylinders, conical flasks) and utensils (spatulas, forceps) were washed thoroughly and baked at 180°C for 2.5 hours to eradicate RNases. Any plastic-ware used was taken from unopened sterile packaging. Surgical gloves were worn at all times while handling reagents and equipment. A sprayer with RNase-away [ICN Pharmaceuticals, Inc., Costa Mesa, CA, USA] was used to clean benches and equipment that could not be autoclaved.

The solutions required for *in situ* hybridisation were all prepared under RNase-free conditions using unopened reagent bottles and baked spatulas and glassware. Water, 20x Standard Sodium Citrate (SSC) and 10x PBS were treated with diethylpyrocarbonate (DEPC) [Sigma], a partial inhibitor of RNase, by adding 1ml DEPC to 1L of solution, stirring it overnight then autoclaving to remove the DEPC. Tris buffer could not be DEPC-treated as above so was prepared using DEPC-treated water. Other reagents such as xylene and ethanol were taken fresh from bottles used only for *in situ* hybridisation.

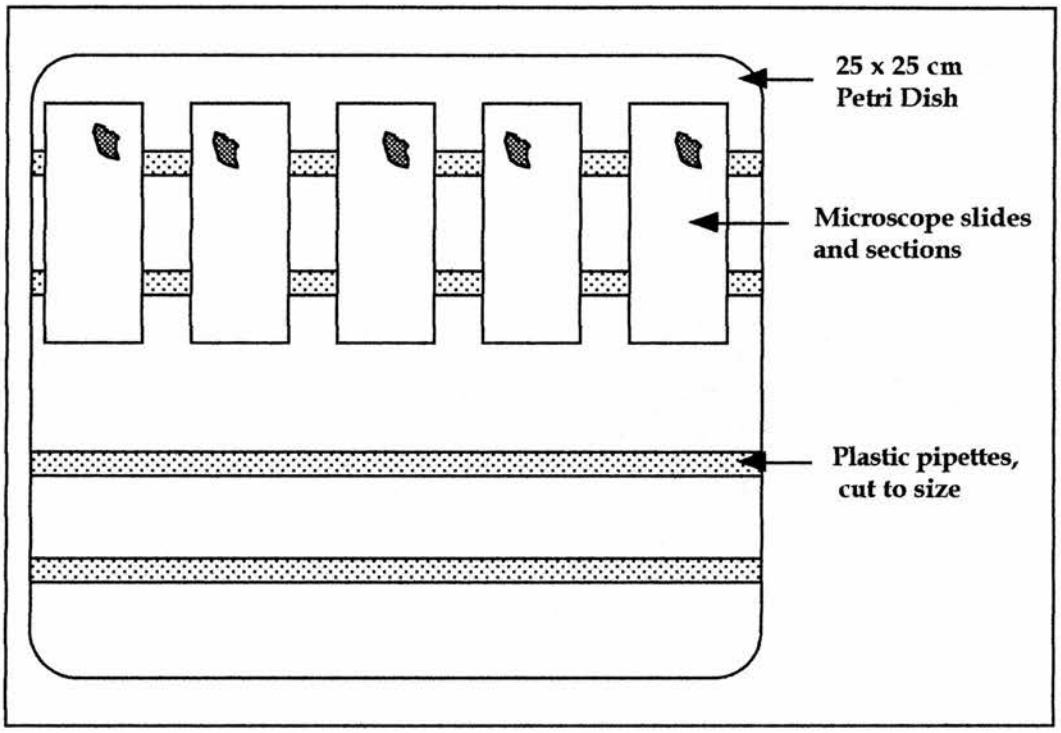


Figure 6.1 Hybridisation Chamber

ii. Assessment of Optimal Proteinase K Concentration

The optimal concentration of Proteinase K (PK) was determined with a titration experiment. Control sections of breast tissue and tonsil were dewaxed, rehydrated and incubated at 60°C in 2x SSC for 10 minutes to increase probe penetration. After 5 minutes in fresh DEPC H_2O, the sections were washed in 0.05M Tris buffer then incubated with PK in 0.05M tris. The concentrations of PK were 50μg/ml, 25μg/ml, 10μg/ml, 5μg/ml and 0μg/ml, incubated at 37°C for 30 minutes. Hybridisation was carried out as described below using 3ng/ml TGFβ DNA probe. The sections were assessed under light microscopy.

iii. *in situ* Hybridisation with DNA Probes

By way of learning the technique of *in situ* hybridisation, an optimised kit was used [R&D Systems, Abingdon, England]. The kit contained wax-embedded, rat pancreas sections and an anti-insulin DNA probe. The protocol resulted in positive hybridisation with the insulin-producing cells of the pancreas.

TGFβ Oligonucleotides: The probes were commercially synthesised by R&D Systems as a cocktail of 3 single-stranded oligonucleotides. The probes were based on the antisense strand of 3 exons from the TGFβ gene (Exon 6, Exon 7A and Exon 7B) and so, were complementary to TGFβ mRNA. All 3 probes were end-labelled with Digoxigenin [Licenced from Boehringer Mannheim GmbH].

Optimisation of Oligonucleotide Concentration: The stock solution of probe was 5μg/ml in distilled, sterile water and the working concentration of 100ng/ml was determined by titration experiments on control tissue.

Dewaxing and Dehydrating of Tissue: The experimental tissue sections and control slides were loaded, back-to-back, into a baked glass slide carrier [BDH] and washed in the following solutions:- xylene (2 x 5 minutes), 100% ethanol (2 x 1 minute), 96% ethanol (2 x 1 minute) and DEPC-H_2O (1 x 5 minutes). They were incubated at 60°C in 2x SSC (10 minutes), to increase probe penetration. After 5 minutes in fresh DEPC H_2O, the sections were washed in 0.05M Tris buffer before being put into a hybridisation chamber (refer to Figure 6.1).

Protease Digestion: The slides were incubated for 30 minutes at 37°C in a 7μg/ml solution of Proteinase K [Boehringer Mannheim, Lewes, England] in 0.05M Tris. The protease action of the Proteinase K breaks down any formaldehyde-induced protein crosslinks, allowing better probe penetration.

Control RNase: RNase treatment of a control slide was carried out using Ribonuclease A [Sigma] at a concentration of 100μg/ml in 0.5M Tris at 37°C for 30 minutes in a separate hybridisation chamber while the experimental slides were incubated in 0.05M Tris under the same conditions. This is a negative control step which aims to destroy all RNA so that the final result should be negative for stain. If stain is present, the probe is binding to something other than RNA.

After these treatments, the sections required post-fixation in 0.4% paraformaldehyde [BDH] in PBS to keep open the pores which were created by Proteinase K. Paraformaldehyde was a solution of 400mg of formaldehyde added to 10ml of 10x PBS then heated to 70°C so that the powder would dissolve. Before use, the formaldehyde concentrate was diluted with 90ml of water to give a final concentration of 0.4% paraformaldehyde in 1x PBS. Post-fixation was carried out at 4°C for 20 minutes. The slides were washed in DEPC-H_2O and returned to the hybridisation chamber.

Prehybridisation: To counteract non-specific staining, a 3-hour incubation with prehybridisation solution was carried out at 37°C. Prehybridisation solution consisted of 0.4M NaCl, 10% w/v Dextran Sulphate, 30% v/v deionised formamide, denatured salmon sperm (2.25μg/ml) [Sigma] and 10% v/v 10x PE (Tris [Sigma], Sodium Pyrophosphate [BDH], PVP [BDH], Ficoll 400 [Sigma], EDTA [BDH]).

Hybridisation: Probe was added to prehybridisation solution at a concentration of 100ng/ml and pipetted onto the slides after removal of the probe-free prehybridisation solution. The hybridisation conditions were 37°C, overnight in a humid hybridisation chamber. Plastic coverslips [Nunc] were put on the slides to prevent any drying out.

Post-hybridisation treatment of sections: Non- or weakly-bound probe was washed from the sections in 2 x 5 minute stringency washes of SSC and formamide, increasing the stringency each time (4x SSC, 2x SSC and 0.2x SSC, all with 30% formamide) at 37°C.

Detection of the probe was done by recognising the incorporated Digoxigenin using an anti-Digoxigenin antibody [Boehringer Mannheim]. The antibody was conjugated to the enzyme, Alkaline Phosphatase, which causes a colour change when in the presence of its substrate. The sections were blocked with a modified Tris Buffered Saline (TBS containing 1% w/v Bovine Serum Albumin (BSA) [Sigma], 1.5M NaCl, 20mM $MgCl_2$) and 0.1% Triton-X-100 for 30 minutes at room

temperature. The TBS modified was drained off and the slides returned to the hybridisation chamber. The antibody was diluted in antibody diluent recommended by Boehringer Mannheim (100mM Tris-HCl, 150mM NaCl, pH 7.5) at a ratio of 1:600 for 2 hours at 37°C. Unbound antibody was washed off in 2 x 5 minutes washes of TBS modified followed by 1 x 5 minutes in distilled water.

Colour Development: The colour developer used was Western Blue [Promega] which is a mixture of 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and Nitroblue Tetrazolium (NBT), the substrates for alkaline phosphatase. Alkaline Phosphatase reacts with BCIP and the product of that reaction reacts with NBT to form NBT Formazan, a solid precipitate which is coloured blue. The Western Blue was supplemented with 0.03M Levamisole, an inhibitor of endogenous alkaline phosphatase activity. Colour development was carried out at 37°C for 4 hours in the dark.

Counterstaining and Mounting: The sections were washed in distilled water after the Western Blue step and then counterstained with Ehrlich's Haematoxylin, 'blued' in tap water, dehydrated for 1 hour at 37°C and then mounted in an aqueous mountant, Aquamount [BDH]. Xylene-based mountants should not be used as they dissolve the colour precipitate.

Viewing and recording results: The mounted slides were viewed under a Zeiss 'Axioskop' light microscope and scored for presence and location of stain.

iv. *in situ* Hybridisation with Riboprobes

TGFβ/GAP-DH Probe in vitro Transcription: This technique was carried out using a DIG-RNA Labelling Kit (SP6/T7) from Boehringer Mannheim. It contained reagents for creating Digoxigenin-labelled RNA transcripts from the TGFβ cDNA and GAP-DH cDNA. TGFβ cDNA lies downstream of the T7 promoter so T7 RNA polymerase was used to initiate transcription whereas GAP-DH cDNA was downstream of the SP6 promoter so required SP6 RNA polymerase. Digoxigenin-labelled UTP was present in the nucleotide mix and was incorporated into the RNA every 20-25 bases, giving a total of 30-40 labelled nucleotides along the length of one TGFβ transcript and 17-21 labelled nucleotides in the GAP-DH transcript. DIG-labelled probes have the advantages of being defined unity length, single-stranded and unlikely to renature. The method was as follows:-

Reagents were kept on ice and added to an RNase-free microfuge tube in the following order - 1μg of template DNA, 2μl of 10x dNTP labelling mix, 2μl of 10x transcription buffer, 1μl RNase inhibitor and enough nuclease-free water to make up to 18μl. Finally, 2μl T7 or SP6 RNA polymerase was added. The tube was mixed and centrifuged briefly then incubated in a 37°C water bath for 2 hours. To remove the plasmid DNA template from the mixture, 2μl of RNase-free DNase1 was added to the mix and the tube was incubated for a further 15 minutes at 37°C. The reaction was stopped by the addition of 2μl of 0.2M EDTA and made up to 50μl with nuclease-free water.

Probe Hydrolysis: The riboprobes were hydrolysed to cut them into lengths of about 200 bases. To the transcripts, 30μl 200mM Na_2CO_3 and 20μl 200mM $NaHCO_3$ were added. The TGFβ probe mix was incubated for 45 minutes and the GAP-DH riboprobes for 23 minutes at 60°C, based on the following equation:-

hydrolysis time (t) = $(L_o - L_f)/(K.L_o.L_f)$, where L_o is the original length of riboprobe in kilobases (0.77kb for TGFβ and 0.413kb for GAP-DH), L_f is the approximate finished length of riboprobe (0.2kb) and K is the constant rate, in this case, 0.11kb/minute.

Probe Purification: The riboprobe was purified by addition of 5μl 10% acetic acid, 11μl 3M sodium acetate (pH 6.0), 1μl 10mg/ml tRNA, 1.2μl 1M magnesium chloride and 300μl cold ethanol. The mix was incubated at -20°C overnight. The probe was centrifuged for 15 minutes at 4°C (20,000*g*) and the supernatant discarded. The RNA pellet was desiccated by vacuum in an Eppendorf Concentrator then resuspended in 20μl of nuclease-free water.

Optimal Probe Concentration: Titration experiments to determine the optimal probe concentration were carried out on fresh-fixed breast tissue, using a range of concentrations of 0.01ng/μl, 0.05ng/μl and 0.5ng/μl. The highest concentration gave a positive result.

Hybridisation: The slides were treated in the same way as for oligonucleotide probes until the hybridisation step (refer to 6.2i-ii) when probe was heated to 95°C for 2 minutes to denature any bound single strands and quenched on ice before addition to prehybridisation solution at the appropriate concentration. The probe mix was vortexed to ensure complete mixing. The probe-free prehybridisation solution was tipped off the slides and probe was pipetted on.

The hybridisation conditions were 50°C overnight in a humid hybridisation chamber. Plastic coverslips [Nunc] were put on the slides to prevent any drying out.

Post-hybridisation treatment of sections: Non or weakly bound probe was washed from the sections in 2 x 30 minute stringency washes of SSC and formamide, increasing the stringency each time (4x SSC, 2x SSC and 0.2x SSC, all with 30% formamide) at 50°C.

RNase Control: To eliminate unbound probe, which may have interfered with the detection step, all slides were incubated with RNase at a concentration of 100μg/ml for 60 minutes at 37°C. The RNase diluent was a solution of 0.5M NaCl, 10mM Tris-HCl (pH 7.5) and 1mM EDTA.

Hot SSC: Slides were incubated with 2x SSC at 65°C for 2 x 30 minutes to unmask the targets of the DIG antibody.

Detection was carried out as before with the anti-DIG antibody at a concentration of 1:600, for 2 hours at 37°C. The colour developer used was Fast Red [Sigma] which was supplied as 2 tablets - a Tris buffer tablet and a Napthol tablet which, when dissolved in 1ml of distilled water, gave the appropriate substrate for alkaline phosphatase. The substrate solution was incubated with the slides overnight at 37°C. The resultant colour was red.

Counterstaining and Mounting: The sections were washed in distilled water after the Fast red step and then counterstained with Ehrlich's Haematoxylin, 'blued' in tap water, dehydrated for 1 hour at 37°C and then mounted in an aqueous mountant, Aquamount [BDH]. Xylene-based mountants should not be used as they dissolve the colour precipitate.

Viewing and recording results: The mounted slides were viewed under a Zeiss 'Axioskop' light microscope and scored for presence and location of stain.

6.3 Resúlts of Research

i. Proteinase K Evaluation

Tissue structure was poor after treatment with 50μg/ml and 25μg/ml and probe penetration was poor in the 0μg/ml section. A second experiment was carried out using concentrations of 10μg/ml, 7μg/ml, 5μg/ml and 2μg/ml. The concentration of proteinase K best suited to paraffin-embedded breast tissue, cut

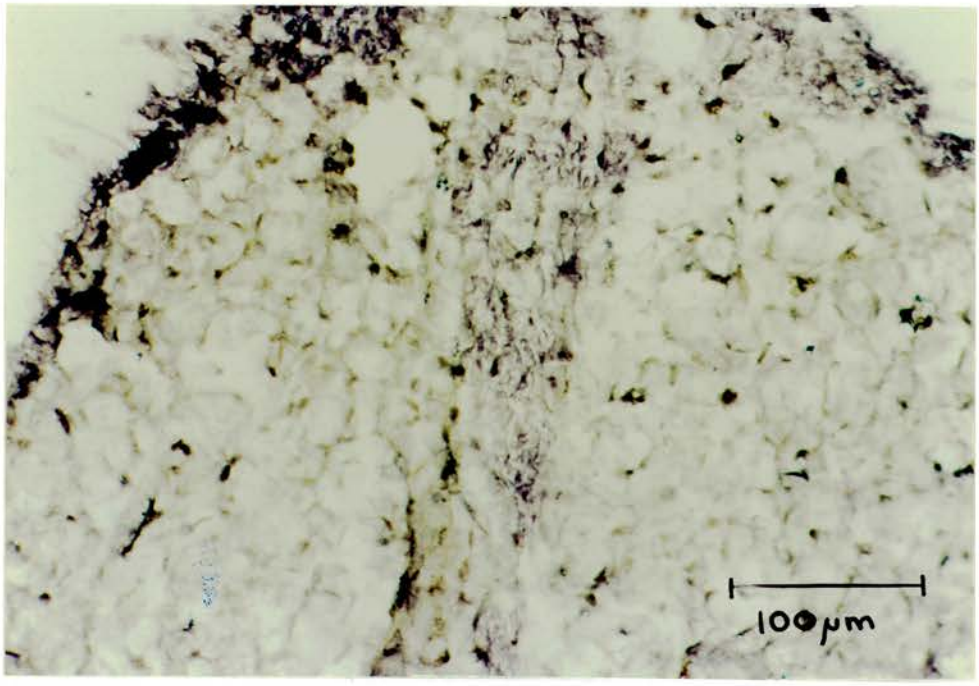


Figure 6.2 50µg/ml Proteinase K on Breast Tissue (x16).

The fibrous structure of the tissue has been digested by the protease. No nuclei are evident.

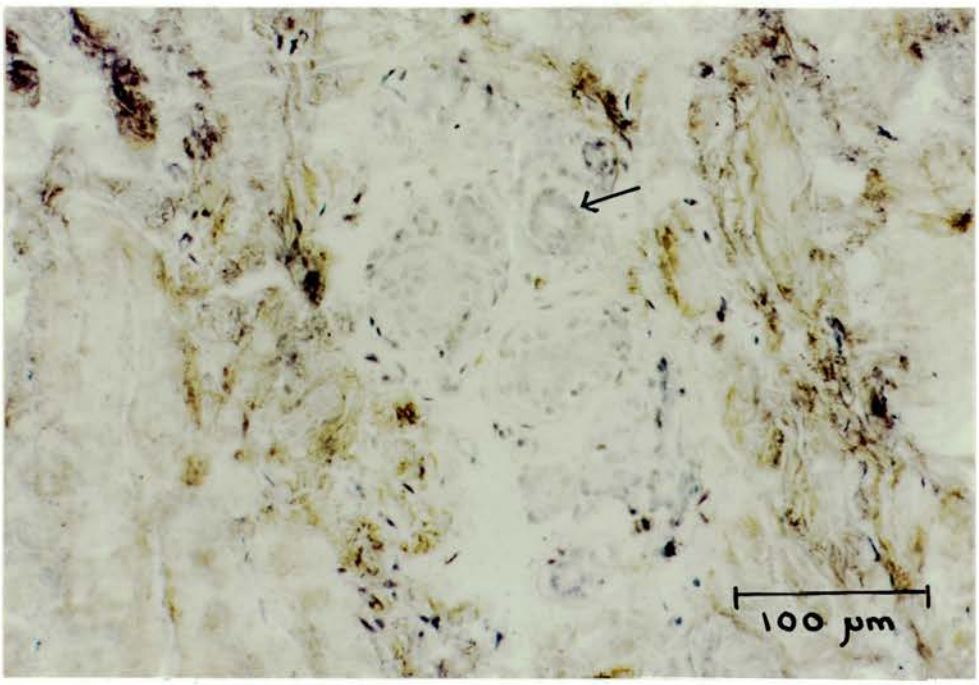


Figure 6.3 7µg/ml Proteinase K in Breast Tissue (x16).

The tissue structure reaims itact and there is faint Western Blue staining (→) in the epithelial cells' cytoplasm, indicating positive hybridisation.

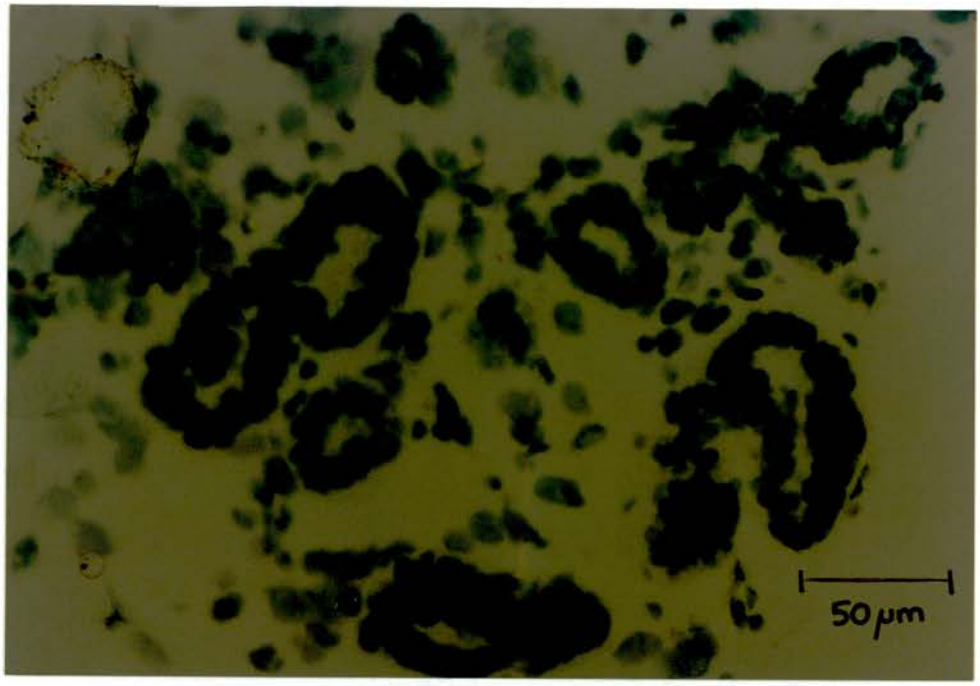


Figure 6.4 0.01ng/µl GAP-DH Probe in Fresh Breast Tissue (x25).
No staining is evident in the low probe concentration.

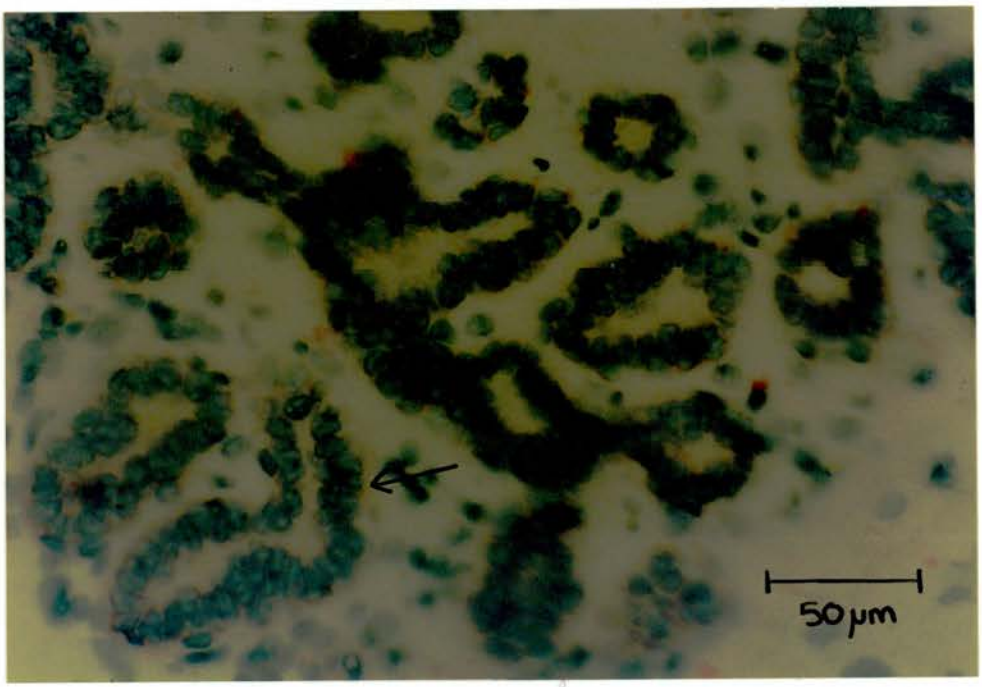


Figure 6.5 0.5ng/µl GAP-DH Probe in Fresh Breast Tissue (x25).
Fast Red staining (→) is evident in the cytoplasm of the epithelial cells.

at 4μm, was 7μg/ml. Figure 6.2 shows the effect of 50μg/ml of proteinase K on breast tissue. The fibrous structure has broken down and no cells can be seen. Figure 6.3 shows the effect 7μg/ml Proteinase K. Faint western blue staining can be seen around the epithelial cluster.

ii. Optimal Probe Concentration

Figures 6.4 and 6.5 show the result of the probe evaluation using the GAP-DH probe. The lowest concentration (0.01ng/μl) did not show positive hybridisation (Figure 6.4) but the highest concentration (0.5ng/μl) did hybridise to the epithelial cells and was detected by Fast Red substrate (figure 6.5).

iii. *in situ* Hybridisation

The staining pattern of the oligonucleotide *in situ* hybridisation was too pale to be conclusive so it was decided to make riboprobes against TGFβ (using cDNA as a template) because RNA-RNA hybrid bonds are stronger than DNA-RNA bonds. Because the rat pancreas/anti-insulin kit had worked, it was assumed that the protocol for the TGFβ1 DNA probes was not the problem.

The TGFβ riboprobe *in situ* hybridisation was also inconclusive as the staining was pale and inconsistent, possible due to an irregular degradation of mRNA. Attempts made to improve the signal, for example hydrolysis of the probe and increasing probe concentration, did not significantly increase the intensity of staining. GAP-DH, a commonly used control probe, also failed to stain its ubiquitous mRNA in the cultured tissue.

6.4 Discussion of Results

i. Proteinase K Evaluation

This concentration was the optimal compromise between probe penetration and tissue preservation. Proteinase K concentration of 7μg/ml falls within the range found to be optimal by other authors using *in situ* hybridisation on human breast tissue, for example Walker and Gallacher (1995).

ii. *in situ* Hybridisation

in situ hybridisation was used with TGFβ riboprobes to detect and quantify the amount of TGFβ1 mRNA present in formalin-fixed, wax-embedded breast cancer tissue which had previously tested positively for TGFβ1 protein. GAP-DH probes were used as a control.

Both the control and experimental probes resulted in low staining levels. Had it been that there was no TGFβ mRNA to be detected, the GAP-DH should still have stained. The poor preservation of mRNA could not have been due to the culture conditions or the presence of drugs as fresh fixed tissue and control explant tissue also did not stain well. This indicates that all mRNA may have degraded during tissue fixation.

Even though the tissue had been handled sub-optimally in the pathology labs, i.e. exposed to various temperatures and left for 24 hours before collection, it was assumed that mRNA had not degraded during this time nor during the culture period, because viable cells, which have to produce RNA, were evident in cultured explant tissue. There was no indication of a problem with the cell culture or immunohistochemistry applications. Since *in situ* hybridisation was the final technique to be tried, it was too late to rectify the preparation of the tissue. This also highlights the importance of optimising all experimental conditions before attempting technically temperamental methods such as *in situ* hybridisation. If circumstances are not optimal, the experiments may not be worth doing because there is a waste of time and resources. However, using human biopsy material introduces significant variation within the experimental tissue itself. What may be optimal for one biopsy may be not optimal for another, but optimising the technique for each biopsy would be nearly impossible.

Future improvements would be to optimise the treatment of the tissue specifically for *in situ* hybridisation. Formalin is the fixative of choice for *in situ* hybridisation but, perhaps, the length of fixation or the subsequent processing could be tailored better to *in situ* hybridisation specifically. RNase inhibitors could also be employed during the initial preservation before culture, during culture or in conjunction with the fixative. Tests for mRNA presence before starting the technique would eliminate those tumours which were mRNA-poor, thus saving on time and resources.

mRNA degradation was also experienced by Walker and Gallacher (1995) when they carried out an *in situ* hybridisation study of TGFβ1 mRNA expression in

histological specimens of breast carcinomas. Their technical findings were that RNA preservation in routinely fixed tissue, as opposed to optimally fixed tissue, was poor, which concurs with the present study as the tissue used was not optimally fixed specifically for *in situ* hybridisation. The experimental tissue was fresh fixed, wax-embedded breast carcinoma. The tissue sections were treated for riboprobe hybridisation and stained with alkaline phosphatase/NBT-BCIP and immunogold technique with silver enhancement. Alkaline phosphatase gave better results than gold staining. They had problems with interpreting the label due to background staining. Nine from 16 carcinomas displayed positive stain in tumour cells but there was no label in tissue where RNA preservation was poor. The level of label varied between individual samples and within the same sample. Stromal cells showed no significant reactivity with either staining technique. Expression of mRNA correlated with positive staining for protein. In the samples that did not stain with TGFβ antibodies, TGFβ mRNA was not observed either. Two samples stained positive for protein but not for mRNA - this was due to RNA degradation. The levels of RNA found in this study are lower than those recorded by MacCallum *et al.* (1994) but similar to those of Barrett-Lee *et al.* (1990), probably due to differences in techniques used.

Thompson *et al.* (1991) investigated expression of TGFβ1 mRNA in estrogen dependent MCF-7 xenografts and in tumours from patients who had received tamoxifen therapy before surgery. The MCF-7 xenografts regressed under tamoxifen treatment and TGFβ mRNA levels increased from low on Day 0 to high from day 7 onwards. In the human tissues, TGFβ1 mRNA was detected in all tumour specimens and in normal tissue. The xenograft tissue treated with tamoxifen gave a consistent and sustained rise of TGFβ1 mRNA, which was only briefly achieved by the tumour tissue. High expression TGFβ1 mRNA was seen in 45/56 of the tumours, almost twice the level observed in the normal tissue. Almost all the premenopausal women had high levels which is a significant correlation but there was no significant correlation between TGFβ and tumour size, ER status of presence or lymph node metastasis. Of 6 post-menopausal patients whose tumours did not respond to tamoxifen therapy, all were ER poor and had high levels of TGFβ1 mRNA, confirming that there is no association of TGFβ with ER status. There is also an association with high TGFβ1 level and tumour progression despite tamoxifen treatment. However, in that study, total RNA was

extracted under optimal conditions from fresh biopsies or freshly excised xenografts. No *in situ* hybridisation was attempted.

The findings here indicate that epithelial cells are the site of TGFβ1 production, contrary to Butta *et al.* (1992), who found that stromal cells were the main producers of TGFβ protein. TGFβ mRNA was not detected in macrophages, fibroblasts or lymphocytes. The conclusions of Walker and Gallacher (1995) were that TGFβ1 mRNA correlates with TGFβ1 protein and that epithelial tumour cells appear to be the site of production.

Had the present *in situ* hybridisation been more successful, the results would have added further evidence to one or the other side of the controversy.

CHAPTER 7

GENERAL SUMMATION OF THESIS

7.1 Discussion of Research Topic

Breast cancer is a devastating group of diseases. It is particularly prevalent in the westernised world. The life time risk of contracting breast cancer is as high as one in 10 in the United States and Europe. Hormonal influences, mainly estrogens, are instrumental in the development of the disease and anti-estrogens are commonly used in the treatment, and secondary prevention, of breast cancer.

Growth factor expression is influenced by estrogen, both during the development of breast disease and during its treatment. TGFβ has a major role in the regulation of normal cell growth, generally promoting stromal cell growth and inhibiting epithelial cell growth. Because breast cancers are of epithelial origin, it has been proposed that TGFβ could be harnessed in the treatment of breast cancer to block cancer cell growth. TGFβ itself cannot be used as a therapeutic agent because it has a very short half life, but an upregulation in its expression can be achieved by anti-estrogens, as demonstrated *in vivo* and *in vitro* studies.

7.2 Discussion of Aims and Purpose of Research

This study aimed to establish a successful organ culture system for the maintenance of fresh breast biopsy tissue, capable of being used to test biological mechanisms of response to therapy. Thereafter, the system was applied to test the TGFβ1 hypothesis, questioning whether anti-estrogens, such as tamoxifen, can upregulate the expression of TGFβ to a clinically significant degree, i.e. in order to cause breast cancer epithelial cell death. Many studies have been carried out on the ER-positive cell line, MCF-7, and on fresh-fixed breast tissue but in the present research, fresh breast cancer tissue was cultured, with minimal disruption, as small explants in a novel soft agar culture system, in hormone-depleted conditions with and without, therapeutic drugs.

7.3 Discussion of Tissue Culture Chapter

Two approaches to culturing fresh breast cancer biopsy material were undertaken.

Enzymatic disaggregation for the establishment of cell lines is associated with low success especially for the culture of breast tissue. Using the simplest of culture conditions for the digested tissue was speculative and subsequently,

resulted in no cell attachments from the cell suspension. However, the simplicity of the experimental design was its main advantage as well as its downfall because had it been successful, the resultant cell line would not have been dependent on exogenous growth factors or hormones. A cell line with few growth requirements would have been excellent research material on which to study the effect of other growth factors and hormones without interference from the medium. Infection of the biopsy material was also a problem with this technique due to the lengthy preparation process and because, understandably, sterility was not a priority for the pathologists who kindly donated surplus material for this study.

Explant culture of the breast tissue in soft agar became the principal research technique. After evaluating tissue health, with regard to apoptosis, it was clear that the soft agar technique was going to be successful at maintaining breast tissue to a satisfactory degree without the need for feeding. The breast tissue was cultured without any drugs and in hormone-depleted conditions (the fetal calf serum was stripped of its undefined quantities of hormones, using the dextran-coated charcoal technique).

The tissue could be manipulated with minimal contact and the effect of the drugs and hormones easily measured once the tissue was out of culture. For simplicity of analysis, it was desirable to use only one initial dose of the drugs to last throughout the 8-day culture period. As it happened, in this research, significant effects were not observed in all the sample groups and future improvements would include using replicates of explant tissue, perhaps, adding an effector into the system and choosing a less variable method of assessing tissue health than scoring apoptosis, for example, measuring thymidine uptake.

Future developments for the culture system itself would include the use of reporter cells in the agar which would give a quantitative way to measure the secretion of growth factors from the tissue. This has already been achieved with the growth stimulation of NRK-49F cells by TGFβ (Macintyre *et al.*, 1988). The soft agar assay could be useful in a clinical setting. It would allow the culture and manipulation of biopsies with several drug therapies. Determining the optimal therapy in individual patients, rather than relying on timely *in vivo* experiments, would not only ensure a quick and efficient treatment regime for the patient, but could in the long run, save on resources.

It would be interesting to investigate the value of agar culture for similar research on other tissue types. The tissue culture system was a success and could

easily be applied for the culture of other tissues. The problem of unpredictability of tissue content for each cultured fragment remains and there are no easy answers.

7.4 Discussion of Immunohistochemistry Chapter

Immunohistochemistry was successfully applied to the cultured tissue. It is a simple and efficient technique. The direct results of the technique in this research were first, that TGFβ1 protein was detectable in almost all experimental and control sections, showing that most cells express the protein at some time. The implication of this for future experiments is that there will be a basal level of the protein in all conditions so that response must be measured on a graded scale; all-or-nothing scores cannot apply.

Second, the protein, although present in some stromal cells, is almost always concentrated in and around the epithelial cells of breast tumours. This implies that the epithelial cells are likely to be the producers of the protein. This being the case, normal epithelial cells could be targeted, as part of a breast cancer therapy, to make more TGFβ1, which could act in a autocrine manner on cancer cells to inhibit their growth.

7.5 Discussion of DNA Cloning and *in situ* Hybridisation Chapters

DNA cloning was necessary because the actin housekeeping gene from which riboprobes were to be transcribed had to be inserted into a vector before this could happen.

Attempting to clone cDNA into a vector was time-consuming and ultimately, unsuccessful. The reasons for the lack of success in cloning were not obvious but it was assumed that the insert DNA had not ligated into the plasmid in the first place. With the increasing availability of commercial vectors/cDNA one should consider resources and time before embarking on DNA cloning.

in situ hybridisation was used in an attempt to determine the location of TGFβ1 mRNA and hence, the site of protein translation. A comparison with the immunohistochemical localisation of the protein would show whether or not the expressed TGFβ mRNA was being translated into protein in the same place. *in situ* hybridisation was attempted with DNA probes and riboprobes, both of which were complementary to TGFβ1 mRNA. No mRNA was detected in the cultured breast

tissue and it was assumed that the fixation process had been detrimental to the mRNA after culture.

7.6 General Summation

By way of an introduction to the thesis presented here, I have described to the reader how cancer arises and the development, structure and function of the normal breast. I then went on to outline the clinical and molecular features of breast cancer followed by an in-depth review of the transforming growth factors. The influence of estrogens and anti-estrogens in the breast was discussed with a reference to the production of TGFβ and a review of the debate surrounding TGFβ production in breast cancer. Lastly, the aims of the research were presented.

The experimental chapters were written, it is hoped, in a logical fashion with results and discussion for each.

I have reviewed the methods used for breast cancer culture, none of which would have been suitable for the present research. My results demonstrate that the soft agar explant culture system was efficient at maintaining fresh breast cancer material. Further more, the tissue could easily be manipulated in culture. The cultured tissue was still suitable for immunohistochemistry but may not have been so suitable for the *in situ* hybridisation due to a lack of knowledge on the optimisation of fixation for such applications.

The present research has furthered the knowledge of culture systems, with particular respect to the culture of fresh breast cancer tissue, in a way which has not been attempted before. This is of importance for the future of research assays and clinical assays.

In retrospect, it has been concluded that measuring the percentage of apoptosis may not be the optimal indicator of drug effect on breast cancer tissue because the drugs may be causing cell cycle arrest rather than cell death. The cultured breast cancer tissue remained an ideal material on which to perform retrospective immunohistochemical studies. The lack of success with *in situ* hybridisation highlights the need for any tissue to be optimally prepared for each technique.

Future developments of this particular research could include an in-depth study of the signalling pathways involved in fresh breast cancer tissue, including the detection of TGFβ1 receptor levels in individual tumours and the analysis of

signalling components, which could be responsible for TGFβ1-insensitivity when mutated.

The system could also be adapted for the measurement of growth with greater sensitivity, and the application of the system to assay other breast cancer therapies, for example, cytotoxic drugs, novel hormone regimes and perhaps, radiotherapy.

Improvements to the project could have been made in the areas of pathology, in order to identify the areas of tumour most likely to produce results after culture. A more consistant and quantiative measuring system, perhaps a computer-aided system, for the assessment of TGFβ1 staining intensity would have been useful.

In short, I have developed and evaluated a novel culture system for the maintenance of fresh breast cancer biopsy tissue. Because tissue can be sustained with adequate cell viability for many days, the culture system is suitable for growth-regulation experiments and the evaluation of protein production.

APPENDICES

APPENDIX 1: ABBREVIATIONS

AMH	Anti-Mullerian Hormone
APES	3-Amino-propyl-triethoxy-silane
BCIP	Bromo-chlor-indolyl-phenol
BMP	Bone Morphogenic Protein
BRCA1/2	Breast Cancer genes 1/2
BSA	Bovine Serum Albumin
CAK	Cdk activating Kinase
CDK	Cyclin-dependent Kinase
cDNA	Complementary Deoxyribonucleic acid
CIAP	Calf Intestinal Alkaline Phosphatase
DAB	Diaminobenzidine
DC-CS	Dextran-Charcoal Stripped Calf Serum
DCIS	Ductal Carcinoma *in situ*
DEPC	Diethylpyrocarbonate
dH_2O	Distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DPPC	Decapentaplegic Complex
DIG	Digoxigenin
dTTP	Deoxy-Thymidine Triphosphate
dUTP	Deoxy-Uridine triphosphate
ECM	Extracellular Matrix
EDTA	Ethylene-diamine-tetra-acetate
EGF	Epidermal Growth Factor
ER	Estrogen Receptor
FCS	Fetal Calf Serum
FGF	Fibroblastic Growth Factor
HRP	Horseradish Peroxidase
GAP-DH	Glyceraldehyde Phosphate Dehydrogenase
G0/1	G0/1 phase of cell cycle
HCl	Hydrochloric acid
IGFI/II	Insulin-like Growth Factors I/II
IgG	Immunoglobulin type G

kD	kiloDaltons
LCIS	Lobular Carcinoma *in situ*
LOWESS	Locally Weighted Regression Scatterplot Smoothing
MCF-7	Mammary Carcinoma cell line
MEM	Minimum Essential Medium
$MgCl_2$	Magnesium Chloride
M-Phase	Mitotic Phase of cell cycle
mRNA	Messenger Ribonucleic acid
NaCl	Sodium Chloride
Na_2CO_3	Sodium Carbonate Anhydrous
$NaHCO_3$	Sodium Hydrogen Carbonate
NaOH	Sodium Hydroxide
NaN_3	Sodium azide
NBT	Nitro Tetrazolium Blue
NRK-49F	Normal Rat Kidney cell line
NRS	Normal Rabbit Serum
PBS	Phosphate Buffered Saline
PCD	Programmed Cell Death
PDGF	Platelet-Derived Growth Facctor
PGR	Progesterone Receptor
PIPES	Piperazine-N,N bis-2, hydroxypropanesulphonic acid
PVP	Polyvinyl pyrolidene
RNA	Ribonucleic acid
SAB	Streptavidin-biotin
ser/thr	Serine/Threonine kinase
SMAD	sma genes/mothers against decapentaplegic
S-Phase	Synthesis phase of cell cycle
SSC	Standard Saline Citrate
TBS	Tris Buffered Saline
TdT	Terminal Deoxynucleotidyl Transferase
TGF	Transforming Growth Factor
VEGF	Vascular Endothelial Growth Factor

APPENDIX 2: SOLUTIONS AND REAGENTS

0.6% Bacto Agar

0.6g Bacto agar powder

100ml dH_2O

Aliquot. Autoclave. Store at 4℃.

Citrate Buffer, pH 6.0

10mM citric acid monohydrate

dH_2O

Dissolve acid. Adjust to pH to 6.0.

Competence Solution 1, pH 5.8

30mM potassium acetate

10mM calcium chloride

50mM manganese chloride

100mM rubidium chloride

15% v/v glycerol

Adjust pH to 5.8 with 1M acetic acid. Sterilise through a 0.2μm filter.

Competence Solution 2, pH 6.5

10mM PIPES (pH 6.5)

75mM calcium chloride

10mM rubidium chloride

15% v/v glycerol

Adjust pH to 6.5 with Potassium Hydroxide. Sterilise through a 0.2μm filter.

DEPC-H_2O

1ml diethylpyrocarbonate

1 litre distilled water (dH_2O)

Add DEPC to water. Stir for at least 1 hour. Leave overnight. Autoclave.

DMEM

Filter-sterilised DMEM

5-10% v/v FCS

50 IU/ml Penicillin/50μg/ml Streptomycin

2μM L-Glutamine

Store at 4℃. Pre-warm before use.

DIG-antibody Diluent, pH 7.5

100mM Tris-HCl

150mM NaCl

dH_2O

Adjust pH to 7.5.

Double-strength MEM

Filter sterilised double strength, phenol-red free MEM

20% v/v dextran-charcoal stripped calf serum

100 IU/ml penicillin/100μg/ml streptomycin

4μM L-glutamine

Pre-warm all components before addition. Store at 4℃.

Hybridisation Buffer (300ml)

10.53g NaCl [0.05M]

30g dextran sulphate

150ml DEPC H_2O

Stir and gently heat.

90ml deionised formamide [30%]

30ml 10x PE

Stir slowly.

4.5ml 150μg/ml denatured salmon sperm [2.25μg/ml]

Make up to 300ml with DEPC H_2O. Store at -20℃.

PBS

1 PBS tablet

100ml dH_2O

Autoclave once tablet dissolves.

10x PBS

10 PBS tablets

100ml DEPC-H_2O

Autoclave once tablets dissolve.

10x PE

75ml 2M Tris (pH 7.6)

3g sodium pyrophosphate

6g PVP

6g ficoll

30ml 0.5M EDTA (pH 8.0)

100ml DEPC H_2O

Stir to dissolve. Make up to 300ml with DEPC H_2O.

20x SSC, pH 7.0

3M NaCl

0.3M sodium citrate

Dilute to 1x SSC when required. Adjust pH to 7.0.

TBS, pH 7.6

200mM Tris

1.54M NaCl

20mM $MgCl_2$

dH_2O

Adjust pH to 7.6.

TBS modified

As above but with the addition of 0.1% v/v Triton-X-100

APPENDIX 3: STATISTICAL ANALYSES

The information is the accompanying data, generated by DataDesk®, for the statistics on the cell viability study in Chapter 3.

Pearson Product Moment Correlation

	√% Apoptosis	√Days in Culture
√% Apoptosis	1.000	
√Days in culture	0.627	1.000

Dependent Variable is: √% apoptosis

No Selector

R^2 = 39.4%

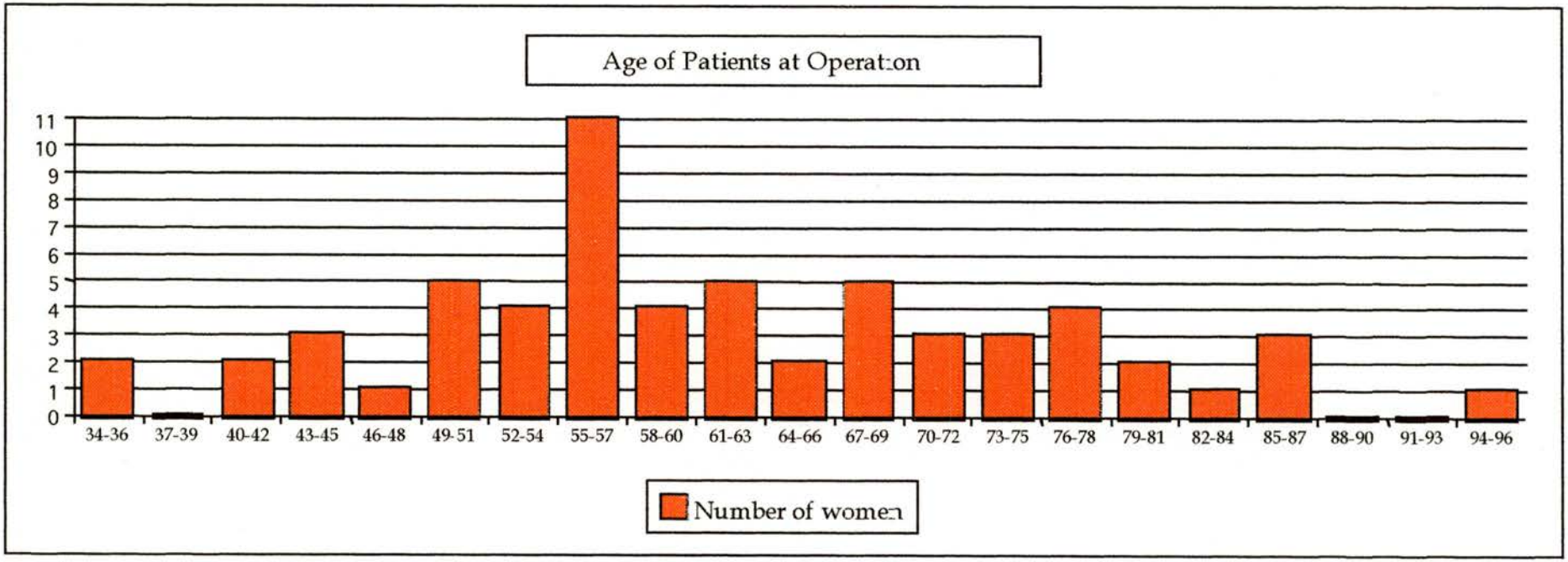
R^2 (adjusted) = 38.5%

s = 1.815 with 75-2 = 73 degrees of freedom

Source	Sum of Squares	Degrees of Freedom	Mean Square	F-ratio
Regression	156.015	1	156.015	47.4
Residual	240.445	73	3.29377	

Variable	Co-efficient	Standard Error of Co-efficient	t-ratio	Probability
Constant	1.98243	0.1484	5.06	≤ 0.0001
√Days in culture	1.02154	0.1484	6.88	≤ 0.0001

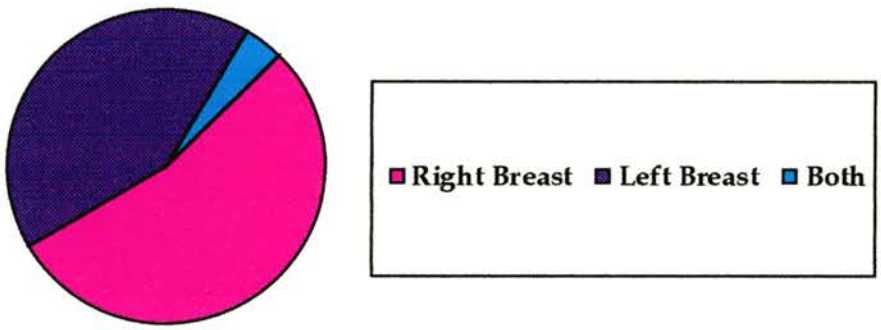
APPENDIX 4: PATIENTS' AGES AT OPERATION



Appendix 4. Chart of Patients' Ages at Operation.

In this subset of women, breast cancer has been diagnosed and treated most frequently between the ages of 55-57.

APPENDIX 5: SITE OF TUMOURS



Appendix 5. Site of Tumours: Right versus Left Breast.

From a subset of 47 patients, 55.3% suffered breast cancer in the right breast and 42.6% had cancer in the left breast. One patient (2.1%) suffered bilateral cancer, i.e. cancer in both breasts.

APPENDIX 6: TREATMENT OF BIOPSIES

Tumour Number	Treatment	Result
001	Collagenase	Infected
002	Collagenase	Infected
003	Collagenase	Infected
004	Collagenase	Infected
005	Collagenase	No viable cells
006	Collagenase	No viable cells
007	Collagenase	No viable cells
008	Collagenase	Infected
009	Collagensae	No viable cells
010	Collagenase	No viable cells
011	Collagenase	Infected
012	Collagenase	No viable cells
013	Collagenase	No viable cells
014	Time course	No blocks
015	Time course	Infected
016	Time course	Infected
017	Time course	No sections
018	Time course	No sections
019	Collagenase, Time course	No viable cells F,1,2,3,4,5,6,11,12,13,14,16,19
020	Collagenase	No viable cells
021	Collagenase	No viable cells
022	Collagenase	No viable cells
023	Time course	F,1,2,3,4,5,6
024	Fresh fix	F
025	Fresh fix	F
026	Fresh fix	F
027	Fresh fix	F
028	Fresh fix	F
029	Fresh fix	No sections
030	Fresh fix	No sections
031	Fresh fix	F
032	Fresh fix	F
033	Fresh fix	F
034	Fresh fix	No sections
036	Time course	F,1,2,3,4,5,6,7,8,9,10
037	Time course	F,1,2,3,4,5,6,7,8,9,10
038	Time course	No sections
039	Time course	F,1,2,3,4,5,6,7,8
040	Time course	F,1,4,5,6
041	Time course	F,1,4,5,6
042	Frozen	
043	Frozen	

044	Agar culture with drugs	F
045	Agar culture with drugs	1,4,8
046	Agar culture with drugs	1
047	Agar culture with drugs	1
048	Agar culture with drugs	1,4,8
049	Agar culture with drugs	1,4,8
050	Agar culture with drugs	F,1,4,8
051	Agar culture with drugs	1,4,8
052	Agar culture with drugs	1,4,8
053	Agar culture with drugs	F,1,4
054	Agar culture with drugs	F,1,4
055	Agar culture with drugs	F,1,4,8
056	Frozen	
057	Frozen	
058 (right)	Agar culture with drugs	F,1,4,8
058 (left)	Agar culture with drugs	F,1,4
059	Agar culture with drugs	F,1,4,8
067	Agar culture with drugs	F,1
069	Agar culture with drugs	1,5,8
071	Agar culture with drugs	1,4,8
073	Agar culture with drugs	F,1,4
075	Fresh fix	F
077	Fresh fix	F
079	Frozen	
081	Fresh fix	F
083	Fresh fix	F
085	Agar culture with drugs	F,1,4,8
087	Frozen	
089	Frozen	
091	Frozen	
093	Frozen	
095	Frozen	
097	Agar culture with drugs	1,4,8
099	Time course	F,2,4,6,9,10,12,14,16,21
101	Time course	F,2,4,6,9,10,12,14,16,21
103	Time course	F,2,4,6,9,10,12,14,16,21
105	Agar culture with drugs	1,4,8
107	Agar culture with drugs Time course	F,1,4,7 F,1,4,7,10,12,14,17,19,21
108	Fresh fix	F
109	Agar culture with drugs	F,1,4,8
111	Frozen	
113	Frozen	
116	Frozen	
118	Frozen	
120	Frozen	
122	Time course	F,1,2,3,4,5,6,7,8,9,12
124	Agar culture with drugs	F,1,4,8
126	Agar culture with drugs	F,1,4,8

128	Time course	F,1,2,4,6,8,12,14,16,18,29,31
130	Time course	F,1,2,4,6,8,12,14,16,22,28
132	Agar culture with drugs Time course	F,1,4,8 F,1,2,4,6,8,12,14,16,22
134	Agar culture with drugs Time course	F,1,4,8 F,1,4,8,14
136	Agar culture with drugs	F,1,4,8
138	Agar culture with drugs	F,1,4,8
140	Agar culture with drugs	F,1,4,8
142	Frozen	

APPENDIX 7: PATHOLOGY REPORTS

These are the tumours for which the pathology reports were made available.

Tumour Number	Treatment of Tissue	ER Status	Viable Sections from Blocks?
025	time course	negative	yes
036	time course	negative	yes
037	time course	positive	yes
039	time course	negative	yes
040	time course	negative	yes
041	time course	negative	yes
050	agar culture with drugs	negative	no
051	agar culture with drugs	negative	yes
055	agar culture with drugs	positive	yes
059	agar culture with drugs	positive	yes
067	agar culture with drugs	positive	no
069	agar culture with drugs	unknown	yes
071	agar culture with drugs	negative	yes
073	agar culture with drugs	negative	yes
097	agar culture with drugs	negative	yes
102	agar culture with drugs	positive	no
105	agar culture with drugs	positive	yes
122	time course	positive	yes
124	agar culture with drugs	positive	yes
126	agar culture with drugs	negative	yes
128	time course	positive	yes
130	time course	unknown	yes
132	time course agar culture with drugs	positive	yes yes
134	time course agar culture with drugs	positive	yes yes
136	agar culture with drugs	negative	yes
138	agar culture with drugs	negative	yes

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