COLLAGEN GENE EXPRESSION IN HUMAN CANCER

Thesis presented by

GAEL FENHALLS

in fulfillment of the degree of

DOCTOR OF PHILOSOPHY

in

MEDICAL BIOCHEMISTRY

in the

FACULTY OF MEDICINE

UNIVERSITY OF CAPE TOWN

SEPTEMBER 1997

CERTIFICATE OF SUPERVISOR

In terms of paragraph eight of the "General regulations for the degree of Ph.D." I, as supervisor of the candidate **G. FENHALLS**, certify that I approve of the incorporation into this thesis of material that has already been published or submitted for publication.

Signed by candidate

signature removed

Professor M. I. Parker
Department of Medical Biochemistry
D. G. Murray Trust Chair of Cancer Research

ACKNOWLEDGEMENTS

My supervisor, Professor Iqbal Parker for guiding me through the many pitfalls presented within this project.

Professor Mario Ehlers, head of the Department of Medical Biochemistry for his support.

Professor Dent from the Breast Clinic, Groote Schuur Hospital for providing the breast tissue and information regarding the breast cancer patients.

Professor Tiltman and Doctor Pauline Close from the Department of Anatomical Pathology for allowing me the use of their microtome apparatus as well as for helping with the interpretation of results.

Sumayah Cornelius and Helen Ilsley from the Department of Anatomical Pathology for cutting the breast and colon sections for in situ hybridisation analysis.

The Department of Anatomy for the use of their microscope and camera facilities.
Ronny Smart from the Department of Cytogenetics for the karyotyping procedure.

To all in lab 105, for the friendships, the lunches in the cafeteria, the laughter and jokes shared, the good and bad times, with thanks to Howard Donninger, Virna Leaner, Julie Rossouw, Malcolm Collins, Ed Brice, Paul Goldberg, Melissa Geyp, Emmerentia Joseph, Hajira Karjiker, Sarah Herman and to the rest of the department; thank you.

My husband who was extremely supportive and understanding, to my children Emma and Tarryn, to my sisters and friends for encouragement, motivation and love.

A very special thanks to Howard Donninger for his help and encouragement and for being such a good friend.

The Foundation of Reseach and Development and the University of Cape Town for funding this project.

CONTENTS

ABBREVIATIONS

ATP- adenosine triphosphate

βAPN- β aminopropionitrile

BIS- N,N'-methylenediacrylamide

bp- base pairs

BSA- bovine serum albumen

cDNA- complementry DNA

CIAP- calf intestinal alkaline phosphatase

CTP- cytosine triphosphate

dATP- deoxyadenosine triphosphate

dCTP- deoxycytosine triphosphate

DEPC- diethyl pyrocarbonate

dGTP- deoxyguanosine triphosphate

DIG-UTP- digoxigenin uridine triphosphate

DNA- deoxyribonucleic acid

dNTP- deoxynucleoside triphosphate

DTT- dithiothreitol

dTTP- deoxythymidine triphosphate

EDTA- ethylenediaminetetra-acetic acid

EGTA- ethyleneglycol-bis-(β-aminoethyl ether) N,N'-tetra-acetic acid

H+E- haemotoxylin and eosin

Kb- kilobases

Kd- kilodaltons

MEM- minimal essential medium

MOPS- (3-[N-Morpholino] propane sulphonic acid)

mRNA- messenger RNA

NBT- nitro-blue-tettrazolium chloride

NEM- N-ethylmaleimide

PBS- phosphate buffered saline

PCR- polymerase chain reaction

PIPES- Piperazine-N,N'-bis(2-ethanesulphonic acid)

PMSF- phenylmethylsulphonylfluoride

RNA- ribonucleic acid

Rnase- ribonuclease

SDS- sodium dodecyl sulphate

SV40- simian virus 40

Taq- Thermus aquaticus

TCA- trichloroacetic acid

TEMED- N,N,N,N'- tetramethylethylenediamine

tris- tris(hydroxymethyl)aminomethane

tRNA- transfer RNA

UTP- uridine triphosphate

X-phosphate- 5-bromo-4-chloro-3-indolyl-phosphate

IN MEMORY OF MY LATE MOM AND DAD, FOR THEIR TREMENDOUS FIGHT AND COURAGE AGAINST CANCER

ABSTRACT

Type I collagen is the predominant collagen within the stroma and plays an important role in the processes of tumour cell invasion and metastasis during which the collagens within the stroma is degraded. Total RNA was extracted from different stages of breast cancer and adjacent normal tissue for analysis of collagen gene expression by Northern blot hybridisation. Stage I breast tumours had increased α1(I) and α2(I) collagen mRNA, whereas stages II and III tumours had decreased mRNA levels when compared to the adjacent normal tissue. This stage-specific change in collagen gene expression was confirmed by non-radioactive in situ hybridisation and the results indicated that α1(I) and α2(I) collagen mRNA was produced by the stromal fibroblasts and not the tumour cells. To determine whether this altered collagen gene expression was manifested in other cancers, α1(I) and α2(I) collagen mRNA levels were analysed in colorectal carcinoma samples by in situ hybridisation. Colon cancer as in the case of breast cancer, also showed stage specific changes in collagen gene expression. Dukes C and D colon cancer samples had decreased collagen mRNA levels compared to Dukes A and B. Mutated Ras has been shown to affect collagen mRNA levels in vitro (Slack et al, 1992), therefore the colon samples were analysed for Ras mutations in an attempt to correlate Ras mutations with the decreased levels of α1(I) and α2(I) collagen mRNA. Colorectal DNA samples were screened for Ras mutations by SSCP and direct sequence analysis. No possible association was found between the presence of Ras mutations and the decreased collagen gene expression.

To gain greater insight into exactly how tumour cells modulate the collagen produced by normal fibroblasts, primary breast fibroblasts (prepared from breast tissue) were co-cultured with various breast tumour cell lines. The fibroblasts were also incubated with conditioned media prepared from the tumour cells. Collagen production was analysed using the collagenase assay and the results showed that co-cultured tumour cells, as well as growth in the presence of tumour cell conditioned media, resulted in decreased type I collagen production by the fibroblasts. Type III collagen is often produced in conjunction

with type I collagen and we have found that the breast tumour cells modulated type III collagen in the same way as type I collagen. These results demonstrated that a factor(s) was secreted by the tumour cells which affected collagen production. This factor was further shown to stimulate the fibroblasts to produce type I collagenase as analysis of the medium from co-cultured fibroblasts and tumour cells indicated the presence of collagenases. The tumour cell conditioned media was subsequently shown by Western blot analysis to contain a protein of similar molecular weight to the tumour cell derived collagenase stimulatory factor (known as EMMPRIN or extracellular matrix metalloproteinase inducer) which stimulates fibroblasts to secrete collagenases and has been shown to play a crucial role in tumour invasion (Biswas 1982, 1984 and Biswas et al, 1995).

In order to determine whether fibroblasts of different origins reacted similarily when co-cultured with breast tumour cell lines, WI-38 lung fibroblasts and FGo skin fibroblasts were co-cultured with breast tumour cells. WI-38 fibroblasts responded in the same way as breast fibroblasts (having decreased collagen production), FGo fibroblasts had no effect or slightly elevated collagen production, depending on the tumour cell line. These results suggested that the response to tumour cells is tissue specific.

The decrease in type I collagen produced by the fibroblasts when incubated with the tumour cell conditioned media was not due to a decrease in α1(I) and α2(I) collagen mRNA as shown by Northern hybridisation. Type III collagen mRNA was affected differently, the levels were either decreased or increased depending on the tumour cell line being used. We postulate that the fibroblasts and tumour cells required contact for type I collagen mRNA to be decreased. Northern hybridisation showed that types I and III collagen mRNA levels were decreased when tumour cells were co-cultured with the fibroblasts. To demonstrate that specific contact was in fact required, the tumour cells were separated from the fibroblasts by a diffusible membrane and the levels of collagen mRNA were not adversely affected.

Tumour cells, therefore can modulate collagen production by normal fibroblasts in two ways 1); cause the fibroblasts to secrete collagenases which will degrade the collagen and 2); decrease collagen mRNA. Both of these mechanisms would aid the tumour in invasion and metastasis.

CHAPTER ONE

INTRODUCTION

Cancer has been described as the invasion of host tissue by a select group of tumour cells. In order for the tumour cells to invade host tissues, they have to cross certain extracellular matrix barriers of which collagen is a major component. The biosynthesis and degradation of collagen is extremely important during the processes of tumour cell invasion and metastasis, it is not yet quite sure whether it actually acts as a barrier to tumour cell growth or whether it facilitates the invasion. The main focus of this thesis was to examine changes in collagen gene expression in two types of cancers; breast and colon carcinomas. An in vitro system was set up to study collagen gene expression and to provide us with a greater understanding of the relationship between fibroblasts and epithelial cells.

1.1 BREAST CANCER

In most countries, the incidence of breast cancer has increased over the past twenty years and appears to be the most common cancer amongst women. This increase in incidence could be due to improved screening techniques, increased frequency of mammography, clinical breast examinations and general breast cancer awareness, so that previously undetected cancers are now registered. In the United States, 185,700 new cases will be diagnosed in 1997 (including 1,400 among men) of which 44,560 deaths are expected (including 260 men) (Rennie 1996). In South Africa, 1 out of 15 white women and 1 out of 60 black women are affected by this disease (Sitas et al,1990 and 1991). This might be an under-representation due to the fact that breast cancer screening is expensive and not affordable to all races and therefore screened mainly in the white population. Other factors to consider would be differences in life style, risk factors, and differences in breast cancer awareness between the population groups. In the United States more white women are also affected by this cancer than black women and it therefore appears a major affliction to women of affluence (Swanson et al, 1993). On the basis of incidence rates for 1983 through 1987 and mortality rates for 1987 in the United States, 12% of all women will be

diagnosed with breast cancer of which 3.5% will die of the disease (Harris et al, 1992). The incidence increases rapidly during the fourth decade and becomes substantial before the age of 50, thus creating a need for continuous screening and encouragement of all races to participate in breast cancer awareness. Due to the fact that the incidence of breast cancer is increasing world-wide, especially among postmenopausal women, it has become clear that extra efforts are required to obtain more information about this disease. Valuable information such as the identification of family members at risk, and whether the risk factors are genetic or sporadic is needed in order to recommend participation in a screening program.

1.1.1. Risk factors

Factors that are generally agreed to increase the risk of breast cancer include early menarche, late menopause, late age at first pregnancy, obesity, history of benign breast disease, a high animal fat and protein diet, exposure to ionizing radiation, use of oral contraceptives, hormone replacement therapy and a positive family history of breast cancer (Lynch et al, 1991; Harris et al, 1992).

1.1.1.1 Hereditary Breast Cancer

The greatest identified risk factor for hereditary breast cancer is having a family history of the disease, particularly when the diagnosis has been made in a mother or a sister at a young age. Those patients with hereditary breast cancer have been defined as having a positive family history for breast cancer, and sometimes, also of related cancers (eg ovarian) and a distribution that is consistent with an autosomal dominant, highly penetrant cancer susceptibility factor (Lynch et al, 1991). Studies indicate that the presence of breast cancer in a first degree relative increases a women's risk of developing breast cancer by two or three fold (Lynch et al, 1991; Hall et al, 1990).

Those individuals with inherited breast cancer have been shown to have mutations in the BRCA1 breast cancer susceptibility gene, which has been localised to chromosome 17q21 and is thought to code for a transcription factor (Miki et al, 1994). The BRCA1 protein

has two ring finger domains near the NH_2-terminus and an acidic COOH-terminal domain (Chen et al, 1995). BRCA1 mutations or deletions occur in most families with multiple cases of both early-onset breast and ovarian cancer and about 45% of families with breast cancer alone (Wooster et al, 1994). BRCA1 therefore appears to confer a high risk of early-onset breast cancer in females and does not play a role in sporadic, nonhereditary forms of breast cancer, which account for 95% of breast cancer cases (Vogelstein and Kinzler 1994).

Another breast cancer susceptibility gene, BRCA2, has been localised to chromosome 13q12-13. BRCA2 confers a high risk of breast cancer alone, unlike BRCA1 which confers a high risk to both breast and ovarian cancers (Wooster et al, 1994). Whether BRCA2 is involved in sporadic breast cancer still remains to be assessed. It is more than likely that sporadic breast cancer may involve several genes and not only one. Studies on LOH in sporadic breast tumours have shown loss of heterozygosity in chromosomes 1, 3, 6, 7, 8, 9, 11, 13, 15, 16, 17, 18 and 22 (Coles et al, 1992; Osborne et al, 1991 and Thompson et al, 1992). This suggests that a number of tumour suppressor genes could be involved, for example mutations in the p53 gene have been found to occur frequently in sporadic breast tumours (Thompson et al, 1992; Coles et al, 1992). Several oncogenes such as myc, neu and int2 have also been implicated in the development of breast cancer (Coles et al, 1992; Tandon et al, 1989).

1.1.2 Types of Breast Cancer

Breast tumours are heterogenous and contain a mixture of several biologically distinct clones of cancer cells, each capable of self replication or clonal growth (Isaacs 1986; Fidler and Hart 1982). There are basically two types of breast carcinomas, those that do not degrade the basement membrane and do not enter the stroma, thus remaining in situ and those that degrade the basement membrane to enter the stroma and become invasive.

1.1.2.1 In Situ Carcinomas

1.1.2.1.1 In situ Ductal Carcinomas

In situ ductal carcinomas are classified as pre-invasive lesions with histological features of proliferating tumour cells within the ducts which have not invaded the surrounding stromal tissue (Harris et al, 1992; Radford et al, 1993). Morphologically, in situ ductal carcinomas have been divided into several subtypes: noncomedocarcinomas; the most common being cribiform (sievelike), micropapillary, solid and comedocarcinoma, although mixtures of the various types have been seen (Pierson and Wilkinson 1991). Comedocarcinomas are classified by large pleomorphic nuclei (irregular shapes), with numerous mitotic figures and necrotic cellular debris in the centre of the ducts. In contrast the noncomedo subtype is characterised by monomorphic nuclei (of similar shapes), few if any mitoses and the absence of necrosis (Harris et al, 1992). The subtypes also differ according to nuclear grade; the comedo is a high nuclear grade (more advanced stage) and more likely to recur after excision than the low nuclear grade (less advacned stage) noncomedo variety. In addition, the comedo subtype is often associated with areas of microinvasion (Radford et al, 1993). The critical, but unanswered question regarding these breast carcinomas, is whether they will progress to invasive cancer. In most cases a mastectomy is performed which results in a cure rate of 98 to 99 percent. However, it is apparently difficult to recommend the mastectomy as routine procedure due to the trauma involved as well as the expense, especially on small lesions, and therefore more data is required about the tumour's invasive potential (Harris et al, 1992).

1.1.2.1.2 In Situ Lobular Carinomas

These lesions are characterised by a solid mass of small epithelial cells within breast lobules, with uniform, round-to-oval nuclei and distinct cell borders.

1.1.2.2 Invasive Breast Carcinomas

1.1.2.2.1 Infiltrating Ductal Carcinomas

Infiltrating ductal carcinomas, account for 75% of invasive breast tumours, and are the most common type of breast tumour. They are characterised by the absence of any specific histological features (Pierson and Wilkinson 1991). They are generally known as infiltrating ductal carcinomas, "not otherwise specified" or scirrhous carcinomas of the breast. Upon palpation they have a stony hardness and when transected are "gritty" in texture. The presence of excess stroma, consisting of collagen types I, III and V and elastin, is often detected within and around these neoplasms and is known as desmoplasia (Harris et al, 1992 ; Hewitt et al, 1993).

1.1.2.2.2 Infiltrating Lobular Carcinomas

Invasive infiltrating lobular carcinomas have been described as being distinctly linear, with a target-like growth pattern as well as having a desmoplastic stroma termed Indian filing (Pierson and Wilkinson 1991). These tumours vary in their gross appearance from being clinically inapparent, microscopic lesions to replacing the entire breast with a poorly defined, firmer lesion. The tumour may mimic inflammatory and benign disease in many instances. Histopathological features include charateristic small cells with rounded nuclei, inconspicuous nucleoli and scanty indistinct cytoplasm. There is controversy concerning the prognosis of infiltrating lobular carcinomas but the overall prognosis appears to be the same as that for infiltrating ductal carcinomas of equivalent stage (Pierson and Wilkinson 1991).

Infiltrating lobular carcinomas account for 5 to 10 percent of breast tumours, presenting as an ill-defined thickening in the breast. Generally, infiltrating lobular and ductal carcinomas show lymph node involvement, however ductal carcinomas metastasize to bone, lung, liver or brain, whereas lobular carcinomas metastasize to meningeal and serosal surfaces (Harris et al, 1992).

1.2 COLORECTAL CANCER

Cancer of the colon is a common malignant neoplasm in Western countries and is thought to be diet related. In the United States, colon cancer is the second highest cause of mortality in men (after lung cancer) and the third highest cause in women (after lung and breast cancer). 133,500 new cases are expected to be diagnosed in the United States this year (1997) of which 54,900 will succumb to the disease (Crissman and Barwick, 1993; Rennie 1996).

1.2.1 Familial Adenomatous Polyposis (FAP)

A genetic alteration which is inherited in FAP is allelic deletion of chromosome 5q, encompassing the APC gene (Fearon and Vogelstein 1990). FAP is inherited as an autosomal dominant trait with high penetrance. It is characterised by the presence of thousands of adenomatous polyps in the colon by the age of 20 and if not treated will progress to colon cancer (Eng and Ponder 1993). FAP accounts for approximately 1% of colon cancers in the Western world (Peltomaki et al, 1993). A germline mutation in the APC gene is thought to be the first hit in the two hit hypothesis of Knudson's model (Eng and Ponder 1993). In some adenomas from patients with polyps, however, no allelic deletions of chromosome 5 have been detected, whereas the deletions were common in adenomas from patients without polyposis. This finding is not compatible with the Knudson model for a tumour suppressor gene which, at the cellular level, functions in a recessive manner so that in order for a tumour to occur, both alleles require inactivation (Vogelstein et al, 1988). It has been hypothesised that deletions in the APC gene induce an increase in proliferation leading to the formation of the adenoma in FAP patients. The mutation in one of the alleles inactivates the APC gene, causing a decrease in normal gene expression and inefficient control of cellular proliferation, even when the other allele remains unaffected. In some patients without FAP, the somatic loss of one allele would also cause normal epithelial cells to proliferate, providing them with a selective growth advantage. Inactivation of the other allele would increase the proliferative effect, but there is still another event which should occur to promote the transition of the abnormal epithelium to the adenoma and this event is not the loss of the wild type allele. This second

event is unknown, but could be a decrease in DNA methylation (Vogelstein et al, 1988; Eng and Ponder 1993).

1.2.2 Hereditary Non-Polyposis Colorectal Cancer

A second form of colon cancer that shows familial aggregation is hereditary non-polyposis colorectal cancer (HNPCC) which is more common than FAP, accounting for 4 to 13% of colon cancers (Peltomaki et al, 1993). Unlike patients with sporadic colon cancer, patients with HNPCC have a mean age of diagnosis of 44 years with 72% of first colon cancers located in the right colon (Eng and Ponder 1993). HNPCC patients are defined as individuals who have at least three relatives in two generations having colorectal cancer, with one of the relatives having been diagnosed at less than 50 years of age (Peltomaki et al, 1993; Eng and Ponder 1993).

1.2.3 DNA mismatch Repair Genes in HNPCC

Genetic linkage analysis has been performed on families with HNPCC to identify the existence of a familial colon cancer gene(s) responsible for this disease. Close linkage using microsatellite markers on chromosome 2 have been demonstrated in two large families (Peltomaki et al, 1993). This gene is not a typical tumour suppressor gene but is the human homolog of the bacterial MutS and S. cerevisiae MSH proteins, and was thus called hMSH2 which maps to chromosome 2p22-21, near the locus implicated in HNPCC (Fishel et al, 1993).

It has therefore been suggested that hMSH2 plays a role in the development of colon cancer. A second mismatch repair gene (hMutL), homologous to the bacterial mismatch repair gene was located on chromosome 3p21.3-23 and has been identified as playing a role in HNPCC (Bronner et al, 1994). These two genes can therefore be used as markers to identify HNPCC patients (Nicolaides et al, 1994). It is also possible that other mismatch repair genes may be involved in HNPCC, and sporadic colon cancer.

1.2.4 Genetic Mutations associated with Colon Cancer

Colon cancer has been described as a multistep process which progresses from an adenoma to a carcinoma and eventually metastasis occurs (Fearon and Vogelstein 1990). Genetic alterations occur and accumulate as the tumour progresses and may be used as markers to identify the stage of the cancer. Four alterations have been described; first is a mutation on chromosome 5q causing a change in the normal epithelium, aiding its progression to an adenoma. Mutation of the Ki-Ras oncogene is the second known gene alteration, which could be the initiating event, allowing for the progression of the adenoma to the carcinoma stage and is therefore involved in the early stages of the disease. The remaining alterations occur in the tumour suppressor genes DCC and p53 which are associated with late adenoma and carcinoma stages respectively. The exact order of these mutations seems not to be the key factor, but it is the accumulation which is important (Fearon and Vogelstein 1990).

1.2.4.1 Ras Gene Mutations in Colon Cancer

The incidence of Ras mutations in colorectal cancer has been reported to be higher when compared to breast, stomach and bladder cancers. The reason for this is unknown but could be due to different pathogenic mechanisms (Bos et al, 1987). A mutation in the Ki-Ras gene is an early event in the adenoma stage. The Ras family of genes consists of three members, Ha-Ras, Ki-Ras and N-Ras, and point mutations in these genes have been shown to occur in about 50% of all human malignancies (Bos et al, 1987). Mutations occur primarily in codons 12, 13 and 61, resulting in amino acid substitutions that reduce or abolish intrinsic GTPase activity (Finkelstein et al, 1993). In colon cancer, mutations in codon 12 of the Ki-Ras gene are more common than mutations in codons 13 and 61 (Bos et al, 1987). The exact role and functional significance of Ki-Ras in tumourigenesis was closely analysed in two human colon cancer cell lines and it was found that cells constitutively active for Ki-Ras were morphologically altered, lost the capacity for anchorage dependent growth, grew slowly in nude mice and showed reduced expression of c-Myc (Shirasawa et al, 1993). It was therefore concluded that activated Ki-Ras plays a key role in tumourigenesis through altered cell growth and differentiation. Transfection of

a mutated Ras gene into normal cells has been shown to confer neoplastic properties (Barbacid 1987; Weinberg 1989; Thorgeirsson et al, 1985). Transformation of rat fibroblasts with mutant Ha-Ras^{VAL2} resulted in cell transformation and a reduction of the levels of α1(I) and α2(I) collagen mRNA of which the exact mechanism is unknown but has been shown to be regulated at both transcriptional and post-transcriptional levels (Slack et al, 1992). The AP-1 site in the first intron of the α1(I) collagen gene has been implicated in the loss of transcription (Slack et al, 1995). The authors relate this decrease in gene expression to the presence of oncogenic Ras which would increase the metastatic potential of the cell.

1.3 TUMOUR INVASION AND METASTASIS

1.3.1 Invasion of the Extracellular Matrix

The extracellular matrix (ECM) acts as a three-dimensional support scaffold that mediates cell attachment and determines tissue architecture (Liotta 1992). It also provides selective permeability for the transport of proteins as well as other molecules between cells, across blood vessel walls and during filtration of the kidney. It influences cytodifferentiation, mitogenesis and morphogenesis. The interstitial matrix, with stromal fibroblasts and myofibroblasts contains predominantly type I collagen as well as collagen types II and III, fibronectin and specific glycoproteins and proteoglycans. The basement membrane, on the other hand, (which separates organ parenchyma cells from the interstitial stroma) contains type IV collagen, laminin, entactin and BM1 proteoglycan. Type V collagen has been identified in the basement membrane and the interstitial matrix, depending on the tissue type (Liotta 1992).

During the process of invasion, the tumour cell(s) must traverse the ECM as they cross tissue boundaries. During the transition from in situ to invasive carcinoma, tumour cells penetrate the basement membranes and subsequently enter the interstitial stroma (Liotta et al, 1983). From the stroma, the tumour cells disseminate further by gaining access to the lymphatics and blood vessels. Intravasation and extravasation through the capillary walls require penetration of the basement membrane by the tumour cells. In the target organ,

where metastases are initiated, the extravasated tumour cells migrate through the perivascular interstitial stroma before tumour colony growth can occur in the organ parenchyma. Therefore the ECM is invaded several times during metastasis (Liotta et al, 1982 and 1983). The ability of tumour cells to invade the ECM is not unique to tumour cells, but also performed by normal cells. For example, trophoblasts, invade the endometrial stroma and blood vessels to establish contact with the maternal circulation during development of the hemocorial placenta (Stetler-Stevenson et al, 1993). Similarily endothelial cells invade basement membranes as well as the interstitial stroma during angiogenesis. The main difference between normal and tumour cells is that tumour cells have lost control over the invasive phenotype expressed by normal cells.

The ECM has often been described as a mechanical barrier to tumour cell invasion and the loss of basement membrane has become a marker for invasive carcinomas which may be due to decreased synthesis or assembly of secreted products (Liotta et al, 1983). Normal epithelial cells require a basement membrane for attachment and growth, whereas actively invading carcinoma cells do not have this requirement. The tumour cell must interact with the basement membrane at all tissue boundaries and the loss of the basement membrane has been depicted as a fundamental difference between malignant and benign tumours (Liotta et al, 1982). Tumour cell interaction with the basement membrane has been defined as the critical event that signals the initiation of the metastatic cascade (Stetler-Stevenson et al, 1993). Other lines of evidence that do not substantiate and support the fact that basement membrane acts as a barrier against tumour cell invasion have also been presented. Many invading carcinomas retain their basement membranes in both primary and secondary sites and the loss of the tumour basement membrane is not always associated with tumour invasion (Nakanishi et al, 1992). It has also been reported that in some tumours, the tumour basement membrane appears to mediate tumour invasion and metastasis where it was shown that more highly metastatic clones had a more highly integrated basement membrane than low metastatic cells (Nakanishi et al, 1991 and 1992). It was speculated that attachment of tumour cells to the vascular basement membrane results in intravasation (Nakanishi et al, 1992). Work done by Hewitt et al, (1994)

demonstrated that colorectal carcinoma metastases tended to have more continuous epithelial basement membranes than corresponding primary tumours and could be explained by local tissue environmental factors having an influence on basement membrane continuity. These results argue against the metastases dogma which infers that metastases arise preferentially from more aggressive tumour cell sub-populations, and being a selective process, more discontinuous basement membranes might be expected in secondary rather than in primary tumours. Metastases are thought to arise from more aggressive tumour cell populations, however, Hewitt et al, (1994) argue that resulting metastases tend to show a less aggressive phenotype than the primary tumour of origin. The authors hypothesize that epithelial basement membrane breaks are associated with invasive activity determined by the local tissue environment at the site of primary tumours and their metastases. These studies demonstrate that the function of basement membrane can be questioned and possibly vary from not only study to study but also from carcinoma type to carcinoma type.

The exact function of tumour basement membrane, whether it aids invasion or acts as a barrier, must still therefore be determined, but its importance in the processes of tumour cell invasion and metastasis cannot be undermined. As already mentioned most of the steps in the metastatic process require that the tumour cells interact with the basement membrane in order to invade and metastasize. A three-step hypothesis was proposed by Liotta and colleagues which describes the sequence of events occuring during invasion of the ECM; first step is attachment or adhesion of the tumour cell to the matrix; followed by the second step which is degradation of the matrix by tumour-cell associated proteases; the third step is tumour cell locomotion or motility into the region of the matrix modified by proteolysis (Liotta et al, 1983; Stetler-Stevenson et al, 1993).

1.3.1.1 Adhesion to the Matrix

A number of specific cell-surface-associated molecules that modulate cell-matrix and cell-cell interaction have been identified, such as the integrins, a 67 Kd laminin binding protein, cadherins, Ig (immunoglobin) superfamily and CD44 (Stetler-Stevenson et al, 1993).

The integrins, of which twenty have been identified so far, are a family of cell-surface receptors that mediate cell attachment and therefore play a role in tumourigenesis, invasion and metastasis. They are formed by various noncovalent associations of fourteen α and eight β subunits and were originally identified as receptors for collagens, fibronectin, laminin and vitronectin. There is redundancy at the level of both receptor and ligand because it has been found that integrin receptors can bind to more than one type of extracellular matrix protein and individual matrix proteins can be recognised by more than one integrin receptor (Stracke et al, 1996). Integrins recognize the arginine-glycine-aspartic acid (RGD) sequence which is the recognition site of many adhesion proteins such as collagen, fibronectin and thrombospondin (Vezediris 1993). Binding of some integrins to the ECM induces their aggregation into focal adhesions which can then activate many signal transduction molecules (Judware and Culp 1996). On the cytoplasmic side of focal adhesions, integrins combine with cytoskeletal components such as talin and vinculin which induces the condensation of actin into F-actin filament bundles, resulting in phosphorylation of both the focal adhesion kinase (FAK) and the β-subunit of the integrin. Intracellular pH increases due to activation of a Na^+ -H^+ antiport, [Ca^{2+}] also increases and c-Src undergoes activation in the presence of phosphorylated FAK. Protein kinase C becomes activated, translocates to the cell membrane, and active NF-kB is liberated which translocates to the nucleus. In the nucleus NF-kB can promote transcription of many genes such as c-Fos, c-Jun, IL-1, MAD-6 and CSF-1 (Judware and Culp 1996). It has been shown that the first intron of the human α1 collagen gene contains an AP-1 motif which is able to bind a heterologous complex composed of members of the Fos and Jun transcription factor families (Slack et al, 1993). In this way activation of the signal transduction pathway which activates NF-kB to promote transcription of Fos and Jun-related transcription factors can modulate the collagen gene.

Laminin is involved in normal cell attachment, spreading, migration, tissue remodelling, embryogenesis as well as tumour invasion and metastases (Stracke et al, 1996). Laminin forms a bridge between the 67 Kd laminin receptor and type IV collagen (Liotta et al, 1983; Liotta 1986). Breast and colon carcinomas have been found to have a large number of exposed laminin receptors compared to benign epithelium. The laminin receptors of normal epithelium may be polarised at the basal surface and occupied with laminin in the basement membrane. This is different to the invading tumour cells, where the receptors may be distributed over the entire surface and be unoccupied, due to absence of basement membrane (Liotta 1986; Stetler-Stevenson et al, 1993).

Recent studies have implied an inhibitory role for cell adhesion molecules (CAMS) of the cadherin family. Cadherins are Ca^{2+} dependent CAMS that mediate cell-cell binding/adherence. Three sub-types (E, N and P-cadherins) have been well characterised and are distinguished by their tissue distribution. Other cell adhesion molecules include the Ig superfamily members, such as N-CAM and VCAM-1, which incorporates a number of proteins that all share the immunoglobulin homology unit consisting of 70 to 110 amino acids organised into 7-9 β-sheets. Family members include molecules involved in cellular immunity, signal transduction and cell adhesion, which makes the exact role of these molecules in tumour invasion difficult to understand. VCAM-1 (also known as INCM-110) was identified on endothelial cells as a cytokine-inducible receptor for the VLA-4 ($\alpha_4\beta_1$) integrin. VLA-4 is found primarily on white blood cells and functions in mediating leukocyte-endothelial cell attachment. Malignant melanoma cells may also express VLA-4, and VCAM-1 therefore may act as a tumour adhesion receptor, facilitating interaction of circulating melanoma cells with the endothelium in advance of tumour cell extravasation (Stetler-Stevenson et al, 1993; Stracke et al, 1996). Attachment to the matrix is only one of the qualities which a tumour cell(s) requires for tumour invasion, and tumour cells that are more adherent (either to a substrate or to other tumour cells) are less tumourigenic, less motile and less invasive. On the other hand receptor function studies (eg, fibronectin, laminin) have indicated that inhibition of tumour cell adhesion to the ECM also results in less aggressive behaviour (Stetler-Stevenson et al, 1993; Stracke et al, 1996).

1.3.1.2 Proteolysis of the Extracellular Matrix

The discovery that proteolytic enzymes play a role in tumour invasion and that inhibitors of metalloproteinases block invasion of the ECM indicated that invasion is not merely a passive growth process but requires active biochemical mechanisms (Liotta et al, 1983). The secretion of matrix metalloproteinases (MMPs) and the tissue inhibitors of matrix metalloproteinases (TIMPs) needs to be balanced and is not a property unique to tumour cells (Stetler-Stevenson et al, 1993; Liotta et al, 1982 and 1983). Proteolysis is utilised during trophoblast implantation, embryo morphogenesis, tissue remodelling, parasitic and bacterial invasion and angiogenesis (Liotta et al, 1991). Lysis of cell membranes is a tightly controlled mechanism used by tumour cells in conjunction with continued attachment and detachment, thus enabling the tumour cell to move. There is now substantial evidence that MMPs are directly involved in tumour invasion and metastasis. The tumour cell secretes proteolytic enzymes or factors which stimulate host cells to secrete these enzymes to locally degrade the matrix proteins. Degradation takes place in a highly localised region close to the tumour cell surface where there is more active enzyme than natural protease inhibitors. Higher levels of proteolytic enzymes have been associated with malignant tumours when compared with normal or benign cells (Stetler-Stevenson et al, 1993).

Connective tissue destruction has been described as an irreversible process, which is why MMP inhibitors constitute viable strategies for interfering with the processes of tumour invasion and metastasis. Several approaches, such as the use of TIMPs or TIMP fragments as selective inhibitors of MMP activation or activity, peptide inhibitors, which mimic the amino terminal MMP motif and maintains the enzyme in a latent state, as well as synthetic compounds which compete for the substrate or bind to the active site, have been described (Kohn and Liotta 1995). An example of a synthetic MMP inhibitor is compound BB94 which has been shown to be active in vitro with a 50% inhibitory concentration of 20 nM for stromelysin and 4 nM for gelatinase A. BB94 inhibited growth of a primary tumour, caused a reduction in the incidence of tumour invasion of local tissue and reduced the number of spontaneous metastases (Kohn and Liotta 1995).

1.3.1.3 Locomotion or tumour cell motility through the matrix

Tumour cells have been found to migrate in response to a number of different stimuli, these include host-derived scatter factors, growth factors, components of the ECM, hyaluronan and tumour-secreted factors (Stracke et al, 1996). Motility stimulated by each of these factors can be either random (chemokinesis) or directed (chemotaxis). In the absence of soluble attractant, tumour cells can also migrate in a directional manner, towards substratum-bound, insoluble ECM proteins and this is called haptotaxis (Stracke et al, 1996; Stetler-Stevenson et al, 1993). Cultured human A2058 melanoma cells produce autocrine motility factors (AMF) which stimulate random and directed motility. Cells in the primary tumour may therefore secrete AMF until the concentration rises sufficiently to stimulate motility via receptors on the responding cells (Stetler-Stevenson et al, 1993). Only two growth factors, hepatocyte growth factor/scatter factor (HGF/SF) and insulin-like growth factor II (IGF-II) and the 125 Kd glycoprotein autoxin (ATF) have been purified to homogeneity and cloned. AMFs are not specific for a given type of cancer cell, but have a wide spectrum of activity on many types of cancer cells. Autocrine motility was based on the hypothesis that certain tumour cells exert autocrine control over their own migration, in this way AMFs would allow tumour cells to initiate, sustain and regulate migration of tumour cells during the processes of tumour cell invasion and metastasis (Stracke et al, 1996).

Tumour cell migration in response to certain matrix proteins has been found to correlate with the in vivo invasive and metastatic potential of melanoma and carcinoma cell lines. Vitronectin, fibronectin, laminin, type I and IV collagen and thrombospondin are all capable of stimulating locomotion in tumour cells. ECM proteins stimulate different types of motility responses, depending on their mode of presentation to the cells; the response will be chemotaxis when they are in solution and haptotaxis when they are insoluble or bound to the substrate. Both types of stimulation appear to act through different cell surface receptors and post-receptor signal transduction pathways (Stracke et al, 1996). It is not clear how the stimulation of tumour cell motility by ECM proteins plays a role in metastasis but it has been hypothesised that during the initial phases of metastasis,

insoluble matrix proteins provide tumour cells with a means of activation thus allowing the tumour cells to track through stromal tissues and along basement membranes. Proteolytic enzymes (eg type IV and interstitial collagenase, cathepsin B, plasminogen activator) could then result in pools of soluble or partially degraded matrix proteins which could provide an additional chemotactic stimulus to motility (Stracke et al, 1996).

Tumour cell locomotion is the third step in the invasion process and has been described as the protrusion of pseudopodia (ameboid false feet) into the zone of proteoysis, followed by migration of the tumour cell (Liotta 1992). The tumour has two fronts, an advancing one which activates enzymes and a rear one which remains attached to the ECM. Once the path for the tumour cell has been cleared, it advances forward. The invading tumour cell attaches to the matrix in the direction of movement, pulls itself forward and releases any attachments at the rear of the cell. Tumour cells, unlike normal cells which stop once the stimulus for movement has been removed, migrate and penetrate tissue barriers and only stop when the host dies (Liotta 1992; Weiss and Ward 1983; Vezeridis 1993; Strauli and Haemmerli 1984).

Even though the three steps of tumour invasion are linked and much is known about them, we still do not know how each step is controlled. If the genes that control the different stages of tumour invasion and metastasis could be determined, this would contribute to a better understanding of these two processes.

1.3.2 Metastasis

Simplistically, metastasis is the process by which tumour cells break away from the primary tumour, enter the host stroma and travel to distant sites, where secondary tumours are formed. Metastasis has been defined as the transfer of disease from one organ to another not directly connected with it (Fidler 1978). It is due to this process that individuals with cancer die, because in most cases by the time the cancer has been diagnosed, metastasis has already occurred. Early diagnosis, surgical removal, general

patient care, local and systemic adjuvant therapies do not prevent metastases from taking place and most cancer deaths are due to metastases that are resistant to conventional therapies (Fidler 1990). The major barrier or hindrance to the treatment of metastases is the biological heterogeneity of cancer cells in primary and secondary tumours.

1.3.2.1 Biological Heterogeneity of Tumour Cells

The term biological heterogeneity is used to describe cells within a tumour cell population where they have different cell surface receptors, enzymes, karyotypes, cell morphologies, growth properties, sensitivities to therapeutic agents and the ability to invade and metastasize (Fidler 1978). Only 0.1% of tumours survive the metastatic process, indicating that metastasis is an inefficient process, carried out only by certain tumour cells which have the ability to do so (Fidler and Kriepe 1977). Studies on a B16 melanoma cell line showed that unique metastatic tumour cells are present in the original tumour population and that cells of both low and high metastatic potential exist in the primary tumour. Nicolson and Custead (1982) showed that these metastatic properties did not arise during metastasis by a process of adaption to local environmental conditions. A cell that acquires an increased ability to survive all the facets of the metastatic process has an increased metastatic potential which enables that cell to "land" in organs with a suitable environment permitting their growth (Fidler and Kriepe 1977). It has now been established that tumour cell invasion and metastasis are not passive processes and a new hypothesis consisting of three principles has evolved which encompasses all aspects of these processess: 1) metastasis is not random, but selective; 2) tumours are not uniform entities and contain cells with heterogeneous metastatic capabilities and 3) the outcome of metastasis depends on properties of tumour cells as well as host factors (Fidler and Balch 1987).

The influence of the environment on the growth of tumour cells was originally proposed in Paget's "seed and soil" hypothesis (Gutman et al, 1995). Experimental data have subsequently shown that the organ environment can also influence tumourigenesis, production of degradative enzymes, angiogenic factors as well as other activities. For example IL-8 enhances the growth of malignant melanomas and melanoma cells growing

in the skin produced high levels of IL-8, whereas those in the liver expressed low levels (Gutman et al, 1995). This differential expression was found to be due to adaptation to the local organ environment and regulation by different cytokines expressed in the specific environment.

The cellular heterogeneity of a tumour population is not unique to neoplastic cells. Normal cells and tissues are also heterogeneous for certain characteristics such as cell surface antigens and enzymes. Malignant cells, however, are more heterogeneous than benign or normal tissue. Tumour heterogeneity, therefore, is a normal characteristic and is exploited by tumour cells, enabling them to survive the metastatic process (Nicolson 1987).

Phenotypic instability is a cellular phenomenon that appears to contribute to normal and tumour cell diversification and heterogeneity (Nicolson 1987). This property includes epigenetic and micro-environmental changes that can result in tumour cell diversity. The micro-environments of tumour cells can be a determining factor in their phenotypic stabilities. Differences in tissue inervation, stroma, nutrients, growth factors, oxygen, hormones, enzymes, inducers, ions and other tumour regulators may play roles in destabilising tumour cells and determining their susceptibilities to genetic and epigenetic changes (Nicolson 1988). The extracellular micro-environment of tumours is determined by the matrix synthesised by normal and tumour cells. The host stromal components or infiltrating host fibroblasts and other cells also determine the type of tumour matrix. Modulation in the extracellular elements synthesised by parenchymal cells, endothelial cells, mesothelial cells, fibroblasts and other host cells could lead to altered gene expression and cell phenotype (Nicolson 1987; 1988).

1.3.2.2 The Metastatic Process

The metastatic process consists of a series of sequential, interrelated steps such as angiogenesis, motility, invasion, survival in the circulation, adhesion, extravasation and proliferation (Fidler 1990; Ellis and Fidler 1995). The major steps after the initial transforming event are the growth of the neoplastic cells, followed by extensive

vascularisation caused by the synthesis and secretion of angiogenic factors which allow the tumour mass to exceed 2mm in diameter. The host stroma is degraded by proteolytic enzymes and subsequently invaded by malignant tumour cells from the primary tumour. The tumour cells, either as single cells or multiple cell emboli can then penetrate lymph nodes or blood vessels, known as intravasation. While in the circulation, the tumour cells must survive mechanical trauma such as blood turbulence and evade host defenses such as lymphocytes, complement, antibodies, natural killer cells and phagocytes. The tumour cells are then transported to distant sites in the body whereby those tumour cells which have survived in the circulation will arrest in the capillary beds of distant organs. Certain tumour cells exit from the capillaries, adhere to endothelial cells or exposed basement membranes and extravasate into the surrounding parenchyma of the tissue. These cells will proliferate, if the environment is suitable for their growth, thus completing the metastatic process (Fidler and Poste 1985; Fidler 1990). To complete the process, tumour cells develope a vascular network, evade the host immune system and respond to specific factors that influence their growth. Failure to complete any of the above steps in the metastatic process leads to the elimination of the desseminating tumour cell (Fidler and Poste 1985).

1.3.2.2.1 Angiogenesis

Angiogenesis is the process of generating new blood capillaries resulting in neovascularisation. The onset of angiogenesis permits rapid growth of a tumour population and is a normal process which occurs during embryonic development, ovulation, wound healing, and in nonmalignant diseases (ocular neovascularisation, psoriasis/hypervascular skin lesions, arthritis) and cancer (Folkman 1974a; Folkman 1989; Folkman 1995).

Some tumours can remain in situ for extended periods without neovascularisation and become vascularised by switching to an angiogenic phenotype. In the prevascular stage, the tumour is rarely bigger than 2-3mm and may contain a million or more cells, and can remain dormant but proliferate until the cells reach an equilibrium and die (Folkman 1995).

The switch to the angiogenic phenotype involves a change in the local equilibrium, whereby tumour cells may start overexpressing one or more of the positive regulators of angiogenesis as well as bring about down-regulation of inhibitors of angiogenesis. The angiogenic phenotype could then cause a benign tumour to become malignant and is influenced by the interaction of stimulatory and inhibitory molecules produced by the tumour cells as well as the organ specific enviroment (Singh et al, 1995). There are 12 known angiogenic proteins, of which basic fibroblast growth factor (bFGF) and vascular permeability growth factor (VEGF) are the most common in tumours. Other factors include angiogenin, TGF-α and TGF-β, TNF-α and IL-8 (Folkman 1995). Thrombospondin-1, TIMP-1, 2 and 3 and angiostatin are a few examples of negative regulators of endothelial-cell proliferation. Removal of a primary Lewis lung carcinoma tumour in mice resulted in increased growth of lung metastases, and the presence of the primary tumour was therefore synonymous with potent anti-angiogenic activity, in the form of angiostatin which inhibited growth of the Lewis lung metastases (Holmgren et al, 1995). Angiostatin is clearly a potential therapy against tumours, in fact, it has been systemically administered and found to inhibit growth of human breast, colon and prostrate cancers (O'Reilly et al, 1996). This inhibition was observed without detectable toxicity or resistance and the tumours were found to regress to microscopic dormant foci in which tumour proliferation was balanced by apoptosis in the presence of blocked angiogenesis. This was the first demonstration of dormancy therapy, whereby malignant tumours are prevented from growing by inhibition of angiogenesis (Ellis and Fidler 1995; O'Reilly et al, 1996).

1.4 COLLAGEN

The largest group of ECM molecules is represented by the collagen family (Judware and Culp 1996). Collagens are large glycoproteins (about 300 Kd for the basic unit) that comprise 30% of the total body protein mass and their primary function is to maintain the three-dimensional shape of tissues. The word collagen derives from the Greek word kolla meaning glue and the term "glue former" was originally used in the 19th Century for the component in skin, bone, cartilage and tendon, which when boiled, produced glue (Weiss

and Ayad 1982). There are now at least 19 different collagens, and at least another 10 have collagen-like domains (Prockop and Kivirikko 1995). Mature collagens are composed of three α chains that intertwine into a triple-helical conformation.

Type I collagen, a fibrillar protein, is the most common form of collagen making up 90% of the total collagen found in the body. Collagenous domains (found predominantly in the fibrillar collagens I, II, II, V and XI) are characterised by a distinct tri-peptide repeat within the three α chains, Gly-X-Y, where X and Y is usually lysyl-hydroxylysine and/or prolyl-hydroxyproline (Prockop et al, 1979). These amino acids allow for crosslinking which confers greater rigidity to the collagen fibrils and a necessary support to tissues, organs, tendons and ligaments (Judware and Culp 1996; Prockop and Kivirikko 1995). Each α chain is coiled into a left-handed helix with three amino acids per turn (Prockop et al, 1979). The three helical chains are subsequently twisted into a right-handed super helix to form a rigid-rope like structure. The conformation of the triple helix places the amino acid side chain in the X- and Y- positions on the surface of the molecule, thus allowing collagen to polymerise (Prockop and Kivirikko 1995).

The fibril-forming collagens have a more selective tissue distribution than the other collagens (Prockop and Kivirikko 1995). Type I collagen is found in most connective tissues, as is type III collagen, except that type III collagen is located in only the soft connective tissue matrix, whereras type I collagen is also found in mineralised bone (Prockop and Kivirikko 1995; Zhu et al, 1993). The ratio of these two collagens varies from tissue to tissue and also changes in various tissues during development. The ratio of type III collagen to type I collagen in cultured fibroblasts is roughly 1:4 (Liau et al, 1985).

1.4.1. Regulation of type I collagen synthesis

Regulation of collagen production, and thus the formation of a functional collagenous matrix, can occur at the level of transcription, translation, or at some step prior to secretion (Paglia et al, 1979; Aycock et al, 1986).

1.4.1.1. Transcriptional Regulation

The transcriptional mechanisms which regulate collagen gene expression are generally poorly understood (Slack et al, 1993). The α1 and α2 collagen genes are co-ordinately expressed, and this is probably mediated by interactions between tightly regulated transcription factors and cis -acting DNA elements. The cis elements include TATA and CCAAT sequences and the trans-acting factors include a CCAAT box binding factor (CBF), which binds to both promoters and appears to be related to, or identical to, other CCAAT binding factors. In addition to factors which activate the collagen promoter, repressors also interact with them, and in the case of the α2(I) collagen promoter, there are overlapping binding sites for activators and repressors suggesting tight control in the regulation of these genes (Collins 1994).

1.4.1.2. Regulation by Cytokines

Fibroblasts are the predominant cells which produce type I collagen and they are continuously exposed to extracellular signaling molecules such as Transforming Growth Factor-β1 (TGF-β1), Interleukin-1 (IL-1), Tumour Necrosis Factor α (TNFα) and Epidermal Growth Factor (EGF). These cytokines are capable of modulating the synthesis of collagen by affecting mRNA synthesis, turnover or stability (Mauviel et al, 1988). It would appear that the actions of these cytokines are best understood in the context of a network, whereby the effect of one cytokine on a target tissue can be altered by the presence of another cytokine (Elias et al, 1990).

In the case of TGF-β, it has been shown that TGF-β stimulates transcription of the human α2(I) collagen (COL1A2) promoter by increasing the affinity of an Sp1-containing protein complex for its cognate DNA-binding site (Inagaki et al, 1994). A TGF-β-responsive element (TbRE) of the COL1A2 promoter has been mapped to a 131 bp region that contains at least two cis-acting elements. DNA binding assays showed that the TbRE contains one box (box 3A) occupied by Sp1 and the other (box B) occupied by unknown factors(s). These results exclude a role for NF-1 and implicate Sp1 in mediating TGF-β

stimulation (Inagaki et al, 1994). It is postulated that TNF-α counteracts the TGF-β-elicited stimulation of collagen gene expression through overlapping nuclear signaling pathways (Inagaki et al, 1995). The one pathway modifies the TGF-β-targeted transcriptional complex (possibly by decreasing its stimulatory effect on collagen gene expression) and the other pathway acts on the binding of the adjacent factor (possibly by increasing its effectiveness in repressing the activity of the collagen promoter).

1.4.1.3. Regulation by oncogenes

v-Src, v-Mos, c-Myc, v-Fos and Ras have all been reported to decrease transcription of either one or both the α1(I) and α2(I) collagen genes (Slack et al, 1992). In the case of v-Mos it was postulated that mutations in this gene may alter cellular transcription factors which either indirectly or directly control the expression of the collagen gene (Setoyama et al, 1985). These oncogenes are members of a network of cellular proteins that play a role in the regulation of cell growth and division in normal and neoplastic cells. Functional Ras, a member of this network, has been reported to be involved in Src-induced changes in gene expression and Src transformation (Cai et al, 1990; Nori et al, 1991 and Smith et al, 1986). Slack et al, (1992) found that levels of both α1(I) and α2(I) collagen mRNAs were decreased in Rat 1 fibroblasts overexpressing either the N-ras^{LYS-61} or the H-ras^{VAL-12} oncogene. Decreased transcription began within 8 hours of Ras induction and it was shown for the α1(I) gene that the decrease was caused by a decrease in transcription as well as by a reduction in stability of the α1(I) transcript. Oncogenic Ras can therefore regulate type I collagen at the transcriptional and post-transcriptional levels and this effect (at least for the α1(I) gene) may perhaps be mediated by sequences located either within the body of the gene or in the distal 3'-flanking region. It has been subsequently shown that Ras transformation blocks the function of an intronic AP1 site in the α1(I) gene (Slack et al, 1995).

1.4.1.4. Post-transcriptional Regulation

The amount of collagen deposited in the ECM could also be regulated by enzymes that modify the polypeptide chains. Specific modifications of the peptides include enzymatic hydroxylation of certain prolyl and lysyl residues to form peptide-bound hydroxyproline and hydroxylysine (Paglia et al, 1979).

Translational inhibition of procollagen mRNAs by the peptides has been shown to play a significant role in the control of collagen biosynthesis. The NH_2 and COOH terminal peptides of type I procollagen were shown to specifically inhibit the synthesis of collagen by affecting polypeptide chain elongation or termination or both (Aycock et al, 1986; Wiestner et al, 1979 and Horlein et al, 1981). This feedback inhibition by these peptides may play a role in vivo, but exactly how they accomplish the inhibition remains to be elucidated (Bornstein and Sage 1989).

1.4.2. Degradation of Collagen

The physical and chemical stability of the collagen triple helix depends on the hydrogen bonds between hydroxyproline residues and amino acids on adjacent alpha chains. Intact collagen is resistant to attack by most proteases except the specific collagenase. (Pauli et al, 1983).

Collagenase is defined as an enzyme specifically capable of degrading either individual collagen molecules in solution or insoluble collagen fibers under physiological conditions (Harris and Krane 1974). The molecular weight of collagenases derived from normal tissues range between 33,000 and 80,000 daltons (Liotta et al, 1983). Tumour-derived collagenases fall into the same molecular weight range and are also calcium and zinc dependent. Collagenases are members of the metalloproteinase (MMP) family which have been found to be involved in the normal processes of tissue remodelling such as wound healing, trophoblast implantation and organ morphogenesis. They have also been implicated in disease states such as tumour invasion and metastasis, rheumatoid arthritis

and osteoporosis (Wilson et al, 1995; Judware and Culp 1996). More than ten MMP's have been classified into five major types; the interstitial collagenases, which degrade fibrillar collagens; gelatinase/type IV collagenases which hydrolyse gelatin, type IV and V collagen; stromelysins which are active against proteoglycans, laminin, fibronectin, type IV collagen and telopeptides of other collagens; matrilysin and macrophage metalloelastase and the membrane-bound enzymes (Imai et al, 1995).

The first vertebrate collagenase was isolated from primary cultures of tadpole tails by Gross et al, (1962). The enzyme was found to be most active at neutral and/or slightly alkaline pH and its activity was inhibited by metal chelating agents such as EDTA. Native collagen is cleaved into two fragments of molecular weight 24,000 daltons (1/4 size) and 71,000 daltons (3/4 size) at 25°C. These collagen products are thermally unstable and can denature at 37°C and can be degraded further into smaller fragments by other proteinases such as plasmin or trypsin (Harris and Krane 1974; Liotta et al, 1982; Pauli et al, 1983).

MMP's have a cys sulfhydryl group in their active site and are produced and secreted from cells as inactive zymogens (Judware and Culp 1996). The enzyme is held in an inactive state because of its association with zinc, which in the zymogen is associated with the cys SH group in the active site. Disruption of this association, probably due to limited proteolysis by a serine protease such as plasmin, is a possible mechanism for MMP activation (Judware and Culp 1996).

Natural protease inhibitors which play a significant role in regulating the degradation of collagen are present in serum and in the extracellular matrix (Liotta et al, 1983). Matrix-derived collagenase inhibitors are a family of cationic proteins found in cartilage, and have molecular weights between 11,000 and 40,000 daltons. Tissue-derived collagenase inhibitors (tissue inhibitor of metalloproteinases or TIMP) are secreted by fibroblasts, vascular smooth muscle cells, endothelial cells and chondrocytes (Liotta 1992). TIMPs produced in normal tissues, such as cartilage and bone, may guard against excessive breakdown of the ECM as well as protecting nerves from injury by preserving the

basement membrane that surrounds nerve fibres (Liotta 1992). TIMP-1 is a glycoprotein of about 28,500 daltons and has been shown to block the invasion of human amniotic membranes by B16 melanoma cells as well as lung colonisation by the same cells (Alvarez et al, 1990). TIMP-2 has been shown to block the formation of new blood vessels required by the growing tumour cells. TIMPs have been described as metastasis suppressor proteins, implying that TIMP analogues may prevent tumour invasion or metastasis (Liotta et al, 1991; Liotta 1992). It has been suggested that a tumour cell's potential to invade the ECM is related to the balance between the relative activity of the collagenase and the collagenase inhibitor produced by the tumour and obviously a fine balance between the two is required (Liotta et al, 1983; Alvarez et al, 1990).

1.5 STROMAL-EPITHELIAL INTERACTIONS AND CANCER

Cancer is a multi-faceted disease involving many different cells and their interactions with each other and the surrounding environment. There are essentially four types: interactions by diffusible factors, cell surface interactions, matrix interactions and direct interactions (Haslam 1991). It is possible that during the process of tumour invasion, the tumour cell interacts with the surrounding environment in a combination of different interactions. Collagen is synthesised, predominantly, by fibroblasts even though some studies have demonstrated that type I collagen is produced by epithelial cells (Ohtani et al, 1992; Al-Adanani et al, 1975; Sakakibara et al, 1982; Roesel et al, 1978). As discussed above the collagen produced by the fibroblasts has to be degraded for metastasis to occur, therefore tumour cells interfere with fibroblast function (in some unknown way) to achieve their ultimate goal. The connective tissue stroma can modulate epithelial cell growth in such a way as to prevent the tumour from growing or to bring about degradation and thus aid tumour cell invasion and metastases. This can be done by the secretion of soluble factors (either by the tumour cells or the fibroblasts) or by any of the other modes of interactions between host cells and the tumour cells. In desmoplasia the excess connective tissue has been shown to slow down tumour growth and the mechanism (ie whether the tumour cells or fibroblasts secrete factor(s) or whether the cells require direct interaction) is not yet known. The possible mechanisms are discussed below.

1.5.1 Modification of the ECM by soluble factors

ECM degradation may be dependent on specific interactions between tumour and host cells. Tumour cells may indirectly alter the ECM by interfering with fibroblast functions by secreting factor(s) which stimulate(s) the fibroblasts to produce collagenases (Biswas 1982). Co-culture of rabbit fibroblasts and mouse epithelial tumour cells resulted in increased collagenase production, compared to cultures of the individual cells and was shown to be dependent on the ratio between normal fibroblasts and tumour cells. Increased collagenase production was proposed to be mediated by a soluble factor produced by the tumour cells (Biswas 1982). Biswas (1984) and Ellis et al (1989) isolated and purified tumour cell-derived collagenase-stimulatory factor (TCSF) or more recently extracellular matrix metalloproteinase inducer (EMMPRIN) which is present on the surface of several human tumour cells in vitro and in vivo and stimulates production of interstitial collagenase in human fibroblasts (Biswas et al, 1995). TCSF was localised in human bladder tumours and normal urothelium, suggesting a role in tumour progression and invasion in vivo. TCSF was evaluated as a possible tumour marker for bladder tumours (Muraoka et al, 1993). Northern blot analysis demonstrated that EMMPRIN increases the expression of interstitial collagenase (MMP-1), stromelysin and 72 Kd gelatinase/type IV collagenase (MMP-2) indicating that this factor could enhance tumour invasion (Kataoka et al, 1993). EMMPRIN was also found on the surface of normal human keratinocytes, where it is thought to play a role in regulating stromal MMP's (Biswas et al, 1995). EMMPRIN has now been identified as a member of the immunoglobin superfamily and its sequence is identical to that of the other members of the group, such as gp42 in the mouse, OX-47 or CE-9 in the rat, HT7, neurothelin or 5A11 in the avian system and M6 in the human. Chinese hamster ovary cells were transfected with the putative cDNA for EMMPRIN and it was shown that EMMPRIN is extensively post-translationally processed and plays a role in tumour cell invasion and processes of tissue remodelling by regulating the production of interstitial collagenase, stromelysin-1 and gelatinase A (Guo et al, 1997). The exact physiological role of these members of the immunoglobin superfamily are poorly understood and whether they all have EMMPRIN activity remains to be assessed. Various roles have however been postulated including cell

adhesion or signaling as well as a molecule that stimulates MMP synthesis (Biswas et al, 1995; Schuster et al, 1996). The rabbit protein was investigated in detail and localised to the basal membranes of the tubule epithelia and the papillary surface epithelium in the kidney (Schuster et al, 1996). Exactly how these results demonstrate a concise and clear cut role for EMMPRIN is not known and whether EMMPRIN is involved in development or cell-cell signaling still needs to be determined.

Other factors which are involved in stromal-epithelial interactions are hormones which stimulate the cells to secrete TGFβ's which may affect the regulation of growth and differentiation of both tumour cells and fibroblasts either positively or negatively (Sakakura 1991). These include TGFα, TGF-β, insulin-like growth factor (IGF), platelet-derived growth factor (PDGF) and basic fibroblast growth factor. TGF-β is the one growth factor which is supposed to play an important role in controlling interactions between tumour cells and the surrounding mesenchyme, and can stimulate fibroblasts to proliferate while inhibiting the proliferation of epithelial cells (Sakakura 1991). TGF-β functions could be mediated by the extracellular matrix, for instance TGF-β increases the expression of type I collagen, fibronectin and tenascin (Rossi et al, 1988; Ignotz et al, 1987).

1.5.2 Direct interactions between fibroblasts and tumour epithelial cells

This is classified as an interaction mediated by small signal molecules which are transported from the epithelium into the stroma, and/or vice versa, through the plasma membrane by direct contact (Sakakura 1991). It has been noted that cytoplasmic processes come from both epithelium and fibroblasts which then make direct contact by penetrating the other tissue throught gaps in the basal lamina. At these contact zones, the cell plasma membranes are 50-150 nm apart (membrane fusion has not yet been detected) and a band of dense material has been found beneath the contact zones. It is thought that these contact zones between the cells act as sites for transfer of signal molecules (Sakakura 1991). Mammary epithelial cell-fibroblast interactions were analysed by their response to oestrogen in vivo and cultures containing both epithelial cells and fibroblasts,

on exposure to oestrogen, were found to have a three-fold increase in progesterone receptor concentration which was associated with the presence of mammary stromal fibroblasts (Haslam 1991). In this instance, the fibroblasts secreted a factor which affected the epithelial cells which facilitated oestrogen responsiveness. Fibroblasts needed to be in close contact with the epithelial cells to promote DNA synthesis. It has been hypothesised that cell-cell interactions occur via gap junctions which allow direct transfer of informational molecules across cell membranes by specialised channels (Haslam 1991).

1.5.3 Desmoplasia

Desmoplasia, otherwise known as increased deposition of stromal collagen is found around tumour cells or glands (Hewitt et al, 1993). Desmoplasia has been shown to occur in cancers such as diffuse infiltrative gastric carcinomas, and in infiltrating ductal (scirrhous) carcinomas of the breast (Ohtani et al, 1992). In the desmoplastic stroma of scirrhous breast carcinomas, collagen types I, III and V and elastin have all been identified, of which collagen type I is the most abundant (Barsky et al, 1982). A common feature of the desmoplastic reaction is that the fibroblasts have similar morphological features to smooth muscle cells and have therefore been termed myofibroblasts. These cells arise during injury and by producing excess collagen participate actively during tissue repair, suggesting that desmoplasia might represent an exaggerated response of tissue remodeling associated with malignancy (Haslam 1991). The cause of the desmoplastic response is not known and controversy exists as to whether the response is a host response to tissue injury, similar to wound healing, or that the response is brought about by the tumour stimulating the recruitment and proliferation of fibroblasts. The response can benefit the host by "walling off" the tumour, thus preventing it from growing and resulting in necrosis. Alternatively, the response can also benefit the tumour by reducing access of host lymphocytes, macrophages and other immune cells, in this way protecting the tumour from the immune system, until it is ready to invade the host tissue (Barsky and Gopalakrishna 1987). To support the hypothesis that the desmoplastic response is a protective mechanism, Barsky and Gopalakrishna (1987) found that when the desmoplastic response was inhibited, there was an increase in spontaneous metastasis from

experimental murine melanomas. Studies performed on colorectal carcinomas by Hewitt et al (1993) showed the desmoplastic response to occur in carcinomas but absent in non-invasive adenomas. Little evidence of a desmoplastic response was detected at the invasive edge of the carcinomas, where active invasion was prominent. Active collagen synthesis was seen in the tumour centre, whereas very little type I collagen mRNA was evident in host tissues ahead of the invasion front. These findings, although on the one hand providing evidence that desmoplasia limits tumour invasiveness, also demonstrate that the desmoplastic response fails to prevent the spread of colorectal cancer because of its absence at the invasive edge (Hewitt et al, 1993).

Nakanishi et al, (1994) have developed a tumour cell line that secretes a factor(s) which causes fibroblasts to produce excess collagen which could be disadvantageous to tumour cells by preventing access to the vascular basement membrane, an important step in hematogenous metastasis. It has subsequently been found that this stroma-inducing tumour cell line expresses a syndecan-like proteoglycan (mouse syndecan-2) which exhibits specific binding to fibronectin via its heparan sulphate chains (Itano et al, 1996). Mouse syndecan-2 was shown to be phosphorylated at its cytoplasmic domain and was hypothesized to be involved in stress-fiber formation.

It has also been suggested that the desmoplastic response, rather than inhibit invasion of scirrhous breast carcinomas, may facilitate their growth by acting as a means of contact guidance for malignant cells (Pucci Minafra et al, 1986). Luparello et al (1991) showed that ductal infiltrating carcinomas were characterised by the reappearance of "embryonic" type I-trimer collagen and an increase in type V collagen content in the matrix. These two collagen types were compared with type I collagen as culture substrata for the spreading, cytoskeletal organisation and motility of 8701-BC breast carcinoma cells. Cells grown on type I- trimer were found to be motile (whereas on type I collagen, they were stationary) indicating that this collagen may favour penetration and scattering of neoplastic cells into the surrounding stroma. Type I-trimer collagen is synthesised during embryogenesis, is depressed in adult tissues and is restored in cancer (Luparello et al, 1991). The breast

carcinoma cell line (8701-BC) used by Luparello et al, (1991) was able to produce type I-trimer and it was therefore hypothesized that the re-deposition of "embryonic" collagen in scirrhous carcinomas, produced by the carcinoma cells themselves, may be instrumental in providing the tumour cells with stromal pathways allowing them to infiltrate the host tissue by acting to guide the tumour cells by contact. This work was refuted by Hewitt et al (1993) when no α1(I) collagen mRNA was detected in the vicinity of neoplastic cells showing invasive activity where the type I-trimer collagen was found. Hewitt et al (1993) state that due to the fact that collagen-I consists of 2 identical α1(I) chains linked with one distinct α2(I) chain and type-I-trimer is composed of three identical α1(I) chains, their probes and/or antibody should have recognised the type-trimer molecule.

The type I trimer found in ductal infiltrating carcinomas has been resolved into three distinct 100 Kd chains, where one has an unusual acidic component and the other two chains are basic with the same electrophoretic behaviour as α1(III) and α1(I). The collagen was also found to bind laminin and due to this fact as well as its presence in both tumour and embryonic-fetal tissues, it was re-named onco-fetal laminin-binding collagen (OF/LB collagen). The possible presence of OF/LB collagen during development and cancer, and its absence in normal adult tissues, makes this protein a potential stromal marker of malignancy, and could in fact induce tumour promotion (Pucci-Minafra et al, 1993; Van Den Hooff 1988). Sequence analysis has been performed on the trimer chains and it has been found that the acidic chain represents a gene product distinct from either type I, type III and other known collagen chains. The identity of the other two chains remains unknown (Pucci-Minafra et al, 1995). These results do not explain why this collagen could not be detected in the work done by Hewitt et al, 1993, taking into account that the polyclonal antibody used should have at least detected one basic chain that is similar to α1(I) collagen chain of type I collagen. And as for the collagen mRNA which was not detected at the invasive front of the colorectal tumours, it is possible that trimer mRNA is rapidly degraded and therefore not detectable.

In summary fibroblasts can effect tumour cells by modifying the extracellular matrix (by proteolysis), by production of soluble factor(s) as well as by direct contact and transfer of molecules via gap junctions or receptors. A combination of these different mechanisms is also possible. All aspects of tumour invasion and metastasis are finely balanced and events are appropriately timed so that in most instances the host is destroyed, the aim of all tumour cells.

The aim of this thesis was to analyse the interaction between fibroblasts and tumour cells in vitro and in vivo and interesting and in some cases novel findings are presented. Firstly, fibroblasts (and not tumour cells) produce collagen, secondly; tumour cells as well as the tumour cell conditioned media decreased collagen production and finally tumour cells need to be in contact with the fibroblasts to bring about a decrease in collagen mRNA. It is hypothesized that a decrease in collagen mRNA and therefore collagen protein assists in tumour invasion along with the secretion of collagenases by facilitating movement of the tumour cells through the ECM.

CHAPTER TWO

ANALYSIS OF TYPE I COLLAGEN GENE EXPRESSION IN BREAST AND COLORECTAL CANCER

2.1 INTRODUCTION

Collagen gene expression was analysed in two types of cancers (breast and colon cancer), both of which are relatively common in certain areas of South Africa and therefore tissue is readily available. Breast cancer is a heterogeneous disease, and as yet no one specific gene is associated with nonhereditary ("sporadic") breast cancer. It is still very much a disease linked to "risk factors" such as family history, age at menopause, use of oral contraceptives, hormone replacement therapy and obesity (Harris et al, 1992). Genetic factors such as p53 mutations, overexpression or mutations in the Ras oncogene and nm23 have all been shown to play a role in breast cancer, but whether they are direct causes of the disease still remains to be assessed (Varley et al, 1991; Coles et al, 1992; Osborne et al, 1991; Bevilacqua et al, 1989; Spandidos 1987; Rochlitz et al, 1989).

Type I collagen is the most abundant protein in the stroma, how it affects tumour cell invasiveness is unknown, an attempt was made in this chapter to obtain a better understanding of the relationship between tumour cells and collagen. Once the tumour cells have entered the stroma, they then have to travel to other parts of the body and they can do so by degrading type I collagen as well as by modulating the functions of fibroblasts (such as causing a shut down in mRNA production).

Total RNA was isolated from breast tumours and the adjacent normal tissue and a stage-specific correlation was identified; stage I breast tumours had increased collagen gene expression, whereas stages II and III breast tumours had decreased expression. These results were confirmed by in situ hybridisation analysis. Colon cancer tissue was subsequently analysed in order to ascertain whether the breast cancer stage-specific relationship was unique to breast cancer or whether it occurred in other cancers.

Similarily, early stages of colon cancer were characterised by high levels of collagen mRNA, whereas the more advanced stages had very little collagen mRNA. Due to the fact that mutations in the Ras oncogene have been shown to occur frequently in colorectal cancer and transfecting cells with mutagenic Ras have been known to cause decreased collagen gene expression, Ras mutations could indirectly or directly affect collagen gene expression in colon cancer. Therefore the colon cancer samples were analysed for Ras gene mutations by means of SSCP analysis and direct sequencing.

2.2 RESULTS

2.2.1 Collagen mRNA in cancer tissues

Type I collagen mRNA was quantitated by Northern blot hybridisation of RNA and then localised in collagen synthesising cells by non-radioactive in situ hybridisation.

2.2.1.1 Northern Analysis

In order to analyse the levels of collagen mRNA in breast cancer tissue, total RNA from breast tumour samples and the adjacent normal tissue was subjected to Northern blot analysis. The breast tumours in this study were obtained from the Breast Clinic at Groote Schuur Hospital. They were staged according to the TNM system, where T= tumour size; N= lymph node involvement and M= presence of distant metastases (Harris et al, 1992). In this study stage I tumours were < 2cm, stage II tumours were > 2cm but < 5cm and stage III tumours were > 5cm. The tumours were all of the invasive ductal type, specifically infiltrating ductal whereby tumour cells are in direct contact with the extracellular matrix (consisting predominantly of type I collagen), and may exert an effect on the surrounding fibroblasts. Total RNA was extracted from each tissue sample (section 6.1), electrophoresed on 1% agarose gels containing 8% formaldehyde and transferred onto nylon membranes. The membranes were first probed with a ^{32}P-labelled α1(I) collagen cDNA probe (Chu et al, 1982), stripped and reprobed with a ^{32}P-labelled α2(I) collagen probe (Meyers et al, 1981) as described in section 6.2.

Analysis of α1(I) and α2(I) collagen mRNA in stage I breast tumours indicated an increase in the level of these mRNAs when compared to the adjacent normal tissue. ß-actin was used as an internal control to correct for loading of RNA samples (figure 2.1). From the ß-actin signal, it can be seen that equal amounts of RNA were loaded onto the gel and that the transfer to the nylon membrane was even. Figure 2.1(C) and (D) shows the collagen signal relative to ß-actin for several stage I breast tumours.

Similarily, stage II breast tumours were analysed for α1(I) and α2(I) collagen mRNA as shown in figure 2.2. These results indicated that in stage II breast tumours, the

levels of α1(I) and α2(I) collagen mRNAs were decreased relative to the adjacent normal tissue. ß-actin was again used in order to monitor loading of the RNA. Figure 2.2(D) and (E) summarises the data for several stage II breast tumours relative to ß-actin.

RNA from stage III breast tumours was analysed in the same way for α1(I) and α2(I) collagen mRNA (figure 2.3). As for stage II breast tumours, the stage III tumours also had decreased levels of α1(I) and α2(I) collagen mRNA. The amount of ß-actin RNA between the normal and tumour samples remained relatively unchanged, indicating that the decrease in collagen RNA within each patient was specific and not due to fluctuations in total RNA. Expression of the results relative to ß-actin is shown in figure 2.3(D) and (E). These results indicated that the level of collagen mRNA was increased in stage I breast tumours compared to the adjacent normal mRNA, whereas in stages II and III breast tumours there was a decrease in the level of collagen mRNA when compared to the adjacent normal mRNA.

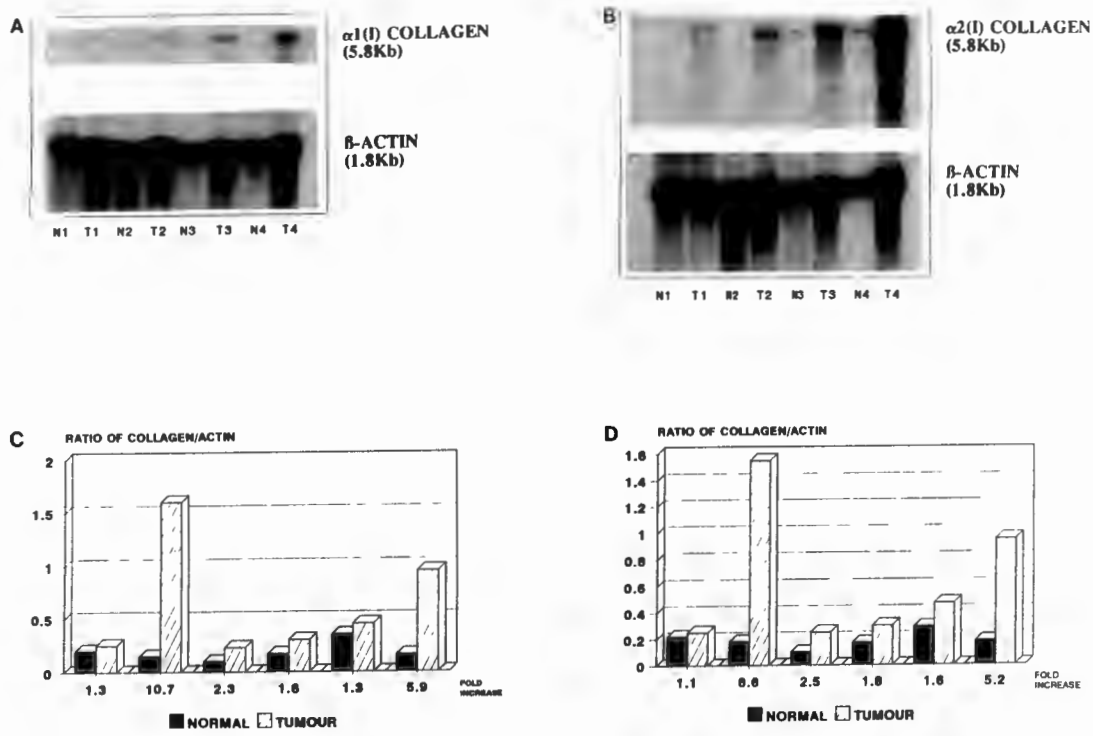


FIGURE 2.1

Analysis of collagen mRNA in stage I breast tumours and adjacent normal tissue

Total RNA was extracted from breast tumour (T) and the adjacent normal (N) tissue of each patient. 5μg of RNA was resolved on a 1% agarose gel containing 8% formaldehyde, blotted onto nylon membranes and hybridised to ^{32}P-labelled cDNA probes as discussed in section 6.2 of Materials and Methods. The sizes of the hybridising bands, in kilobases, are shown on the right. The nylon membrane was first hybridised with nick-translated Hf677 containing the full length α1(I) collagen cDNA probe **(A)**, stripped and hybridised with nick-translated Hf32 containing the full length α2(I) collagen cDNA **(B)**. Both membranes were stripped and hybridised with a nick-translated ß-actin cDNA probe. The duration of exposure to X-ray film was 18-24 hours. The expression of α2(I) and α1(I) collagen mRNA relative to ß-actin in some samples are shown in **(C)** and **(D)**, respectively.

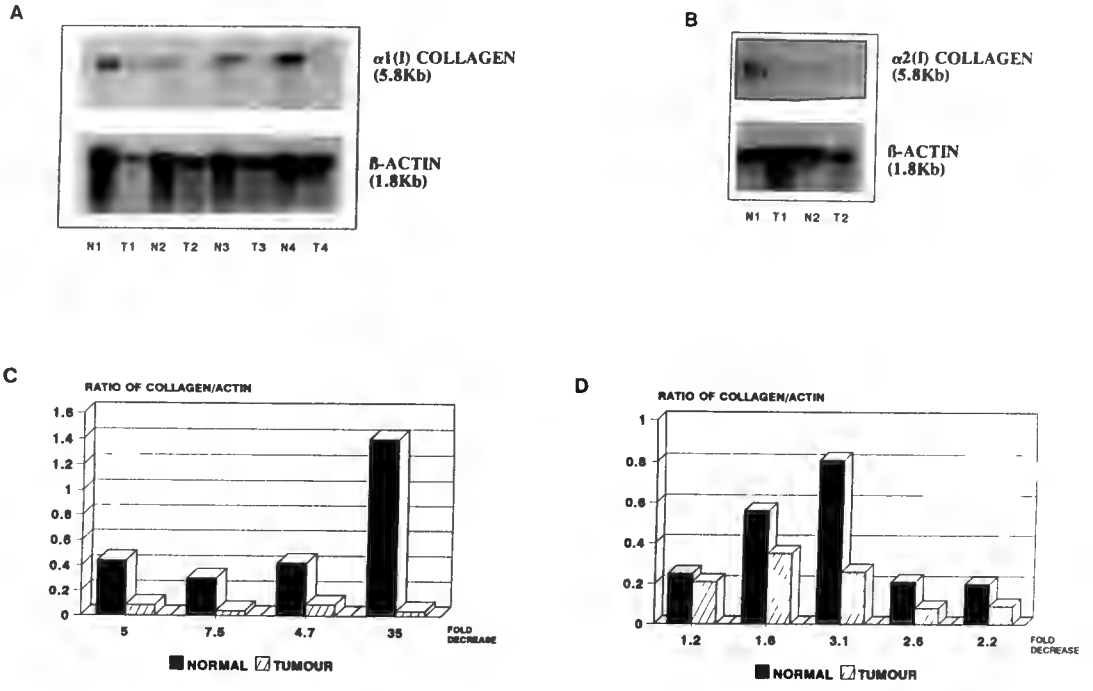


FIGURE 2.2

Analysis of collagen mRNA in stage II breast tumours and adjacent normal tissue Total RNA was extracted from breast tumour (T) and adjacent normal (N) tissue of which 5µg was resolved on a 1% agarose gel containing 8% formaldehyde. The RNA was blotted onto nylon membranes and hybridised to ^{32}P-labelled cDNA probes as discussed in section 6.2 of Materials and Methods. The membrane was hybridised with the α1(I) collagen probe **(A)** as described in the legend to figure 2.1, stripped and hybridised with the α2(I) collagen probe **(B)**. After autoradiography, the filter was stripped and hybridised with the ß-actin probe. The expression of α2(I) and α1(I) collagen mRNA relative to the ß-actin signal in some samples are shown in **(C)** and **(D)**, respectively.

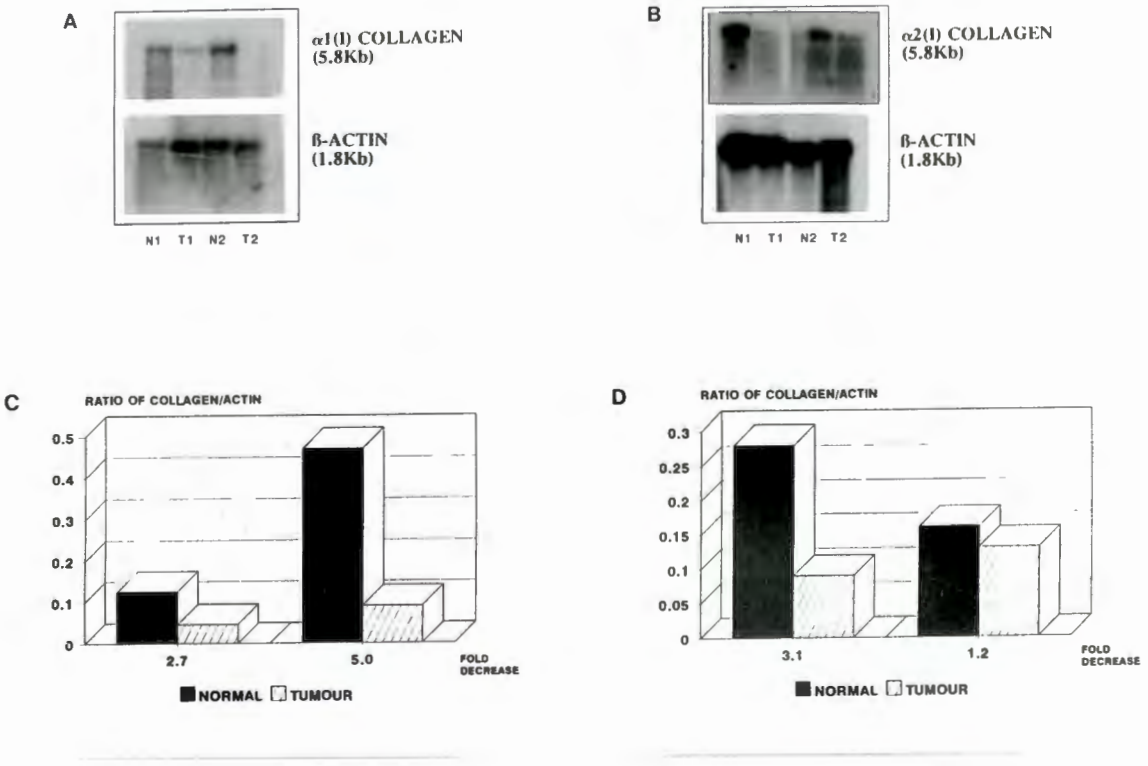


FIGURE 2.3

Analysis of collagen mRNA in stage III breast tumours and adjacent normal tissue Total RNA was extracted from stage III breast tumours (T) and the adjacent normal tissue (N). 5µg RNA was resolved on a 1% agarose gel containing 8% formaldehyde, blotted onto a nylon membrane and subsequently hybridised with ^{32}P-labelled cDNA probes (as described in section 6.2 of Materials and Methods). **(A)** represents the membrane hybridised with the α1(I) collagen probe (Hf677), as described in the legend to figure 2.1, and **(B)** represents the α2(I) collagen probe (Hf32). Both membranes were stripped and hybridised with a nick-translated β-actin cDNA probe. The corrected results for α2(I) and α1(I) collagen mRNA relative to ß-actin is shown in **(C)** and **(D)**, respectively.

2.2.1.2 In Situ Hybridisation

Northern blot analysis is not only limited by the problem of heterogenous cell populations in solid tumours, but also by different proportions of tumour to normal cells in the tissues which can cause under or over estimation of RNA transcripts. In situ hybridisation circumvents this problem, providing a correlation between gene expression and histological data. This technique allows for the direct localisation of specific mRNA species in tissue sections. In this study non-radioactive in situ hybridisation was used to identify cells specifically producing collagen mRNA. Non-radioactive in situ hybridisation (Ohtani et al, 1992) has an advantage over radioactive in situ hybridisation (Hewitt et al, 1993) in that it is safer and allows morphological preservation of tissue structure, enabling better resolution of cell types. Radioactive in situ hybridisation, however is more sensitive and quantitative, in that it allows quantitation of radioactive "spots" compared to the non-radioactive assay which is based on a colour reaction. In this study, the digoxigenin non-radioactive labelling system from Boehringer Mannheim was used. It has to be emphasised that non-radioactive in situ hybridisation is not a quantitative technique, in fact it is an empirical method and was used to detect the absence or presence of collagen mRNA. A positive signal was demonstrated by a blue-purple colour only, other colours such as a faint brown or green was caused by the different chemical reagents used.

In order to confirm the stage specific collagen gene expression observed by Northern blot analysis (section 2.2.1.1), 10 tissue samples from each stage of breast tumours and the adjacent normals were subjected to non-radioactive in situ hybridisation (section 6.3). In order to ascertain whether this stage specific gene expression occurred in other cancers, 32 colorectal carcinoma samples were also subjected to in situ hybridisation.

All tissue sections were hybridised with digoxigenin labelled RNA probes. A 160 base pair XbaI-SacI fragment was released from Hf32 (α2(I) cDNA) and cloned into the vector pGEM3. A 581 base pair EcoRI-XhoI fragment was released from Hf677 (α1(I) cDNA) and cloned into the EcoRI-SalI sites of pGEM3 (refer to Appendix A for cloning details). ß-actin, which had already been cloned into pGEM3, was released by digestion with restriction enzymes PvuII and SalI. The DNA fragments were eluted

from agarose gels using the Qiaex kit from Qiagen as described in section 6.3.2. DNA fragments, as well as the α2(I) and α1(I) collagen clones (after linearisation or releasing of the appropriate fragment) and the vector pGEM3 were used as templates to transcribe digoxigenin-labelled RNA using T7 RNA polymerase (section 6.3). ß-actin was used as a positive control since it should be present in all cells, whereas only fibroblasts have been shown to produce α1(I) and α2(I) collagen RNA. The vector pGEM3 was used as a negative control to ensure that the collagen signal was specific and not due to non-specific hybridisation.

Figure 2.4 shows consecutive sections of a stage I tumour and its adjacent normal tissue probed with an α2(I) collagen riboprobe. Sequential sections were probed with a ß-actin riboprobe (positive control). Figures 2.4(A) and (D) were stained with haemotoxylin and eosin (H+E), which allows for the identification of the different cell types. This stain also differentiates between normal and tumour tissue. Eosin stains the cytoplasm pink and haemotoxylin stains the nuclei purple thus allowing the identification of multinucleated cells in tumours. The normal tissue, figure 2.4(A), was classified as histologically normal clearly depicting intact mammary ducts containing two different types of epithelial cells; epithelial cells on the inside of the duct and myoepithelial cells on the periphery. The tumour tissue, figure 2.4(D) is characterised by a fragmented extracellular matrix (stroma) consisting of collagen fibrils and a number of fibroblasts (also present in normal tissue, but not shown here). The stroma also contains numerous multinucleated tumour cells. Figures 2.4(B) and (E) represent sections hybridised with an α2(I) collagen riboprobe. The normal section, 2.4(B) shows non-specific binding of the collagen riboprobe which tended to stick to the edges of the epithelial cells because of the slightly greater thickness of this section. The majority of fibroblasts within the stroma, figure 2.4(E) surrounding the tumour cells were positive for α2(I) collagen mRNA as seen by the blue staining within the fibroblasts (arrowheads). The tumour cells (t) on the other hand, were not positive for α2(I) collagen mRNA. Figures 2.4(C) and (F) represent sections hybridised with the ß-actin riboprobe and as can be seen, the epithelial cells, fibroblasts (arrowheads) and tumour cells (t) all stained positive for ß-actin mRNA. These in situ hybridisation

in situ hybridisation results indicate that the staining for collagen mRNA was specific to the fibroblasts in the stroma and not the epithelial cells.

Staining of consecutive sections of a stage II tumour and its adjacent normal tissue with H+E clearly showing fibroblasts (f) between intact collagen fibrils in the normal tissue (figure 2.5A). The tumour section, figure 2.5(D) contained many tumour cells (t) within the stroma as well as numerous fibroblasts (of which one is shown by an arrow). Upon hybridisation of adjacent normal and tumour sections with an α2(I) collagen riboprobe the fibroblasts present in the normal stroma were shown to be positive for α2(I) collagen mRNA, whereas those fibroblasts and tumour cells present in the tumour section were negative, a negative fibroblast is indicated by an arrow (figures 2.5B and E respectively). Figures 2.5(C) and (F) represent adjacent normal and tumour sections hybridised with a ß-actin probe. All cells (tumour cells and fibroblasts) were positive for ß-actin mRNA, indicating that those fibroblasts present in the tumour tissue were producing ß-actin mRNA but not collagen mRNA and that the absence of a collagen signal in the tumour section was not due to degradation of the RNA in these sections. An arrow represents one of the many fibroblasts present which was positive for B-actin mRNA.

FIGURE 2.4

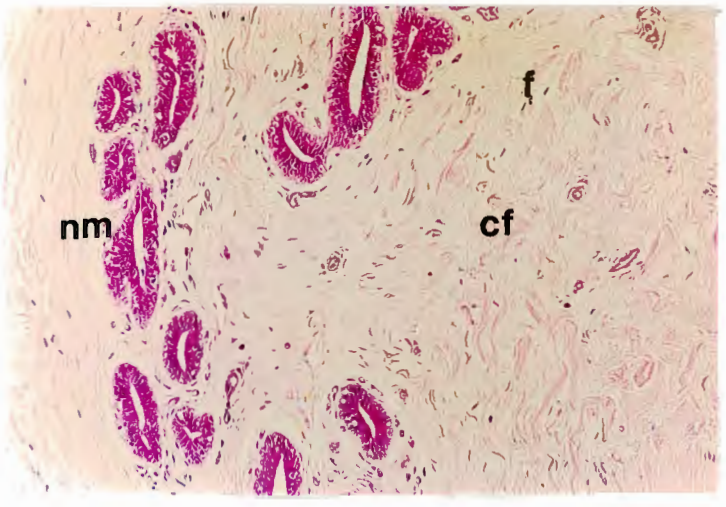
Collagen mRNA in a section of a stage I breast tumour and adjacent normal tissue. Five micron sections were cut from wax blocks using a microtome. **(A)** and **(D)**; sections stained with haemotoxylin and eosin (H+E). (A) is characterised by several normal mammary ducts (nm) as well as intact collagen fibres (cf) containing numerous stromal fibroblasts (f). (D) is characterised by tumour cells (t) which have infiltrated the stroma which also contain stromal fibroblasts (arrows). There is a blood capillary (bc) in the left hand corner of the section. **(B)** and **(E)**; sections hybridised with a digoxigenin-labelled α2(I) collagen riboprobe (section 6.3). The stromal fibroblasts stained blue-purple, (arrows) indicating a positive result for collagen mRNA. The tumour cells (t) in section (E) were not positive for collagen mRNA. **(C)** and **(F)**; sections hybridised with a digoxigenin-labelled ß-actin riboprobe. The tumour cells (t) and stromal fibroblasts (arrows) were positive for ß-actin as detected by blue-purple staining. Those tumour cells in section (E) which were negative for α2(I) collagen were clearly positive for ß-actin. Total magnification for each section was 100X except for section (E) which was 200X. These are consecutive sections and were cut from the same block.

STAGE I

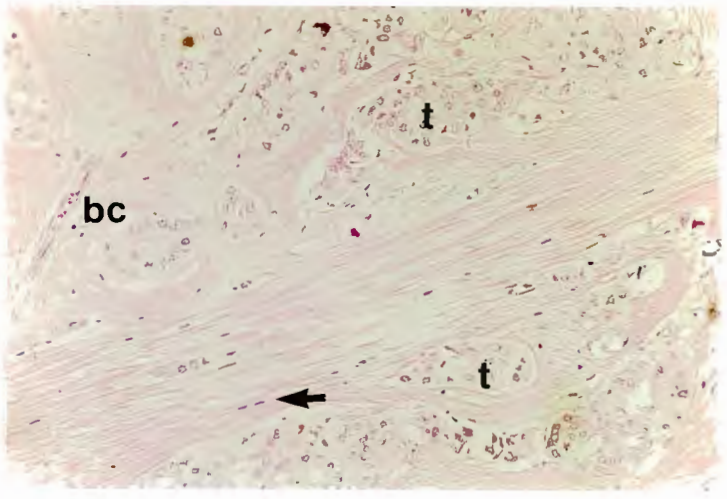
NORMAL

TUMOUR

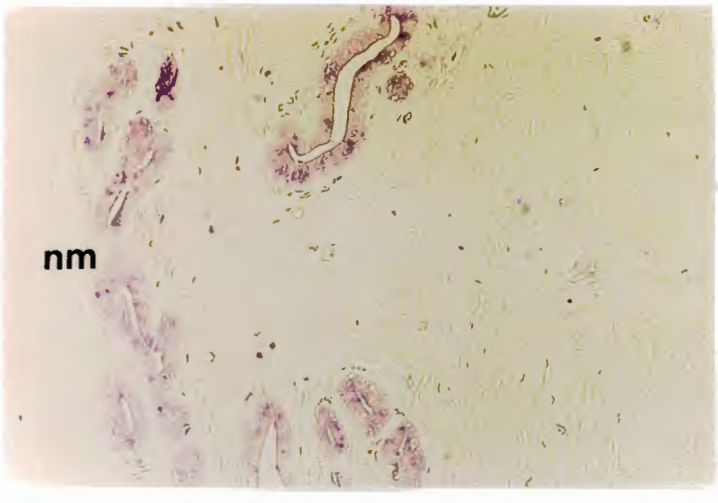
A



D

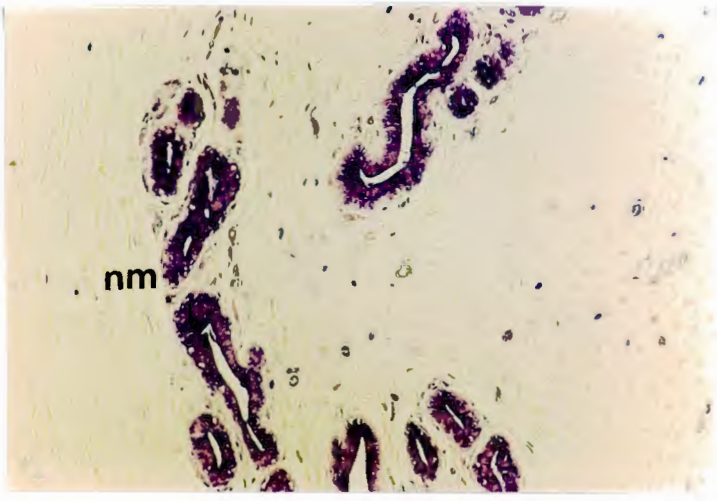


B



E

C



F

FIGURE 2.5

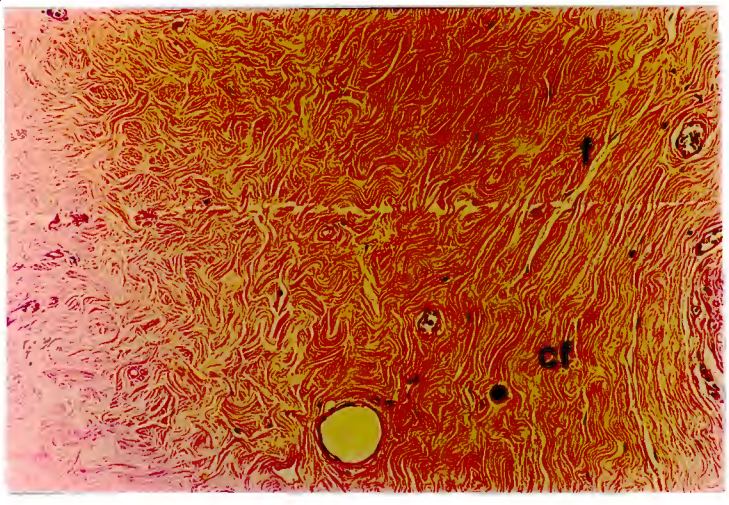
Collagen mRNA in a section of a stage II breast tumour and adjacent normal tissue. (A) and **(D)**; sections stained with H+E. (A) shows intact collagen fibres (cf) interspersed with numerous fibroblasts (f). The tumour cells (t) in (D) have infiltrated the collagen stroma and an arrow indicates one fibroblast in this section. **(B)** and **(E)**; sections hybridised with a digoxigenin-labelled α2(I) collagen riboprobe (section 6.3). The normal section (B) shows a few blue-purple stained fibroblasts (f) positive for collagen mRNA amongst the intact collagen fibres. The fibroblasts (of which one is indicated with an arrow) and the tumour cells (t) in section (E) are negative for collagen mRNA. **(C)** and **(F)**; sections hybridised with a digoxigenin-labelled ß-actin riboprobe. Section (C) shows fibroblasts (arrow) positive for ß-actin as indicated by the blue stain. Both tumour cells (t) and fibroblasts are blue-purple in the tumour section when hybridised with ß-actin. Total magnification for each section was 100X except for sections (D) and (F) which were 200X. These sections are consecutive and were cut from the same block.

STAGE II

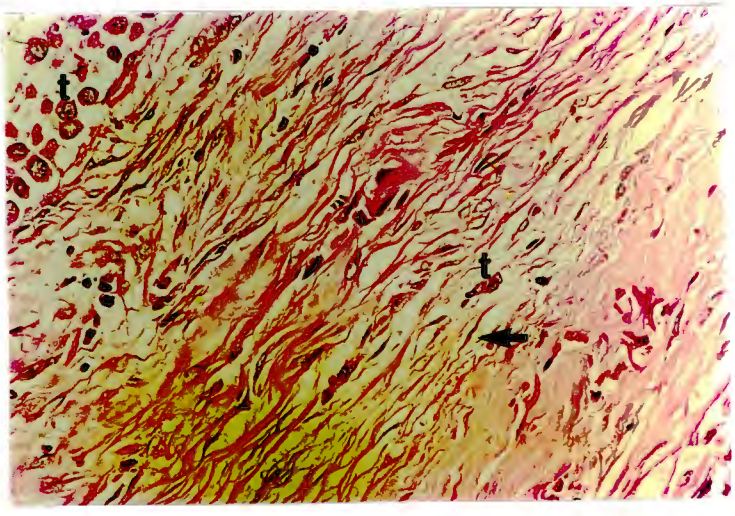
NORMAL

TUMOUR

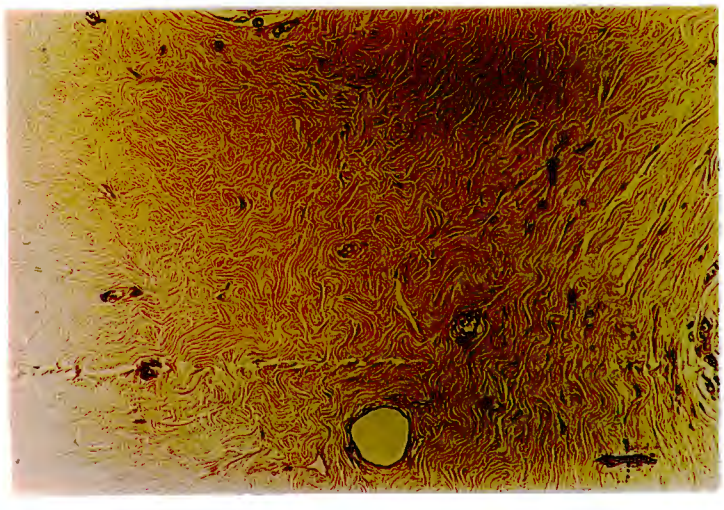
A



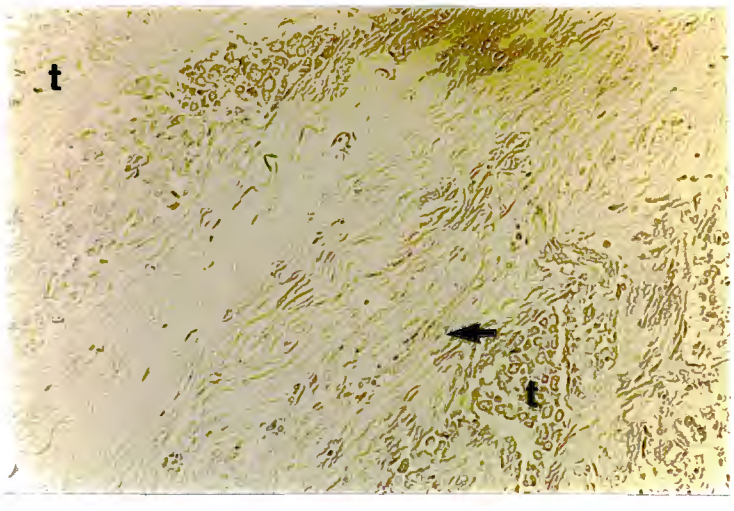
D



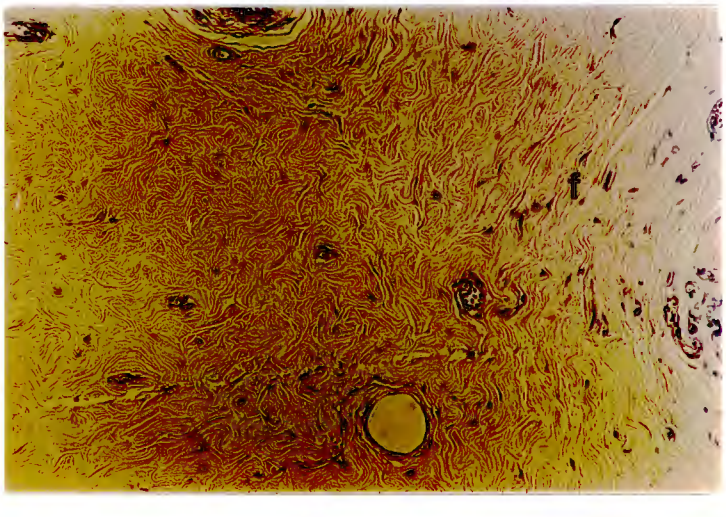
B



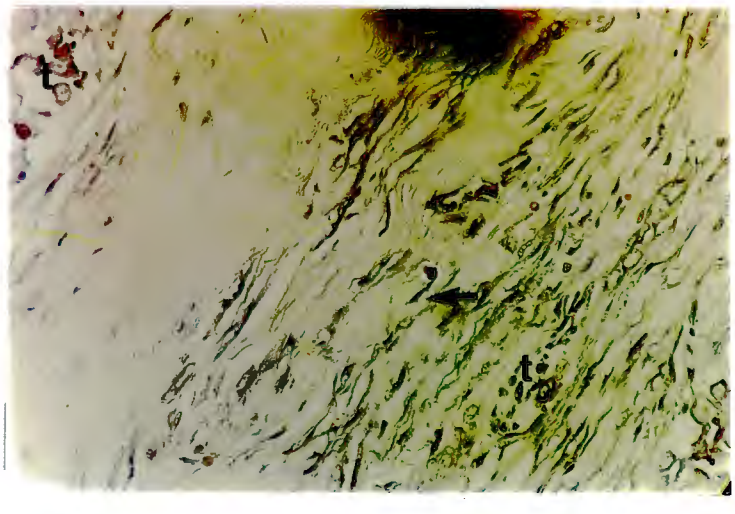
E



C



F



Haemotoxylin and eosin staining of a stage III tumour showed a stroma with infiltrating tumour cells (t), lymphocytes (l) and some fibroblasts (arrows) (figure 2.6D). The normal section contained collagen fibrils and fibroblasts (f) in the stroma (figure 2.6A). The mammary duct (md) in figure 2.6(D) is filled with tumour cells. The fibroblasts (f) in the adjacent normal tissue figure 2.6(B) were positive for α2(I) collagen mRNA whereas those present in the tumour (arrows), figure 2.6(E), did not produce α2(I) collagen mRNA. The fibroblasts (arrows) and tumour cells (t) did produce ß-actin mRNA, (figure 2.6C and F) including the fibroblasts (arrows) that did not produce collagen mRNA. There is clearly no staining of collagen mRNA (2.6E) when compared to the signal obtained with the β-actin probe (2.4F). The staining also shows definitive cells within the stroma as well as the tumour cells which cannot be seen in 2.6(E).

A summary of the in situ hybridisation results is shown in Table I. The majority of normal fibroblasts in the tumour tissue of all 10 stage I patients were positive for α1(I) and α2(I) mRNA as compared to sections from stages II and III patients which were negative.

TABLE I: Summary of the in situ hybridisation results in breast tumours.

TUMOUR STAGE	NO.OF PATIENTS	COLLAGEN GENE EXPRESSION	β-ACTIN GENE EXPRESSION
I	10	+++	+++
II	10	+	+++
III	10	+	+++

Where intensity of signal by visualisation is shown as:

+++ strong; + weak

FIGURE 2.6

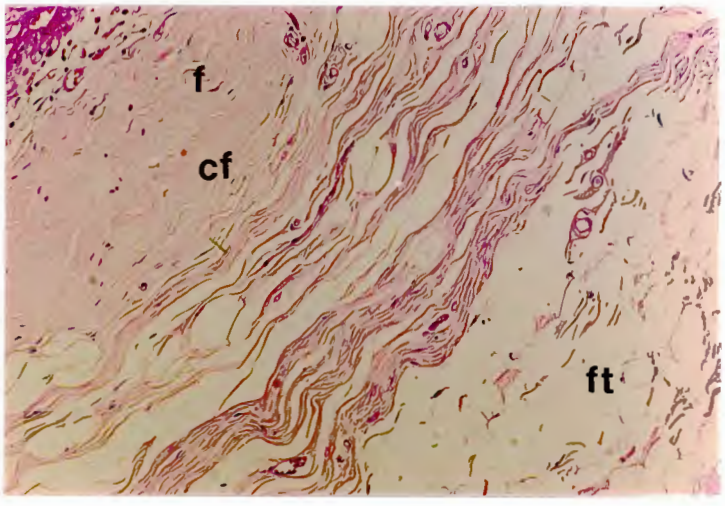
Collagen mRNA in a section of a stage III breast tumour and adjacent normal tissue. (A) and **(D)**; sections stained with H+E. Intact collagen fibres (cf) as well as fragmented tissue (ft) due to processing are indicated in (A). Amongst the collagen fibres are several fibroblasts (f). Section (D) consists primarily of tumour cells (t) and lymphocytes (l). A region of intact stroma (s) is present next to the mammary duct (md), which is filled with tumour cells. Several fibroblasts (arrows) occur in this stroma. **(B)** and **(E)**; sections hybridised with a digoxigenin-labelled α2(I) collagen riboprobe (section 6.3). Section (B) is characterised by blue-purple staining fibroblasts, (f) positive for collagen mRNA. The fibroblasts (arrows) on the periphery of the mammary duct filled with tumour cells in section (E) were negative for collagen mRNA. The tumour cells (t) in the same section were also negative. **(C)** and **(F)**; sections were hybridised with a digoxigenin-labelled ß-actin riboprobe. Section (C) shows fibroblasts (f) positive for ß-actin and that the tumour cells (t) and fibroblasts (arrows) which were negative for collagen mRNA in section (E) do produce ß-actin mRNA as shown in (F).Total magnification for each section was 100X. These are consecutive sections and were cut from the same block.

STAGE III

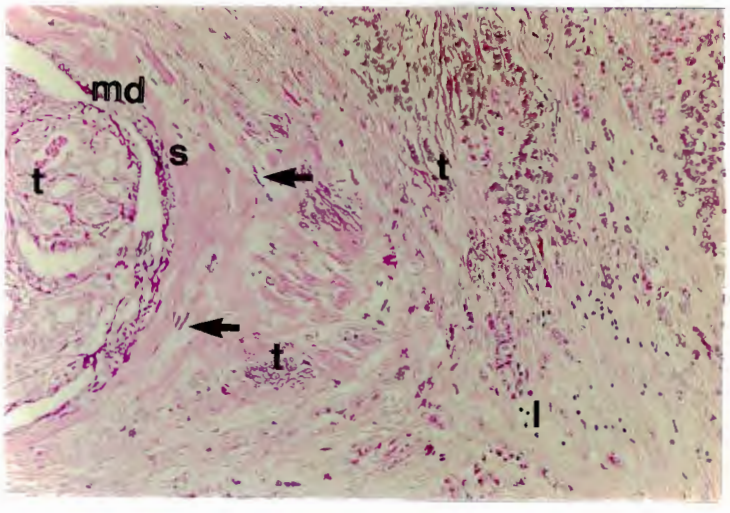
NORMAL

TUMOUR

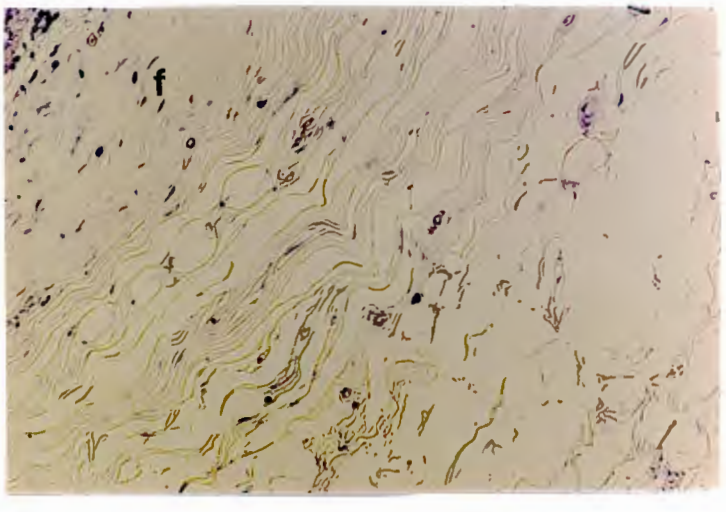
A



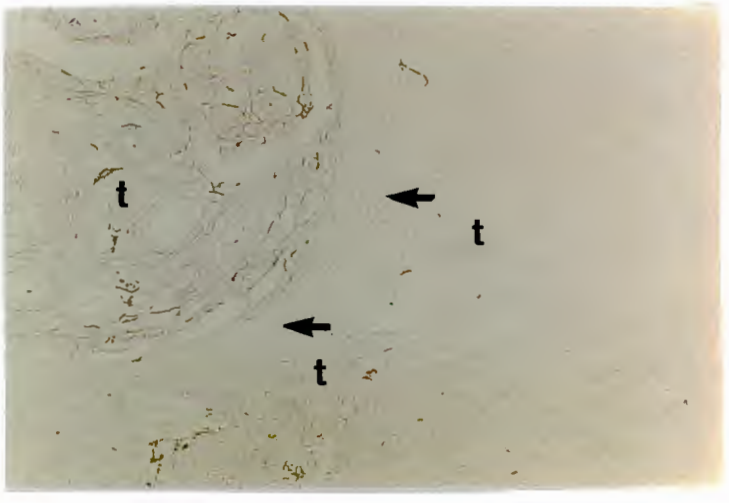
D



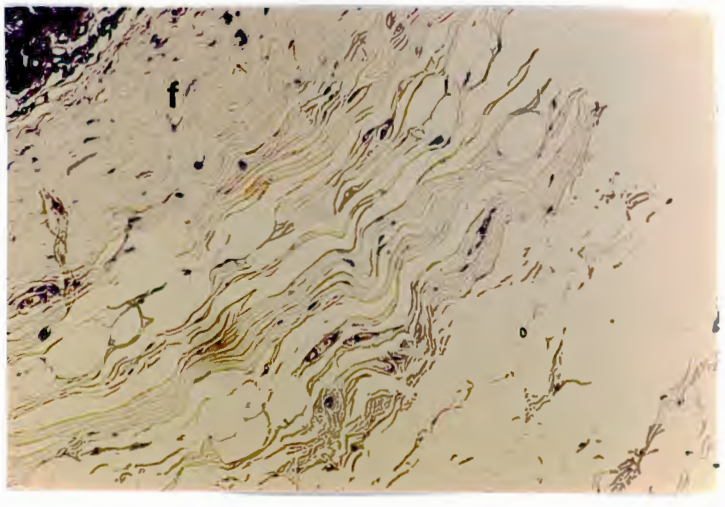
B



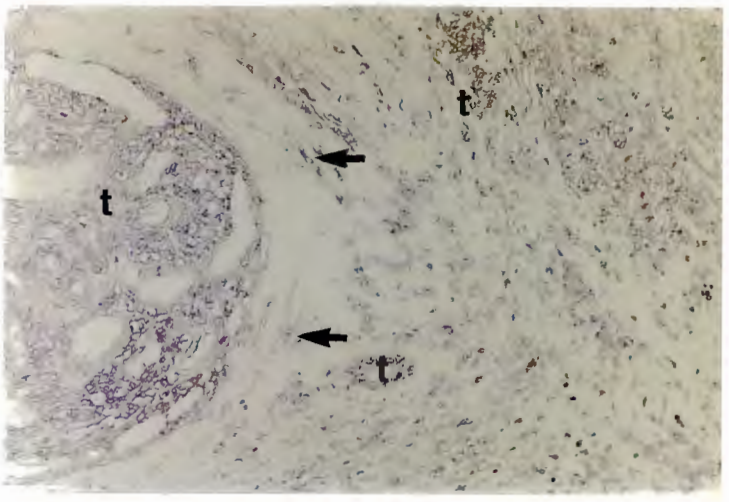
E



C



F



2.2.2 Collagen Gene Expression in Colon Cancer

Collagen mRNA levels in 32 colorectal carcinoma samples were analysed by non radioactive in situ hybridisation as described in Materials and Methods (section 6.3). In this study colon tumours were staged according to the Dukes system of staging (Basic Pathology 1992). The four main stages are described as follows: Dukes A, the neoplasm is located to the mucosa; Dukes B1, the neoplasm has extended into the muscularis propria but has not penetrated it, lymph nodes are not involved; Dukes B2, the tumour has penetrated through the entire wall, but without lymph node involvement. In Dukes C1 the tumour is limited to the wall with lymph node involvement; Dukes C2, the tumour has extended through all layers of the wall, also with lymph node involvement. Dukes D involves distant metastases. Consecutive paraffin embedded sections, consisting mostly of normal colon tissue, from each sample were analysed for collagen and ß-actin mRNA's using digoxigenin labelled riboprobes as described in section 2.2.1.2. Only two Dukes A samples were available, and both were found to be positive for α1(I) and α2(I) collagen as well as ß-actin mRNA (results not shown).

An example of a Dukes B colon carcinoma sample is shown in figure 2.7, stained with H+E. The sections consisted of a serosa containing stroma (s) in which is a diverticulum (d) and an elongated blood vessel (bv) (figure 2.7A). The fibroblasts are indicated by arrowheads and are located within the stroma. Sections were hybridised to an α1(I) collagen riboprobe which produced an intense blue signal in the majority of fibroblasts (figure 2.7B). The same fibroblasts were positive when the section was hybridised to a ß-actin riboprobe (figure 2.7C). A riboprobe was produced from the vector pGEM3 as a negative control and, no signal was detected, showing no non-specific binding of the riboprobes (data not shown). The fibroblasts in Dukes B tumours therefore clearly were producing type I collagen mRNA.

A Dukes C carcinoma, stained with H+E, was found to consist of a layer of muscle (m), called the muscularis propria, in which is situated a colon crypt (c) as indicated in figure 2.8(A). This layer of muscle is surrounded by the submucosa (s), which is interspersed with several fibroblasts, marked with arrowheads, as well as individual

tumour cells (t) and lymphocytes (l). The section hybridised to an α1(I) collagen riboprobe, produced no blue signal, indicating that the fibroblasts present in this section did not produce any α1(I) collagen mRNA (figure 2.8B). These fibroblasts, however, stained positive for ß-actin (figure 2.8C) demonstrating that the fibroblasts were still intact and that there had been no general RNA degradation. The vector pGEM3 riboprobe showed negative staining, indicating no non-specific hybridisation (data not shown).

Analysis of Dukes D colon carcinoma samples are shown in figure 2.9. The serosa consisted of fragmented collagen fibres (cf) as well as numerous elongated fibroblasts (arrowheads) and rounded darkly stained lymphocytes (l) and a blood vessel (bv) (figure 2.9A). This section was negative for α1(I) collagen mRNA (figure 2.9B), while the ß-actin hybridisation signal was positive (figure 2.9C). The negative control showed no non-specific hybridisation (data not shown). All in situ hybridisation analyses were also performed with the α2(I) collagen riboprobe and the results were identical to those shown for the α1(I) collagen mRNA.

These in situ hybridisation results suggest that although the fibroblasts were viable, that they no longer produced type I collagen mRNA as the tumour progressed from a Dukes A through to a Dukes D (Table II). This decrease in collagen mRNA could facilitate growth of the tumour as well as eventual metastatic spread. Possible mechanisms for this modulation of collagen mRNA will be discussed in chapters three and four.

Table II: Summary of the in situ hybridisation results in colorectal tumours

TUMOUR STAGE	NO. OF PATIENTS	COLLAGEN GENE EXPRESSION	β-ACTIN GENE EXPRESSION
DUKES A	2	++	+++
DUKES B	10	+++	+++
DUKES C	10	+	+++
DUKES D	10	—	+++

Where intensity of signal by visualisation is shown as:

+++ strong; ++ moderate; + weak; — no signal

2.2.3. Analysis of Ras Gene Mutations

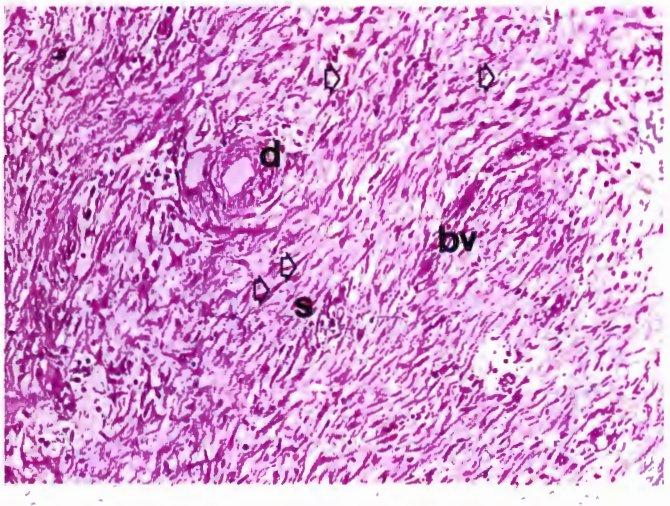
Mutations in the Ras gene are commonly associated with various cancers (Barbacid 1987; Forrester et al, 1987). In particular specific mutations in codons 12, 13 and 61 have been shown to be prevalent (Bos et al, 1987). Mutations in the Ki-Ras gene have been found to occur in the adenoma stage of colorectal cancer patients, this mutation therefore could be the initiating event leading to the formation of a carcinoma (Fearon and Vogelstein 1990). It is also possible that the mutation in the Ras gene affects the synthesis of collagen mRNA in colon cancer, because cells transfected with mutagenic Ras have been shown to decrease collagen gene expression (Slack et al, 1992). A possible correlation between Ras mutations and collagen gene expression was subsequently addressed to ascertain whether mutations in the Ras gene could cause a decrease in collagen mRNA levels. 32 carcinoma samples of different stages were therefore analysed for Ras mutations, the breast carcinoma cell line T47D was used as a positive control for mutations in the Ha-Ras gene.

FIGURE 2.7

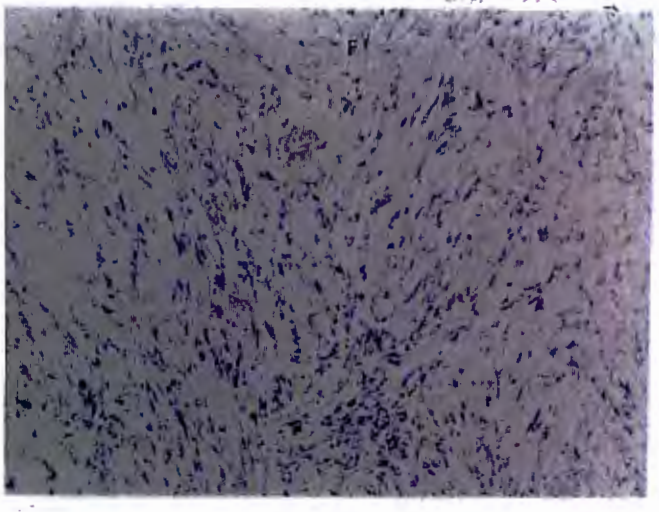
Analysis of collagen mRNA in sections of a Dukes B colorectal carcinoma Section **(A)** was stained with haemotoxylin and eosin (H+E), depicting a diverticulum (d) and an elongated blood vessel (bv) within the dense collagen stroma (s). There are numerous fibroblasts, some of which are indicated by arrows. Section **(B)** was hybridised with a digoxigenin-labelled α1(I) collagen riboprobe (section 6.3) which bound to the fibroblasts indicating a positive result for collagen mRNA. Section **(C)** was hybridised with the digoxigenin-labelled ß-actin riboprobe (positive control) which bound to tumour cells and fibroblasts. All sections were photographed at a final magnification of 100X. These are consecutive sections.

Dukes B

A



B



C

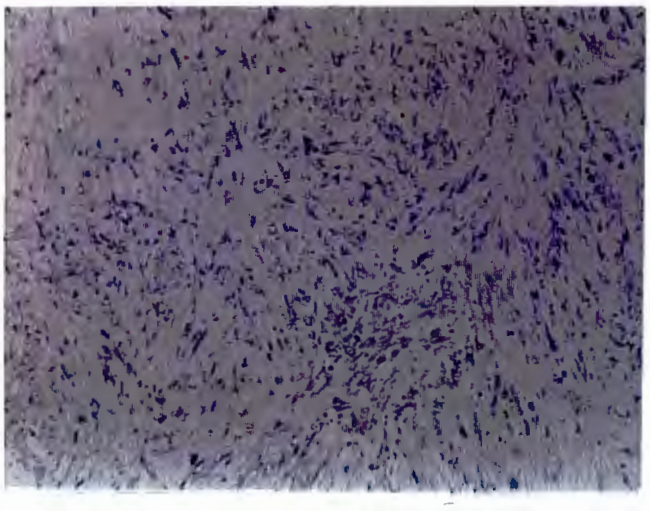
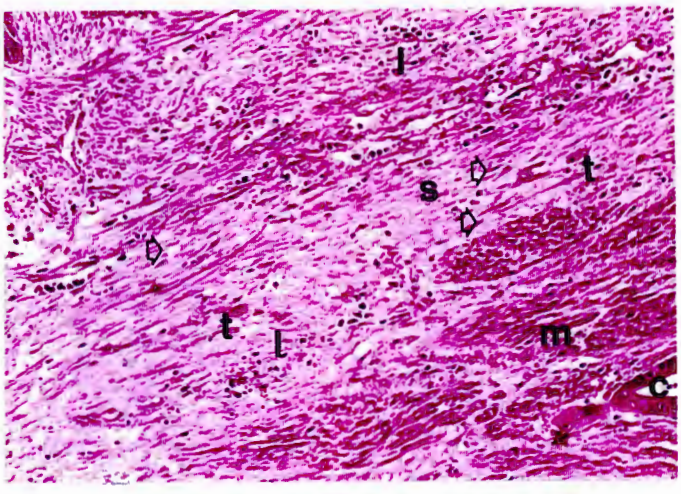


FIGURE 2.8

Analysis of collagen mRNA in a Dukes C colon carcinoma by in situ hybridisation The H+E section **(A)** contains a typical colon crypt (c) within a layer of muscle (m), on top of which are several individual tumour cells (t), lymphocytes (l) as well as fibroblasts (arrowheads). These are all contained within the submucosa layer (s). **(B)** and **(C)** were hybridised with the digoxigenin-labelled α1(I) collagen and ß-actin riboprobes respectively (as described in figure 2.7). The fibroblasts in section (B) are negative for α1(I) collagen mRNA but positive for ß-actin mRNA section (C) indicating a specific down regulation of collagen mRNA synthesis. All sections were photographed at a final magnification of 100X. These are consecutive sections.

Dukes C

A



B

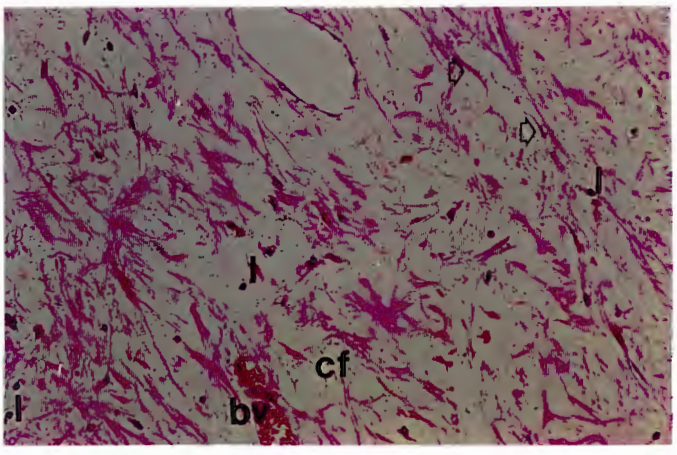
C

FIGURE 2.9

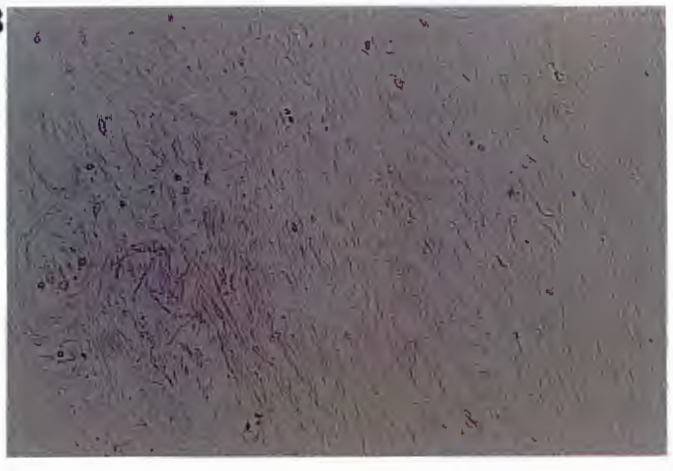
Analysis of collagen mRNA in sections of a Dukes D colorectal carcinoma Section **(A)** stained with H+E consists of the serosa containing fragmented collagen fibres (cf), several lymphocytes (l) and fibroblasts (arrowheads) as well as a blood vessel (bv). Section **(B)** was hybridised with the digoxigenin-labelled α1(I) collagen riboprobe which did not bind to the fibroblasts clearly shown in section (A) indicating that these fibroblasts did not produce type I collagen mRNA. The fibroblasts upon hybridisation with the digoxigenin-labelled ß-actin riboprobe, synthesised β-actin demonstrating that the down regulation of mRNA was specific for collagen **(C)**. All sections were photographed at a final magnification of 100X. These are consecutive sections.

Dukes D

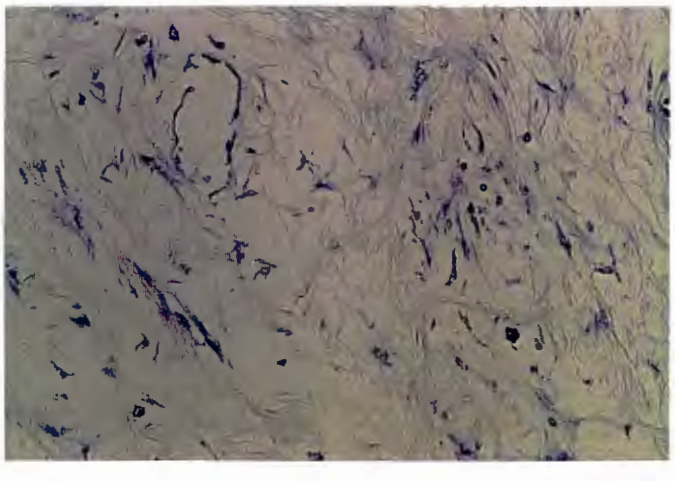
A



B



C



2.2.3.1. Ha-Ras Gene Mutations

DNA from 32 colon carcinoma samples (these were the same samples used for in situ hybridisation and contained a mixture of normal and tumour cells, of which the majority were normal) was extracted from paraffin-embedded sections as described in section 6.12.1. PCR conditions were optimised using primers flanking Ha-Ras exon 1 and 2 (which contains codons 12 and 13). PCR fragments were amplified from genomic DNA in the presence of ^{32}P-dCTP, denatured and electrophoresed on 6% polyacrylamide gels with or without glycerol at 4°C or room temperature (see sections 6.12.2 and 6.12.3). All samples were subjected to all four SSCP conditions and Ha-Ras 12/13 mutations were detected in the T47D cell line (indicated as number 1) and patient number 9 with both missing the top band (figure 2.10A). Patient numbers 34, 44, 43, 39 and 45 were shown to lack either the top, bottom or middle bands and these mutations were presumed different from each other as judged from their different electrophoretic mobility patterns (figure 2.10B). No Ha-Ras 61 mutations were found using any of the SSCP conditions, an example of gels electrophoresed at room temperature with or without 5% glycerol is shown in figure 2.11.

DNA from those colon samples that appeared positive for Ha-Ras 12/13 mutations by SSCP analysis were amplified by PCR and cloned into pUC 19 for subsequent sequencing. PCR products were also sequenced directly as described in section 6.12.5. In total, 9 out of the 32 colon carcinoma samples were found to have Ha-Ras 12/13 mutations; the breast tumour cell line T47D was found to have a mutation in codon 27 (CAT to CAC), which does not cause an amino acid change. Five colon samples, of different Dukes stages, had the same mutation as T47D. Four other colon samples were found to have Ha-Ras mutations, 2 in codon 8 changing the amino acid valine (GTG) to leucine (TTG) and 2 having a deletion of a guanine in the intron. Due to the fact that the colon samples consisted of a mixture of normal and tumour cells, the mutations in the tumour cells were diluted and thus did not photograph well. Therefore the sequencing data is not shown. The data is summarised in Table III.

2.2.3.2. Ki-Ras Gene Mutations

DNA from the 32 colon carcinoma samples were also used for PCR amplification of the Ki-Ras exons 1 and 2 as described in section 6.12.2. Ki-Ras 12/13 mutations were analysed under the four different electrophoretic conditions described for Ha-Ras and no mutations were detected. Due to the fact that a known Ki-Ras 12/13 mutation (cell line MDA-MB-231) did not separate under any of the conditions, other methods of SSCP analysis were attempted such as varying the concentrations of bisacrylamide/acrylamide, longer electrophoretic times as well as "cold" or non-isotopic SSCP analysis, based on the results of Hongyo et al, (1993) (see section 6.12.4 of Materials and Methods). These all proved unsucessful and all samples were therefore subjected to direct sequence analysis.

Of the 32 samples analysed, 10 were positive for Ki-Ras 12/13 mutations, 4 were in Dukes B, 4 in Dukes C and 2 in Dukes D. There were nine codon 12 mutations; four changed glycine (GGT) to valine (GTT), three changed glycine (GGT) to aspartate (GAT), one altered the glycine (GGT) to alanine (GCT) and one changed glycine (GGT) to arginine (CGT). There was only one codon 13 change, from glycine (GGC) to aspartate (GAC). A summary of all these mutations is shown in Table III.

Due to the fact that the SSCP analysis technique proved unreliable for the detection of codon 12/13 mutations in the Ki-Ras gene, codon 61 PCR products were also subjected to direct sequence analysis. PCR conditions were optimised for the primers flanking exon 2 of the Ki-Ras gene, as decribed in section 6.12.2, the samples were sequenced directly and no mutations were detected. Other mutations in the Ki-Ras gene were detected, for instance a mutation in codon 16 of Ki-Ras in a Dukes D colon sample changed serine (AGT) to arginine (CGT). This mutation has not been documented in the literature and is not known whether it will impair the GTPase activity of $p21^{ras}$ (Polakis and McCormick 1993; Boguski and McCormick 1993). There were also three mutations in the untranslated region, two occurring in Dukes C colon samples and one in Dukes D. These mutations consisted of a G-C transversion, an A-C transversion and a GA-CC double transversion. These results are summarised in Table III. It is unlikely that the mutations in the untranslated region will have an

effect on Ras function, however, it may be possible that they affect the initiation of translation by altering the ribosome binding site.

TABLE III: Ras gene mutations in colorectal tumours

RAS GENE	CODON	SAMPLE NO.	BASE CHANGE	AMINO ACID CHANGE	DUKES STAGE
Ha-Ras	8	12	GTG→TTG	Val→Leu	C
		23	GTG→TTG	"	D
	27	6	CAT→CAC	His→His	D
		9	"	"	D
		34	"	"	B
		41	"	"	B
		55	"	"	C
	Untranslated	4	ΔG	—	A
	region	20	"	—	B
Ki-Ras	12	8	GGT→GAT	Gly→Asp	C
		23	"	"	D
		58	"	"	B
		25	GGT→GTT	Gly→Val	C
		29	"	"	C
		44	"	"	D
		46	"	"	B
		17	GGT→GCT	Gly→Ala	C
		18	GGT→CGT	Gly→Arg	B
	13	32	GGC→GAC	Gly→Asp	B
	16	50	AGT→CGT	Ser→Arg	D
	Untranslated	45	G→C	—	D
	Region	49	A→C	—	C
		55	GA→CC	—	C

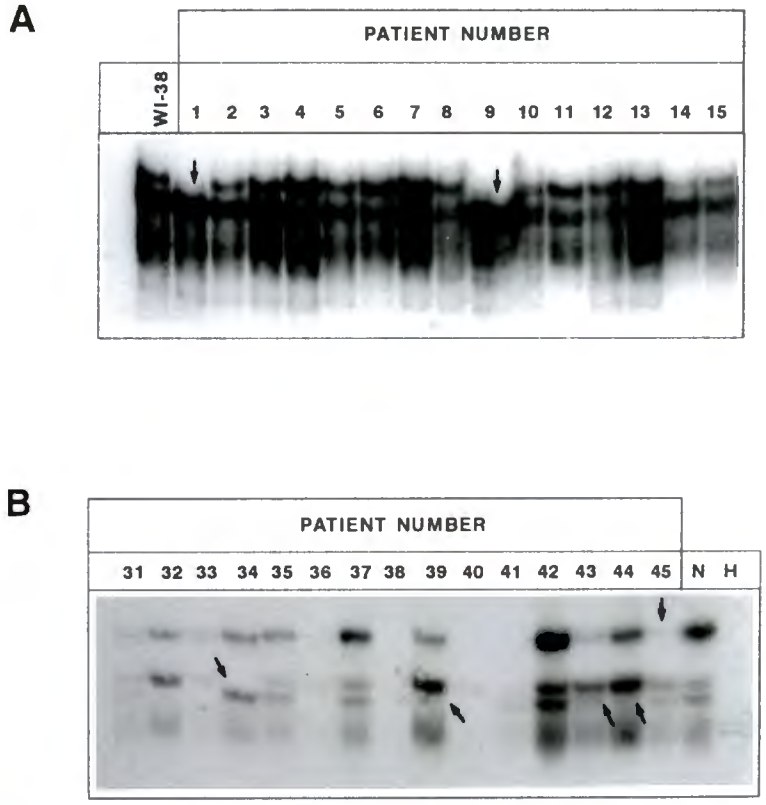


FIGURE 2.10

SSCP analysis of colon samples for Ha-Ras 12/13 mutations at room temperature

(A) DNA from 14 colorectal carcinoma samples was extracted from paraffin-embedded sections. PCR was performed using ^{32}P-dCTP with primers for exons 1 and 2 of Ha-Ras 12/13. The products were electrophoresed on a 6% polyacrylamide gel for 4 hours in 1X TBE at 30 watts at room temperature (see section 6.12.3). DNA from the tumour cell line T47D (1) and patient number 9 lack the top bands, indicated by the arrows.

(B) 15 different samples, after PCR amplification and labelling with ^{32}P-dCTP were electrophoresed on a 6% polyacrylamide gel at room temperature containing 5% glycerol for 6 hours at 30 watts in 1X TBE buffer. The dried gels were exposed for 16-18 hours to X-ray film. Three different mutations are illustrated. Patient number 45 misses the top band, patients 39, 43 and 44 lack the bottom band and patient number 34 missed the middle band. All differences are indicated with arrows. (N) represents normal DNA and (H) (PCR water blank containing no DNA) was used as a negative control.

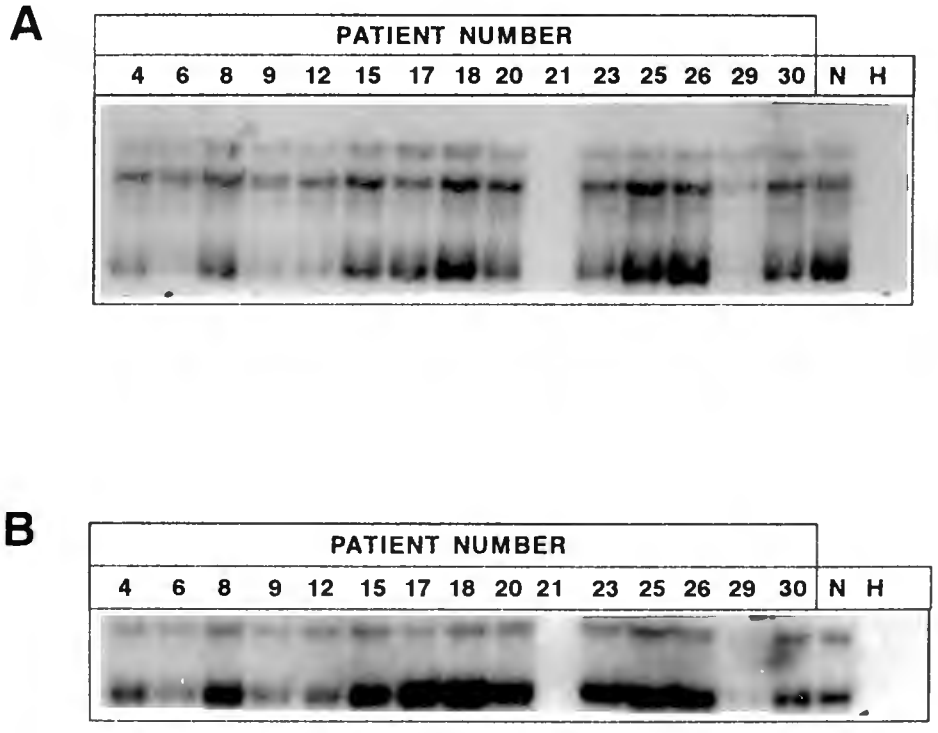


FIGURE 2.11

SSCP Analysis of colon samples for Ha-Ras 61 mutations at room temperature

DNA from 14 colorectal carcinoma samples was extracted, PCR amplified with primers for Ha-Ras 61 (section 6.12.2) and analysed by SSCP on a 6% polyacrylamide gel at room temperature for 4 hours **(A)** or electrophoresed on a 6% gel containing 5% glycerol at room temperature **(B)**, for 4-6 hours at 30 watts in 1X TBE buffer (section 6.12.3). The gels were dried and exposed to X-ray film for 16-18 hours. (H) is the water blank containing no DNA and (N) is normal DNA. These gel conditions failed to detect any mutations.

2.3 DISCUSSION

The stage-specific changes in collagen gene expression detected in infiltrating ductal breast carcinoma and colorectal carcinoma are probably due to a series of events triggered by the onset of the invasive/metastatic process. The stimulation of collagen mRNA production in stage I breast tumours and Dukes A and B colon tumours can be explained by the desmoplastic effect (Noel et al, 1992). Desmoplasia is described as excess production of collagen in the stroma resulting in "hardening" and "encapsulation" of the tumour (Pucci Minafra et al, 1986; Basic Pathology 1992). There is and has been considerable controversy as to whether the tumour cells themselves synthesize collagen (Al-Adnani et al, 1975; Niitsu et al, 1988) or whether fibroblasts around the tumour produce collagen (Ohtani et al, 1992; Barsky et al, 1992). Yoshida et al (1989) showed that gastric carcinoma cell lines produced pro α1(I) mRNA, whereas the breast and colon tumour cells analysed in our study did not produce collagen mRNA. Also, the in situ hybridisation results presented in this study clearly demonstrate that the host fibroblasts were responsible for the production of collagen and not the tumour cells.

A study by Nakanishi et al (1994), using cloned mouse Lewis-lung-carcinoma-derived cell lines with different metastatic potential, found an inverse relationship between the host stromal response and spontaneous lung metastasis. The authors speculate that the host stromal response is disadvantageous to tumour cells by preventing their access to the vascular basement membrane (Nakanishi et al, 1992 and 1994). This work is in agreement with the results presented in this study: stage I breast tumours and Dukes A and B colorectal cancers synthesised increased amounts of type I collagen mRNA which is similar to the low-metastatic P29 tumour with high collagen protein levels. Similarly, stages II and III breast tumours and Dukes C and D colorectal cancers are can be compared with the highly metastatic Lewis carcinomas (which contained smaller amounts, if any, of type I collagen). Although Nakanishi et al, (1994) analysed collagen protein levels, whereas this study concentrated on collagen mRNA, it is reasonable to assume that the mRNA levels would determine the protein content. The authors speculate that the host stromal response is disadvantageous to tumour cells by

preventing their access to the vascular basement membrane (Nakanishi et al, 1992 and 1994). In this study, collagen mRNA was localised by in situ hybridisation to the fibroblasts, which is in agreement with Nakanishi et al (1994) where they showed that the cells positive for type I collagen and fibronectin were not tumour cells but host stromal cells, probably derived from fibroblasts. Our study found that the fibroblasts were present and normal in the more advanced stages of breast and colon cancers, but were not producing collagen, indicating that the tumour cells modulated collagen mRNA production. This process would allow the tumour to invade the stroma and metastasize. The results in this study suggest that desmoplasia correlates with early stages of disease (stage I for breast cancer and Dukes A and B for colorectal cancer) and that collagen mRNA is down regulated in the later stages which more than likely aids in tumour cell invasion. Hewitt et al (1993) have also reported the absence of type I collagen mRNA at the invasive edge of colorectal tumours, which is in agreement with the data presented in this chapter. They also refute the work of Luparello et al (1991) who found a type-I collagen trimer variant deposited close to the neoplastic cells and speculated that the type-I collagen trimer may provide the tumour cells with contact guidance for invasive spread. The in situ hybridisation analysis performed in this chapter showed no α1(I) or α2(I) collagen mRNA in fibroblasts in the later, more invasive stages of disease.

Whether the increased collagen present in the early stages of the disease were of benefit to the tumour or "protecting" the host is impossible to say, but both options are feasible. It is possible that desmoplasia, in certain tumours, could be part of the process of tumour invasion and metastasis. The exact nature of the desmoplastic response is still unknown, and even though studies have shown that growth of tumour cells from early lesions is repressed in the presence of fibroblasts, whereas metastatic cells are stimulated to grow (Cornil et al, 1991), it is possible that tumour cells utilize the desmoplastic response so that they can survive and grow. It is possible that interstitial collagen, due to its abundance, in the early stages of disease can either protect the host or facilitate tumour invasion. This will depend on the tumour type (as not all tumours exhibit the desmoplastic response) as well as the tumour ECM environment. In the more advanced stages of disease, the tumour cells may degrade the collagen by

producing collagenases as well as switch off collagen mRNA synthesis which facilitates and aids in the progression of tumour invasion and eventually metastasis.

The decreased collagen gene expression in stage II and III breast carcinomas as well as the Dukes C and D colon cancers can be linked to any of the many factors involved in tumour cell invasion and metastasis. In order for the process of tumour invasion and metastasis to occur, the tumour cells must penetrate the extracellular matrix (Liotta et al, 1983). It is now well accepted that matrix metalloproteinases (MMPs) are responsible for the degradation of the ECM and thus aid in the processes of tumour cell invasion and metastasis. These MMPs can be secreted by the tumour cells themselves or can be produced by the host fibroblasts in response to factors secreted by the tumour cells (Liotta et al, 1983; Biswas 1984). The factors involved in modulating collagen production in fibroblasts (ie collagen mRNA levels), however, have not been identified and it is not known whether the factor(s) is secreted by the tumour cells, macrophages or fibroblasts themselves. This will be addressed in chapters three and four.

One of the factors known to inhibit collagen gene expression is transformation of cells with oncogenic Ras (Slack et al, 1992). Since these tumours are all of epithelial origin, while the fibroblasts are normal, the effect on collagen synthesis by the fibroblasts would be indirect, and possibly via some signaling pathway. It has been found that cytoplasmic Raf and mitogen-activated protein kinases (MAPK) differentially regulate α1(I) collagen gene expression in activated stellate cells (Davis et al, 1996). A MAPK-stimulatory signal was mapped to the most proximal NF-1 and Sp-1 binding domains of the collagen promoter whereas a Raf-inhibitory signal was mapped to a further upstream binding domain involving a novel 60 Kd DNA-binding protein (p60) (Davis et al, 1996). Since co-culture of oncogenic Ras transformed cells with normal Rat-1 fibroblasts results in decreased collagen synthesis in the normal cells (Geyp and Parker, unpublished data), it is possible that tumours containing mutant Ha-Ras may be indirectly responsible for the decrease in collagen mRNA detected in this chapter. Since colorectal cancer, shows involvement of Ki-Ras mutations as a late to middle event it is possible that these mutations could affect the synthesis of collagen and allow

progression to the invasive stage to occur. The exact reason for the mutation remains unknown, it could be an initiating event, allowing for the transition from adenoma to carcinoma, or those adenomas containing Ras mutations may be more predisposed to progress to carcinomas. It cannot be ruled out, however, that the mutations may just be coincidental. Ras mutations occur with a very low frequency in breast cancer (less than 10%) but overexpression of Ras has been reported as fairly common in breast cancer (Rochlitz et al, 1989; Going et al, 1992). If mutant Ras is indeed involved in invasion in colon cancer, then these results imply that a different mechanism confers invasive properties on the colon cancer cells.

CHAPTER THREE

TUMOUR CELLS ALTER COLLAGEN PRODUCTION IN NORMAL FIBROBLASTS

3.1. INTRODUCTION

This chapter attempts to address the aberrant collagen gene expression detected in breast and colorectal tumours in chapter 2. Tumours are heterogeneous in nature and the exact mechanism(s) involved in the modulation of extracellular matrix (ECM) deposition is therefore not easily studied. The modulation of ECM deposition is vital for tumour cell invasion and metastasis and the effectors can function in a negative or positive manner. Negative modulation can be transcriptional, ie due to a shutdown of collagen gene expression, unstable mRNA, or translational inhibition of collagen synthesis. Alternatively the protein can be degraded which would cause a decrease in the amount of collagen protein.

The degradation of the ECM is thought to lead eventually to metastasis. The exact mechanism for modulating the ECM may be different for different tumour types. Tumour cells can secrete proteolytic enzymes to degrade the ECM directly or the tumour cells may induce the production of proteolytic enzymes by the surrounding normal cells such as macrophages, lymphocytes or fibroblasts. This interaction between cells could not only bring about the production of collagenases to degrade the ECM, but also factors which could switch off collagen gene transcription (which would aid the tumour in its ability to invade and metastasize). It is also possible that this process does not involve a single mechanism, but a combination of the above mechanisms.

There were two major findings in chapter two; (i) increased collagen gene expression (desmoplasia) in the early stages of cancer and (ii) decreased collagen gene expression in the later stages. The aberrant collagen gene expression may be brought about by the tumour cells (which secrete factors) or by the fibroblasts in response to the tumour cells. Tumour cells have been shown to directly alter the ECM by secreting proteolytic

enzymes such as collagenases. In fact, Liotta et al (1979) identified a type IV collagenase in the culture medium of a highly metastatic murine tumour which preferentially digested type IV collagen in basement membranes. Few, if any, inflammatory cells were observed in the vicinity of the tumour and it was deduced that the enzyme is produced by the tumour cells themselves. Alternatively ECM modulation may be dependent on specific interactions between tumour and host cells. Tumour cells may indirectly alter the ECM by interfering with fibroblast functions by secreting factor(s) which stimulate(s) the fibroblasts to produce collagenases and thus cause degradation of the matrix (Biswas 1982 and 1984).

In order to obtain a better understanding of the way in which tumour cells modulate type I and III collagen synthesised by normal fibroblasts, we developed a tissue culture system. A normal breast fibroblast cell line was established and the influence of breast tumour cells on the production of collagen by fibroblasts was analysed using conditioned media as well as co-culture. Tumour cell conditioned media and the tumour cells themselves caused a decrease in the collagen produced by the fibroblasts. This was investigated further by attempting to look for the presence of collagenases within the system. A collagenase could be secreted by the tumour cells themselves or the tumour cells could induce the fibroblasts to produce it. The tissue culture system indicated that tumour cells secrete factor(s) which stimulate normal fibroblasts to produce collagenases.

3.2. RESULTS

3.2.1. Preparation of Normal Primary Breast Fibroblasts

Breast tissue was obtained from a 55 year old female undergoing a mastectomy at the Breast Clinic at Groote Schuur Hospital. The tissue was excised at a distance from the infiltrating ductal tumour, sliced into small pieces, placed in several 30mm petri dishes, covered with a coverslip (see section 6.4) and cultured in 1ml of Eagle's minimal essential medium supplemented with 10% fetal calf serum and antibiotics. After 3 weeks, fibroblasts began to grow out of the tissue and gradually protruded from under the coverslips. After 4 weeks the coverslips were removed and the fibroblasts were allowed to grow to confluence at which stage they were transferred into 60mm petri dishes. Their morphology was similar to that of other normal fibroblasts, having spindle-shaped structures (figure 3.1). To ensure that the fibroblasts were in fact normal, they were subjected to karyotype analysis.

3.2.1.1. Karyotype analysis of Normal Breast Fibroblasts

The breast fibroblasts (BRF fibroblasts) were seeded onto 4 individual coverslips and grown to 80% confluence. They were then treated with colcimid, swollen in a hypotonic solution and fixed with methanol:acetic acid (3:1) (as described in section 6.5). After the cells were air dried, the 4 coverslips were stained with Giemsa for conventional examination and counting of chromosomes. The chromosomes in several cells on all 4 coverslips were counted and found to be 46 in each case (figure 3.2). There were no chromosomal abnormalities and the two X chromosomes could also be seen.

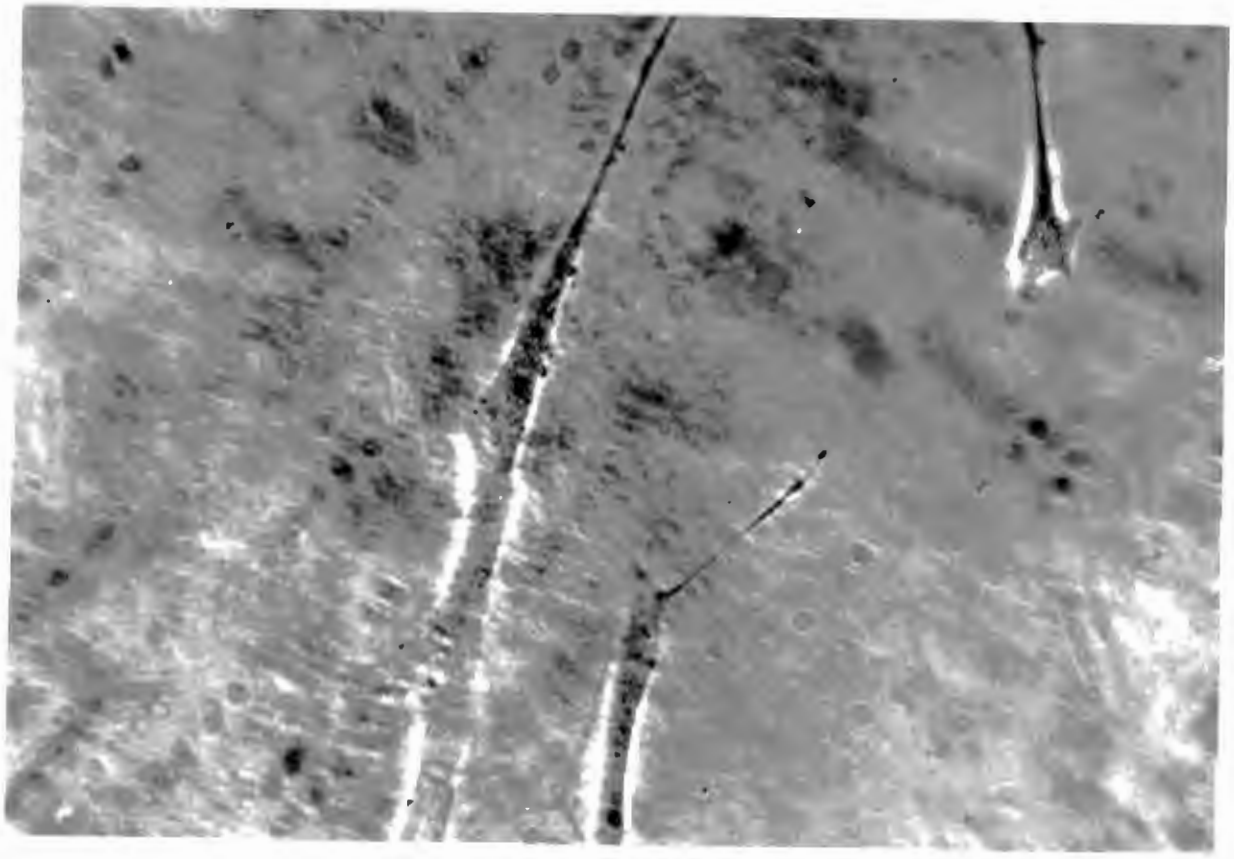


FIGURE 3.1

Establishment of normal primary breast fibroblasts. Breast tissue was sliced into small pieces, placed in 30 mm dishes (as described in section 6.4) and covered with a coverslip to allow attachment of the cells. Fibroblasts eventually grew out of the tissue pieces, under the coverslips and were allowed to grow to confluency. The cells shown in the figure were at their 8th passage, showing spindle-shaped morphology. The cells were photographed at a total magnification of 100X.

FIGURE 3.2

Karyotype analysis of breast fibroblasts. Breast fibroblasts were seeded onto coverslips in 30 mm dishes and allowed to grow to 80% confluence, prior to treatment with colcimid (see section 6.5). The cells were subsequently fixed in acetic acid:methanol (1:4), after which the coverslip was removed from the dish and allowed to dry. The coverslip was stained with Giemsa, once again allowed to dry after which the chromosomes were examined. These fibroblasts were characterised in their 9th passage and show 46 normal chromosomes. The chromosomes were shown to be intact, with no breaks or deletions. The two X chromosomes are indicated by arrows.

3.2.1.2. Analysis of Breast Fibroblast Collagens

Breast fibroblasts (6th passage) were allowed to grow to 80% confluence in 6-well (24 mm diameter) dishes prior to the addition of ^{3}H-proline (see section 6.6). ^{3}H-proline was used as a label, because of the high proline content in collagen. After 16 hours the cells were harvested by trypsinisation and the number of cells determined in a Coulter Counter. Collagen was harvested from the medium, precipitated in 96% ethanol, washed twice with 70% ethanol, vacuum dried and digested with pepsin to remove the propeptides. Radioactive collagen corresponding to equal numbers of cells per sample was electrophoresed on 6% polyacrylamide SDS gels. The fluorogram shown in figure 3.3 clearly shows the product of α2(I) and α1(I) chains of type I collagen by these cells. As a positive control normal WI-38 lung fibroblast cells were treated in the same way as the BRF fibroblasts. BRF fibroblasts produced normal type I collagen, except that the levels were much higher than in WI-38 fibroblasts. This could be due to the fact that BRF fibroblasts were used in their 6th passage, whereas WI-38 fibroblasts were used in their 21st passage.

3.2.2. Modulation of Type I Collagen by Tumour Cells

These experiments were performed in order to determine whether the tumour cells affected type I collagen produced by normal breast fibroblasts. The following breast cancer cell lines were used in this study: MCF-7 (Soule et al, 1973); MDA-MB-231 (Cailleau et al, 1974); ZR-75-2 (Engel et al, 1978) and T47D (Keydar et al, 1979). Conditioned media were prepared from each of the breast cancer cell lines as well as BRF and WI-38 fibroblasts as described in section 6.7.

As a first step, the breast tumour cell lines were assayed to confirm that they do not produce type I collagen. Cells were plated in 6-well dishes at a density of 300 000 cells per dish and allowed to reach confluency. ^{3}H-proline, ascorbate and ßAPN were added 16 hours prior to harvesting, (see section 6.6). After removal of the medium, the cells were harvested and counted in a Coulter Counter. Radioactively-labelled collagen equivalent to 10^{5} cells for each cell line was electrophoresed on a 6% polyacrylamide SDS gel, and the results (figure 3.4) clearly show that the tumour cell lines used in this study did not produce any type I collagen.

The effect of tumour cells on type I collagen produced by the fibroblasts were investigated by two approaches: 1) the addition of tumour cell conditioned medium and 2) co-culture of tumour cells and normal fibroblasts.

3.2.2.1. Conditioned Medium Experiments

Normal breast fibroblasts were incubated with 1X conditioned medium for 2 days before the addition of ^{3}H-proline. The medium was harvested 16-18 hours later and the total collagen determined using the collagenase assay. The collagenase assay allows for quantitative measurement of collagen in the presence of large amounts of other proteins. Bacterial collagenase possesses a specificity for the amino acid sequence R-Pro-X-Gly-Pro-, cleaving between X and Gly. This sequence is found almost exclusively, and with great frequency, in collagen, therefore bacterial collagenase cleaves collagen into acid-soluble peptides (Peterkofsky and Diegelmann 1971). Figure 3.5 shows type I collagen in the medium and the cells taking into consideration the excess proline in collagen compared to other proteins (see section 6.8.1 in Materials and Methods). All the breast cancer conditioned media caused a decrease in type I collagen produced by the breast fibroblasts. BRF conditioned medium on BRF fibroblasts was used as a control. Conditioned media from MDA-MB-231, MCF-7 and T47D cells resulted in approximately 50% reduction of type I collagen production, whereas ZR-75-2 conditioned medium resulted in a smaller decrease of approximately 20% (figure 3.5A). These results suggested that the breast cancer cell lines secrete a factor(s), such as cytokines or even collagenases, into the medium resulting in a reduction in the amount of collagen. Intracellular type I collagen levels varied, but the changes were small (by 7-10%) for all the breast cancer cell lines used (figure 3.5B). It is possible that a type I collagenase was responsible for the degradation of type I collagen in the medium. Alternatively, the breast cancer cells may secrete a factor into their medium which stimulates the fibroblasts to produce collagenases which degrade type I collagen. Other possible explanations are that the decrease in collagen is due to a decrease in collagen gene transcription, decrease stability of mRNA or decreased translation.

3.2.2.2. Co-Culture Experiments

In order to confirm the conditioned medium experiments tumour cells were co-cultured with BRF fibroblasts, or with normal lung and skin fibroblasts to ascertain whether fibroblasts from different origins responded to tumour cells in the same way.

Equal numbers of BRF fibroblasts and tumour cells (30 000) were co-cultured in 24-well dishes for 48 hours at 37°C (see section 6.8.2 in Materials and Methods). ^{3}H-proline, was added to the medium 16 hours prior to harvesting and total collagen was measured in the medium and cell fractions using the collagenase assay.

Co-culturing of BRF fibroblasts with the breast tumour cell lines resulted in a decrease (ranging between 51% and 56%) in the amount of collagen produced by the BRF fibroblasts, compared to when BRF fibroblasts were cultured on their own (figure 3.6A). In each case the decrease was greater in the co-culture experiments than in the tumour cell conditioned medium experiments (figure 3.5). The most pronounced effect was observed in the tumour cell line ZR-75-2, where a decrease of 50% was observed in the co-culture experiments, but a decrease of only 20% in the conditioned media experiments. The levels of intracellular type I collagen, for all the tumour cell lines, was decreased or unchanged as would be expected since the collagen is secreted fairly rapidly into the medium (figure 3.6B). The co-culture results were in agreement with the conditioned medium experiments and confirmed the hypothesis that breast tumour cells can in fact modulate the production of type I collagen by normal breast fibroblasts.

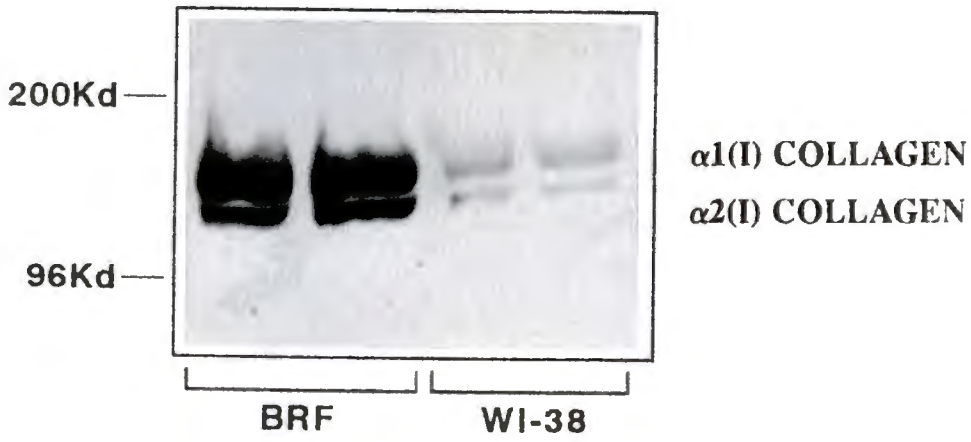


FIGURE 3.3

Analysis of type I collagen in BRF and WI-38 fibroblasts. 300 000 BRF (passage 6) and WI-38 (passage 21) fibroblasts were plated in 6-well dishes in 2ml medium and allowed to grow to confluence. 3H-proline was added and the cells labelled for 16 hours. The cells were harvested by trypsinisation, counted in a Coulter Counter and collagen extracted from the medium. Radioactivity corresponding to 10^5 cells was dried (see section 6.6), resuspended in 2X treatment buffer, denatured for 5 minutes at 95°C and electrophoresed on a 6% polyacrylamide SDS gel for 2 hours at 100 volts. The gel was placed in Enhance^{TM} solution, dried and exposed to X-ray film for 2 days. The sizes of the protein molecular weight standards, are shown on the left. The α1(I) and α2(I) collagen chains are indicated by the arrows. The experiment was performed in duplicate for each cell line.

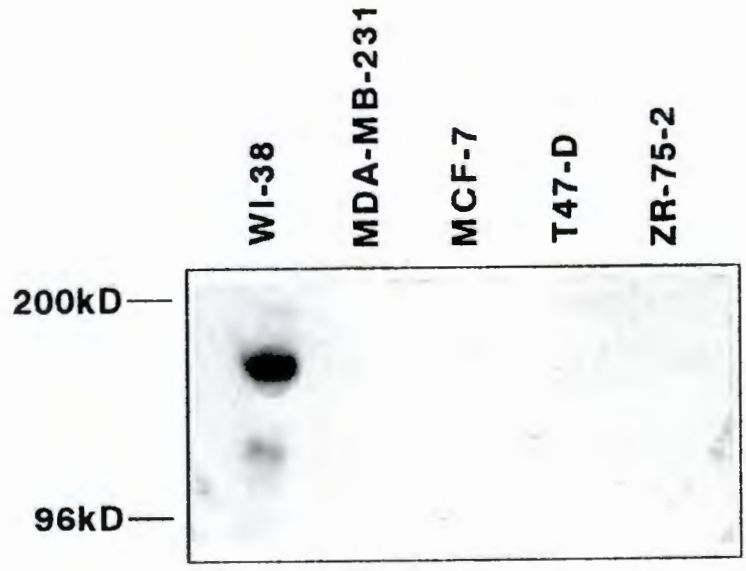


FIGURE 3.4

Analysis of type I collagen in Breast Tumour Cell Lines. 300 000 epithelial cells from the 4 breast tumour cell lines as well as normal WI-38 fibroblasts were plated in 6-well dishes in 2ml medium as described in the legend to figure 3.3. Radioactivity corresponding to 10^5 cells was analysed on a 6% polyacrylamide SDS gel (see section 6.6). The gel was electrophoresed at 100 volts for 2 hours, placed in Enhance^{TM} solution for 1 hour, rinsed in distilled H_20 for another hour, dried at 70°C and exposed to X-ray film for 2 days. Lane 1; WI-38 cells, lane 2; MDA-MB-231, lane 3; MCF-7, lane 4; T47D and lane 5; ZR-75-2. The sizes of the protein molecular weight marker are indicated on the left hand side of the gel.

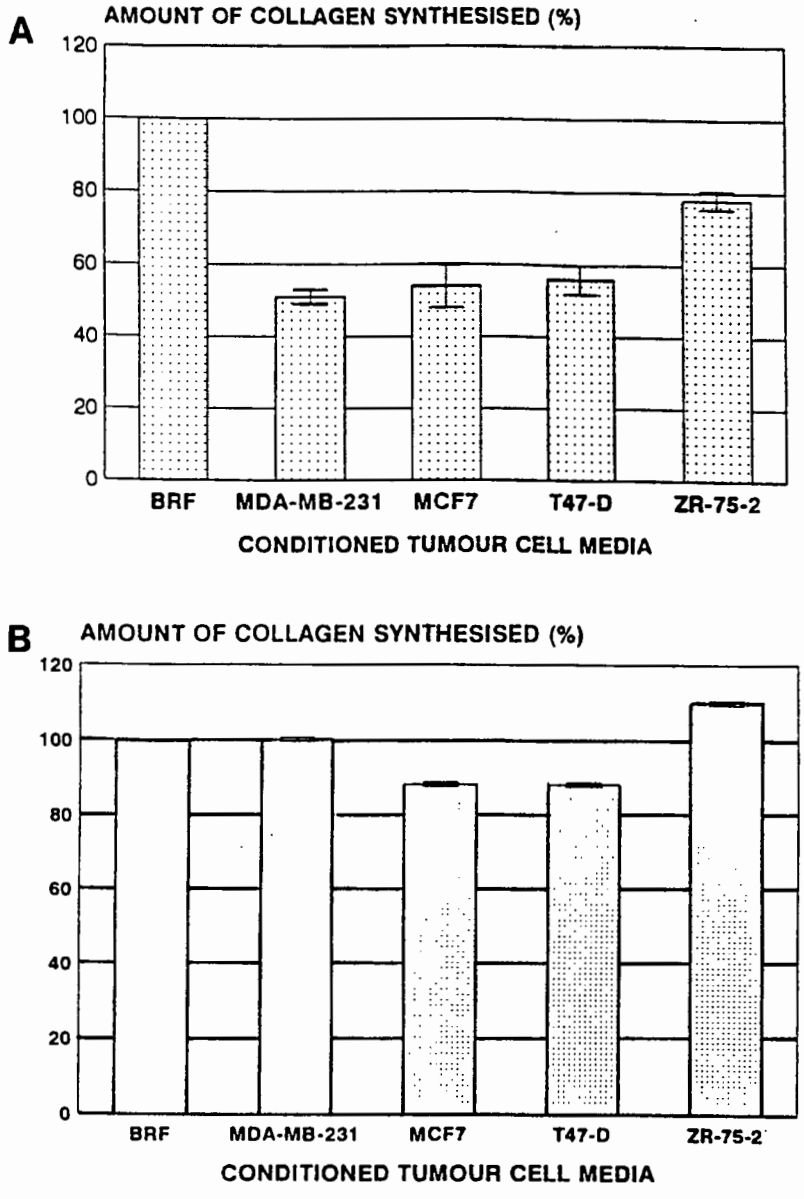
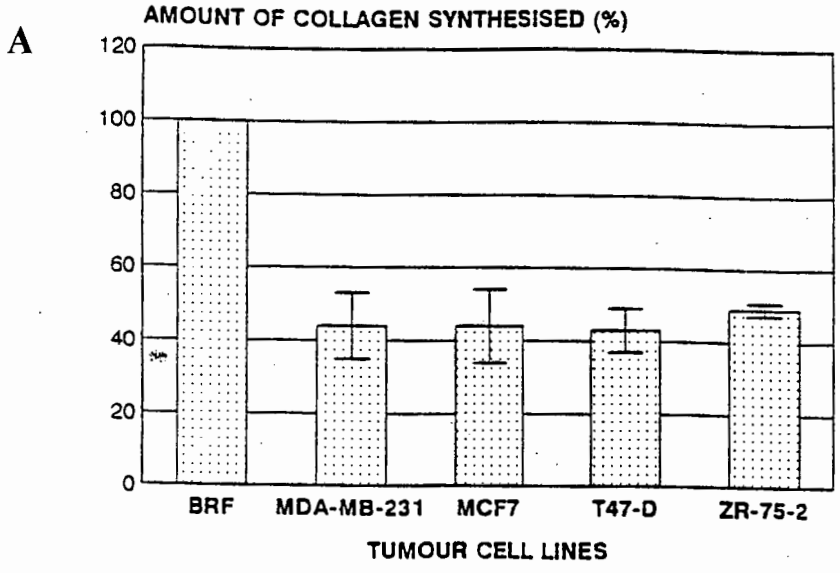


FIGURE 3.5

The effect of tumour cell conditioned media on type I collagen production in normal breast fibroblasts. Conditioned media from BRF fibroblasts and the different breast tumour cell lines (MDA-MB-231, MCF-7, T47D and ZR-75-2) were prepared as described in section 6.7 of Materials and Methods. 500µl of conditioned medium was incubated with 40 000 BRF fibroblasts for a total of 48 hours (see section 6.8). Total type I collagen was determined using the collagenase assay, as described in section 6.9. The results are expressed as the amount of type I collagen upon incubation of BRF fibroblasts in BRF conditioned medium as 100%. The conditioned media experiments were performed in triplicate. Values indicate means and standard deviations. **(A)** represents the amount of type I collagen harvested from the medium and **(B)** within the cells.



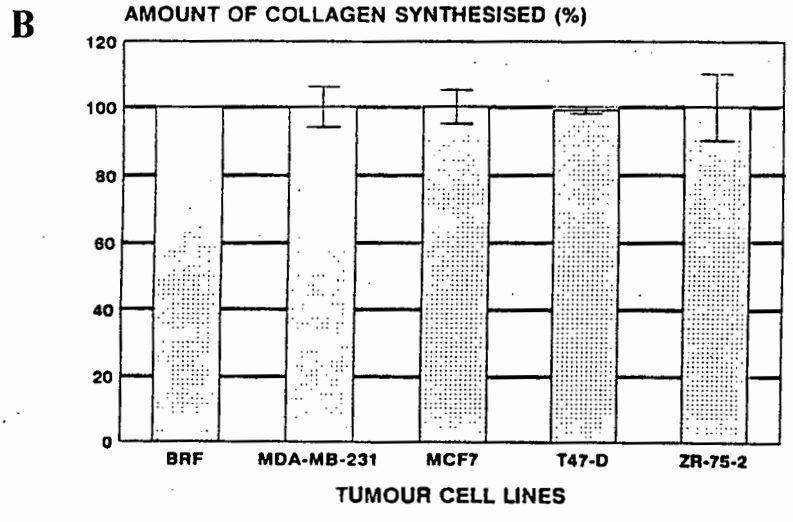


FIGURE 3.6

Type I collagen production in Breast fibroblasts co-cultured with Breast tumour cells. 30 000 breast tumour cells (MDA-MB-231, MCF-7, T47D or ZR-75-2) and 30 000 normal breast fibroblasts were co-cultured for 2 days in 24 well dishes in 500µl medium (see section 6.8.2) and labelled for 16 hours with ^{3}H-proline, prior to quantitation of type I collagen using the collagenase assay (see section 6.9). 30 000 BRF fibroblasts incubated on their own were set as a 100%. Values indicate means and standard deviations. **(A)** represents the amount of type I collagen harvested from the medium and **(B)** within the cells.

The co-culture experiments were repeated with the normal embryonic lung fibroblast cell line (WI-38) or a skin fibroblast cell line (FGo) in order to determine whether skin, lung and breast fibroblasts respond in the same way (figure 3.7). 30 000 cells of each cell type were co-cultured in 24-well dishes for 48 hours, as described for BRF fibroblasts. WI-38 fibroblasts in monoculture was used as a control. Co-culture with all the tumour cell lines caused a decrease in the amount of collagen produced by WI-38 fibroblasts (figure 3.7(A)), whereas when co-culturing the tumour cells with normal skin fibroblasts (FGo's) a different response was observed (figure 3.7(B)). The tumour cell line MDA-MB-231 had no effect on type I collagen by skin fibroblasts, whereas co-culture with MCF-7, T47D and ZR-75-2 actually resulted in increased type I collagen (45%, 12% and 23% respectively). These results are in agreement with studies by Noel et al (1992; 1993) and also suggest that fibroblasts of different origins do not respond in the same manner.

3.2.2.3. Effect of Tumour Cells on Type III Collagen

Type III collagen is often produced in conjunction with type I collagen, and in order to ascertain whether the effect was specific for type I collagen, samples were analysed for type III collagen on SDS polyacrylamide gels containing 10% β-mercaptoethanol (see section 6.6 in Materials and Methods). Type III collagen in WI-38 fibroblasts incubated with tumour cell conditioned media (MDA-MB-231, MCF-7 and ZR-75-2) were also decreased when compared to the fibroblasts incubated with WI-38 conditioned medium (figure 3.8(A)). Type III collagen from fibroblasts incubated with MDA-MB-231 conditioned medium was decreased by 95%, MCF-7 by 75% and ZR-75-2 by 69% (figure 3.8(B)). There clearly was no change in the proportion of type I to type III collagen. These results show that when WI-38 fibroblasts were incubated with tumour cell conditioned media there was a decrease in the levels of both types I and III collagens.

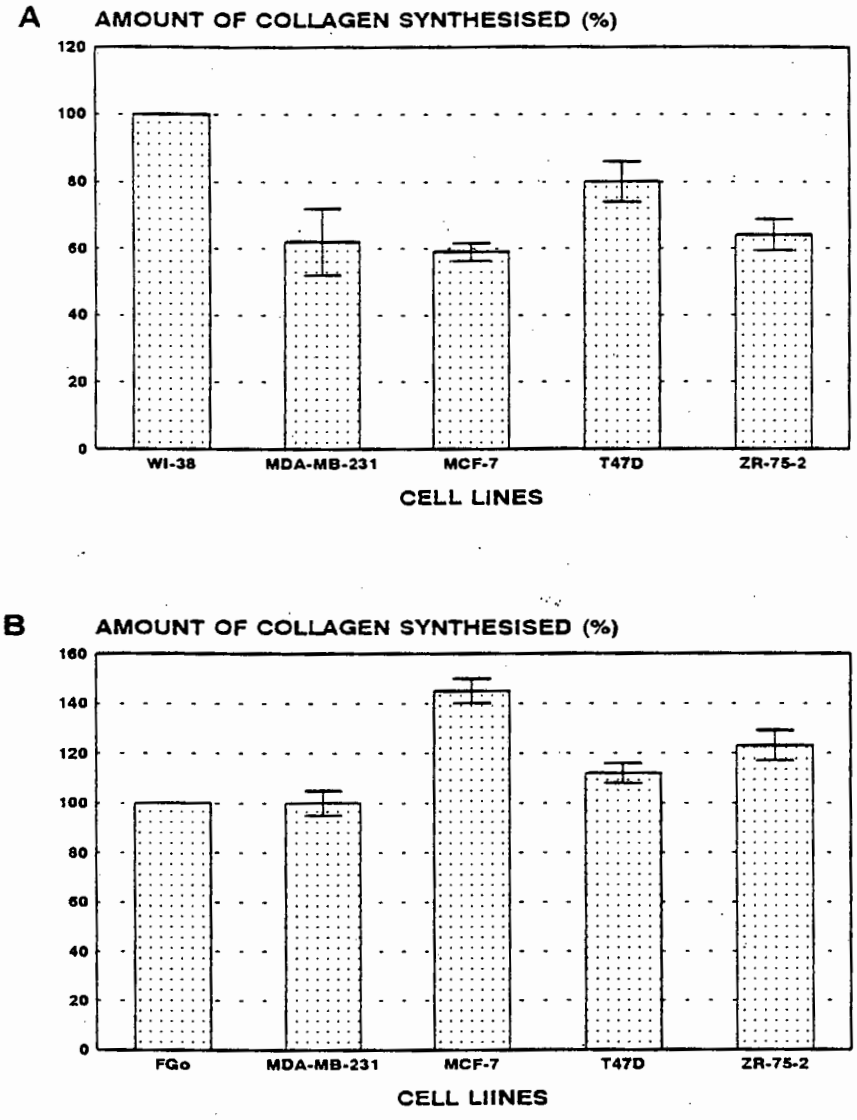


FIGURE 3.7

Type I collagen production in lung and skin fibroblasts co-cultured with breast tumour cells. Equal numbers (30 000) of the breast tumour cells MDA-MB-231, MCF-7, T47D or ZR-75-2 were incubated with normal WI-38 lung fibroblasts **(A)** or normal FGo skin fibroblasts **(B)** for 2 days at 37°C and labelled with ^{3}H-proline for 16 hours prior to performing the collagenase assay (see section 6.9 of Materials and Methods). WI-38 or FGo fibroblasts incubated on their own were set as 100%. All experiments were performed in triplicate. Values are the means and standard deviations.

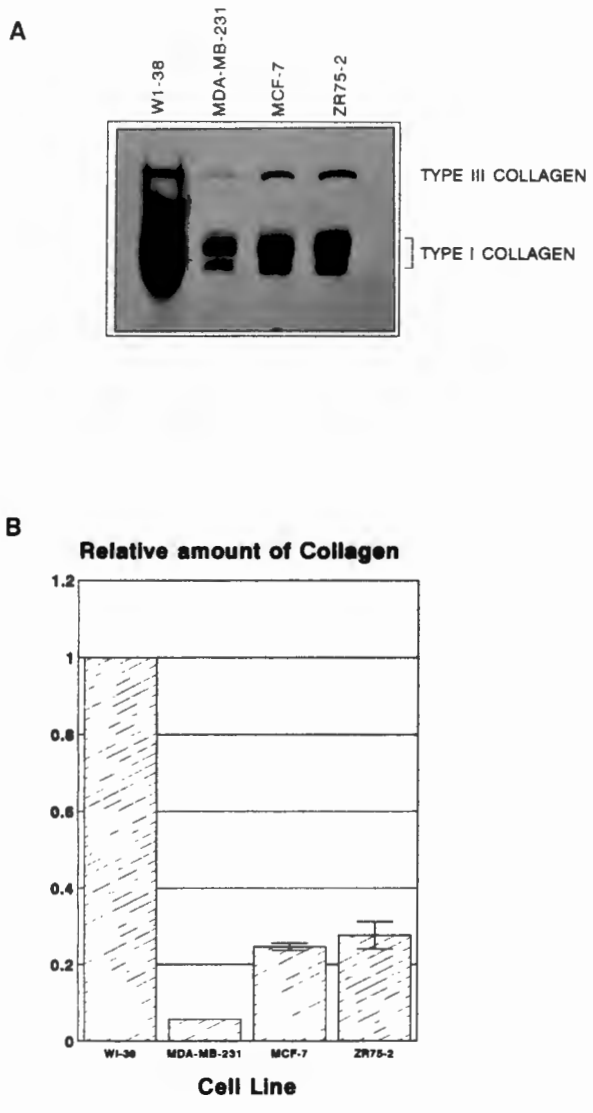


FIGURE 3.8

Analysis of Type III Collagen in WI-38 fibroblasts after incubation with tumour cell conditioned media. 300 000 WI-38 fibroblasts were grown to confluence in 6-well dishes. Tumour cell conditioned media was added 32 hours prior to the addition of ^{3}H-proline, βAPN and ascorbate (see section 6.6). After labelling for a further 16 hours, the collagen was harvested from the medium, the cells counted and radioactivity corresponding to 10^{5} cells was electrophoresed on a 6% polyacrylamide SDS gel containing 10% β-mercaptoethanol (section 6.6). **(A)**, lane 1; cells labelled in WI-38 conditioned medium, lanes 2, 3 and 4, cells labelled in MDA-MB-231, MCF-7 and ZR-75-2 tumour cell conditioned media respectively. The top band represents type III collagen and the lower bands are the α1(I) and α2(I) chains of type I collagen. The intensity of the bands were determined by densitometric scanning and the type III collagen in the tumour cell conditioned media was expressed as a % of the type III collagen in cells grown in WI-38 conditioned medium **(B)**.

3.2.3. Tumour cell conditioned media contains collagenase stimulatory activity

The tumour cell conditioned medium on its own and after incubation with normal breast or lung fibroblasts was analysed for type I collagenase activity. The medium was also analysed by Western blotting for the presence of the tumour cell collagenase stimulatory factor (TCSF/EMMPRIN) which has been shown to stimulate interstitial collagenase (Biswas 1984).

3.2.3.1. Detection of collagenase activity

Type I collagen was extracted from WI-38 cells after labelling with ^{3}H-proline (see section 6.6). 10 000 dpm of labelled collagen substrate was incubated overnight at 25°C with the different tumour cell conditioned media. After digestion, the products were analysed on SDS polyacrylamide gels (section 6.6). The tumour cell conditioned media (figure 3.9) did not contain any collagenase activity, as was also the case with the BRF fibroblast conditioned medium. The positive control was completely digested by the bacterial collagenase. Densitometric analysis of the fluorogram showed slight decreases in the intensity of the bands, but these decreases were not significant.

The tumour cell conditioned media clearly did not contain any collagenase activity (figure 3.9) but it is possible that collagenase activity can be induced in normal fibroblasts. The breast fibroblasts were therefore incubated in tumour cell conditioned medium for 48 hours before the assay in order to determine whether the tumour cell conditioned media could induce collagenase production by the fibroblasts (section 6.10). The results in figure 3.10 clearly show that tumour cell conditioned medium was capable of inducing collagenase activity in breast fibroblasts. ZR-75-2 tumour cells caused the highest stimulation of collagenase as can be seen from the total absence of collagen after the incubation period, whereas MCF-7 tumour cells caused the least stimulation. The 83 Kd and 27 Kd digested fragments of type I collagen were not detected, possibly due to further degradation by other proteolytic activities (eg gelatinase). The same results were obtained with WI-38 fibroblasts (data not shown).

3.2.3.2. Identification of the collagenase stimulatory factor

In an attempt to identify the factor secreted by the tumour cells, the tumour cell conditioned medium was subjected to Western blot analysis using the tumour cell collagenase stimulatory factor (TCSF) mouse antibody (see section 6.11 of Materials and Methods). This antibody was kindly provided by Professor Toole (Tufts University, Boston, Massachusetts). 20µl tumour cell conditioned media from MDA-MB-231, MCF-7, T47D, ZR-75-2, BE and Ht-29 tumour cell lines (see section 3.22 and 6.7) were electrophoresed on a 10% polyacrylamide SDS gel for 1 hour. The proteins were electroblotted onto Hybond C membranes (Amersham), blocked overnight, hybridised with the TCSF antibody as described in section 6.7 of Materials and Methods. A specific protein band of approximately 66 Kd, (figure 3.11) which is in agreement with the size of TCSF, was detected in the tumour cell conditioned media which suggested that the factor which stimulates collagenase in this system could very well be the TCSF discovered and isolated by Biswas and colleagues (Biswas 1982, 1984; Ellis et al, 1989).

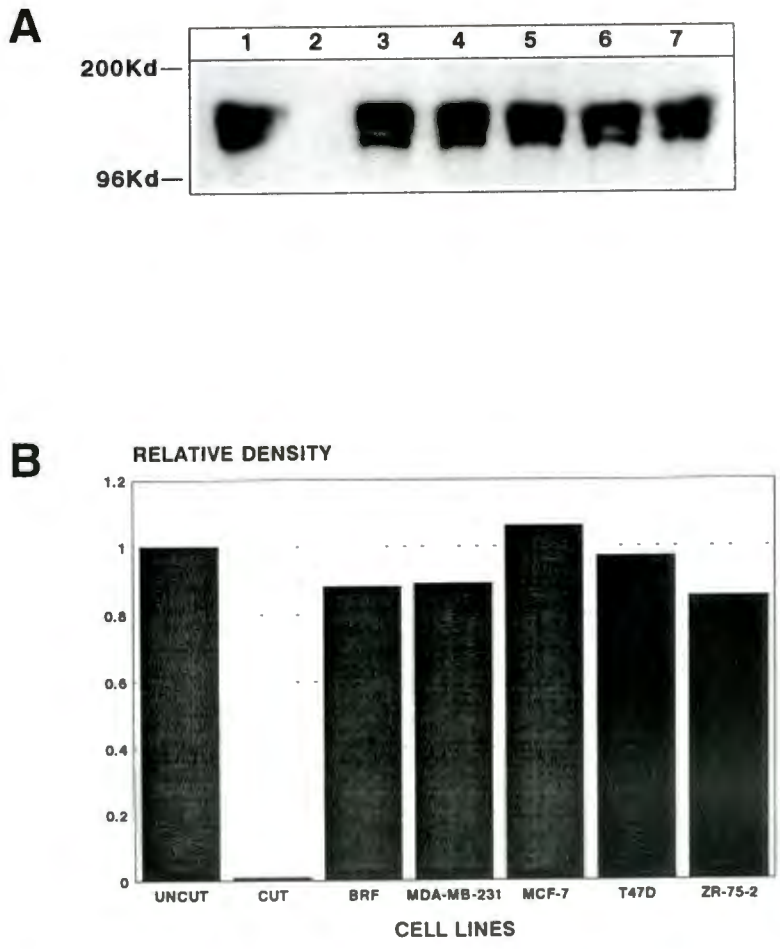


FIGURE 3.9

Absence of collagenase activity in tumour cell conditioned media. (A) ^{3}H-labelled type I collagen (10 000 dpm) was incubated with the indicated tumour cell conditioned media at 25°C for 16 hours (see section 6.10), after which the samples were electrophoresed on a 6% polyacrylamide SDS gel at 150 volts for 2 hours. The gel was soaked in Enhance™ for 1 hour, rinsed in distilled H_20 for another hour and exposed to X-ray film for 2 days. Lane 1; undigested collagen, lane 2; collagen digested with 2 units of bacterial collagenase, lane 3; collagen digested with breast fibroblast conditioned medium, lanes 4, 5, 6 and 7; collagen digested with MDA-MB-231, MCF-7, T47D and ZR-75-2 tumour cell conditioned media respectively. The sizes of the protein molecular weight markers are indicated on the left hand side of the gel. **(B)** shows the densitometric analysis of the gel in (A).

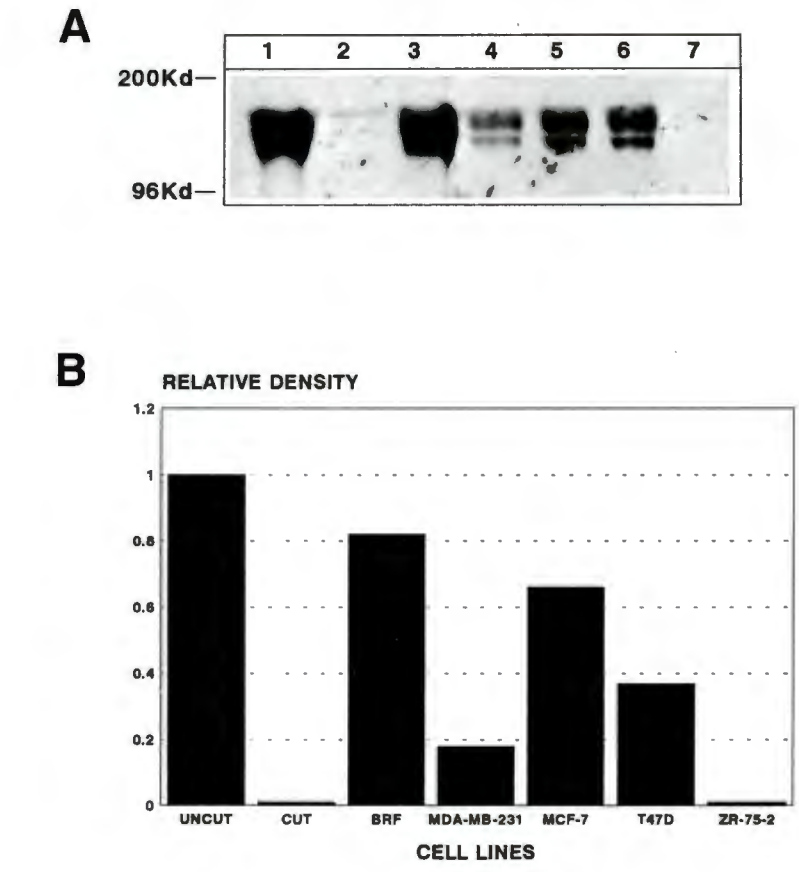


FIGURE 3.10

Stimulation of collagenase activity in breast fibroblasts by tumour cell conditioned media. (A) Tumour cell conditioned media was incubated with normal breast fibroblasts for 48 hours, and incubated with 10 000 dpm ^{3}H-labelled collagen at 25°C for 16 hours (section 6.10). Samples were analysed on a 6% polyacrylamide SDS gels and processed as described in the legend to figure 3.9. Lane 1; undigested collagen, lane 2; collagen digested with 2 units of bacterial collagenase, lane 3; collagen digested with BRF fibroblast conditioned medium, lanes 4, 5, 6 and 7; collagen digested with MDA-MB-231, MCF-7, T47D and ZR-75-2 respectively. The sizes of the protein marker is shown on the left hand side of the gel. The gels were scanned densitometrically and the results are shown in **(B)**.

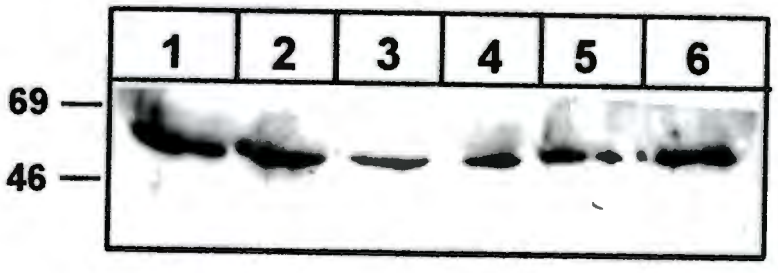


FIGURE 3.11

Detection of a Tumour Cell Collagenase Stimulatory Factor. Tumour cell conditioned media was electrophoresed on a 10% polyacrylamide SDS gel for 1 hour, electroblotted and hybridised to the TCSF antibody as described in section 6.11 of Materials and Methods. Lanes 1, 2, 3, 4, 5 and 6; tumour cell conditioned media from the tumour cell lines: MDA-MB-231, MCF-7, T47D, ZR-75-2, BE and Ht-29 respectively. A 66 Kd sized protein is shown which is of similar size to the TCSF/EMMPRIN. Protein molecular markers are shown on the left hand size of the gel.

3.3 DISCUSSION

The studies described in this chapter analysed the relationship between normal fibroblasts and breast tumour cells. Specifically, whether tumour cells can modulate the production of collagen in fibroblasts, and also whether this modulation is unique to breast fibroblasts. In attempting to analyse the complex relationship between tumour cells and host fibroblasts, we developed a normal breast fibroblast cell line which would be more appropriate than using fibroblasts of another origin. Primary breast fibroblast cultures were established and shown to be normal by karyotype analysis which was necessary because the tissue had been obtained from a ductal infiltrating carcinoma, excised at a distance from the tumour which consisted predominantly of fatty tissue. These fibroblasts were shown to produce type I collagen consisting of α1(I) and α2(I) collagen chains, in contrast to the breast tumour cells which did not produce any type I collagen.

It is uncertain why the particular breast cancer cells used in this study did not produce oncofetal/laminin binding (OF/LB) collagen, it might be due to the environment from which the tumour cells were originally excised. If the breast tumour cells (MDA-MB-231, ZR-75-2, T47D and MCF-7) did produce OF/LB collagen, our gel systems should have identified it because our system is the same as that utilised by Pucci-Minafra et al, (1993). The human breast carcinoma cell line (8701-BC) that produces OF/LB collagen was derived from a primary infiltrating ductal carcinoma, unlike the breast carcinoma cell lines utilised in this study where the epithelial cells were obtained from either ascite fluids or pleural effusions (Minafra et al, 1995; Soule et al, 1973 and Cailleau et al, 1974). This demonstrates the importance of variations in tissue culture systems and also questions the exact nature of the breast cancer cell line 8701-BC as to whether it is epithelial or perhaps it is a transformed fibroblast cell line. The fact that none of the epithelial cells (normal and tumour) in the in situ hybridisation studies (chapter two) produced type I collagen mRNA would substantiate the theory that epithelial cells are not meant to produce collagen and it is possible that when they are cultured in vitro, genes become switched on which in vivo are actually not activated.

Both the co-culture and conditioned media experiments showed that tumour cells were able to inhibit type I collagen in normal fibroblasts. One of the explanations, for the decrease in collagen, is that the tumour cells secrete a collagenase or induce the secretion of collagenases by fibroblasts. The stimulation of fibroblasts to produce collagenases by secreted tumour factors was initially demonstrated by Bauer et al (1979) and then by Biswas (1982; 1984). Ellis et al, 1989 called their factor the Tumour Cell Collagenase Stimulatory Factor which is now termed EMMPRIN (Biswas et al, 1995).

Tumour cell conditioned media incubated with breast fibroblasts generally caused a reduction in type I collagen in the medium. However, a larger decrease was observed when ZR-75-2 cells were in direct contact with breast fibroblasts, unlike the other tumour cell lines which did not require such interaction. This suggested that different tumour cells modulate collagen synthesis via different mechanisms, with some requiring direct interaction with fibroblasts for any significant effect to be achieved.

Co-culture experiments with the lung fibroblast cell line (WI-38) and the different tumour cell lines yielded results similar to those obtained with BRF fibroblasts. Tumour cell conditioned media incubated with WI-38 fibroblasts also resulted in a significant reduction in type III collagen levels. This was not totally unexpected since these two collagens are co-ordinately expressed as well as co-ordinately regulated in many cells (Noel et al, 1992).

In the FGo skin fibroblast cell line, however, co-culture of the MCF-7, T47D or ZR-75-2 tumour cell lines caused an increase in collagen synthesis, whereas MDA-MA-231 had no effect. These results are in agreement with those published by Noel et al, (1992 and 1993) who used the same cell lines to show that the stimulation in collagen synthesis was not related to the fibroblast proliferation rate and that the levels of steady-state collagen mRNA paralled that of the protein. The fact that fibroblasts of lung and breast origin were responsive to the tumour cells and produce collagenase, whereas skin fibroblasts were not, is in agreement with the work done by Biswas (1982) who showed that certain mouse fibroblasts are refractory to stimulatory agents

to produce collagenase and therefore cannot respond to the tumour cells as other fibroblasts (of rabbit and rat origin). These results indicate that "responsive" fibroblasts may not necessarily be species specific but tissue specific.

The intricate relationship between normal fibroblasts and breast tumour cells was assessed in order to gain better insight into the complicated process of tumourigenesis. In vivo studies, discussed in chapter two, demonstrated that cancer growth or progression is associated with decreased collagen gene expression. An in vitro system was therefore developed which allowed analysis of collagen by breast fibroblasts co-cultured with tumour cells. None of the tumour cells or tumour cell conditioned media resulted in an increase in collagen synthesis by breast fibroblasts (correlating with desmoplasia), an increase in collagen was only detected when skin fibroblasts (FGo's) were used, but the reason for this is unknown at this stage. The decrease in collagen types I and III (by conditioned medium and in the co-culture system) was thus shown to be due to induction of collagenase production by the fibroblasts and not because of increased secretion of collagenases by the tumour cells. It is possible that the decrease in collagen could be contributed to by a decrease in collagen mRNA, this is further investigated in chapter four.

CHAPTER FOUR

TUMOUR CELLS CAN MODULATE TYPE I AND III COLLAGEN mRNA IN NORMAL FIBROBLASTS

4.1 INTRODUCTION

Investigation into the interaction between normal breast fibroblasts and tumour cells, showed that tumour cells, either in co-culture with fibroblasts or by the addition of tumour cell conditioned media to normal fibroblasts, caused a decrease in collagen by the fibroblasts. Chapter three showed that normal fibroblasts were stimulated to secrete collagenases which resulted in degradation of collagen. It is also possible that the breast tumour cells, used in this study, also secrete the tumour cell collagenase stimulatory factor (TCSF or now termed EMMPRIN) into their medium, which upregulates the expression of the collagenase gene which then secretes excess collagenases to degrade the collagen (Prescott et al, 1989; Biswas et al, 1995). It has been postulated that EMMPRIN is an important regulator of tissue destruction during tumour cell invasion of the ECM (Biswas et al, 1995). What is not known is whether EMMPRIN affects the collagen gene itself causing a decrease in collagen mRNA levels which would also explain the decreased collagen synthesis. Understanding the modulation of collagen gene expression during the process of tumour cell invasion and metastasis would provide new insights into these complex processes. In situ hybridisation analysis (chapter two) suggested that the fibroblasts most affected during tumourigenesis were those in the vicinity of the tumour cells, whether fibroblasts have to be in physical contact with each other or whether factors are secreted which affect the collagen gene are discussed in this chapter.

The in vitro system established in our laboratory enabled us to investigate the levels of type I and III collagen mRNA when normal fibroblasts were incubated with tumour cells as well as with tumour cell conditioned media. When tumour cell conditioned media was added to the fibroblasts, type I collagen mRNA was shown to be increased or unaffected,

whereas type III collagen was either decreased or slightly increased. When the cells were co-cultured, both types I and III collagen mRNAs were decreased. This demonstrated that either the fibroblasts and the tumour cells required direct interaction in order to exert an effect on collagen mRNA or the "factor" that affected the collagen gene was lost during the preparation of the tumour cell conditioned media. To further analyse this "factor" the tumour cells and fibroblasts were cultured in the same medium but separated by a membrane to prevent direct interaction between the cells but still allowing for the exchange of any factors. In most cases types I and III collagen mRNA levels were unaffected, demonstrating that tumour cells require direct contact with the fibroblasts in order to affect collagen mRNA.

4.2 RESULTS

4.2.1 The Effect of Tumour Cells on Collagen Gene Expression in Normal fibroblasts

The modulation of collagen synthesis in BRF and WI-38 fibroblasts could involve a transcriptional mechanism, as there are many factors, such as cytokines, which are known to affect collagen gene expression. TGF-β1 is known to stimulate the production of collagen mRNA in cultured fibroblasts, whereas tumour necrosis factor-α (TNF-α) decreases the level of α1(I) collagen mRNA (Bornstein and Sage 1989). The question was presented as to whether tumour cells either indirectly (by secreting factors into the medium) or directly (being in contact with fibroblasts) modulate collagen mRNA production by normal fibroblasts. MDA-MB-231, MCF-7 and ZR-75-2 breast cancer cell lines, normal lung fibroblasts (WI-38), and the colorectal cancer cell lines BE and HT-29 were used to prepare conditioned media as described in section 6.7.

The modulation of collagen mRNA by tumour cells was analysed using three approaches: 1) conditioned media, 2) co-culture experiments and 3) culturing the cells in the same medium but separating them by a permeable membrane.

4.2.1.1. The effect of tumour cell conditioned media on type I and III collagen mRNA

Monolayer cultures containing breast or WI-38 fibroblasts ($2X10^6$ cells/100mm dish) were incubated with conditioned medium for 48 hours, followed by extraction of the RNA (see section 6.1.2). 5μg of total RNA was electrophoresed on a 1% agarose gel containing 8% formaldehyde, transferred to nylon membranes and hybridised with a mixture of either ^{32}P-labelled α2(I), α1(I) or α1(III) collagen (Hf 934, Chu et al, 1985) and β-actin cDNA probes. Breast or WI-38 fibroblasts cultured in fibroblast conditioned medium was used as a control. The addition of MDA-MB-231 and ZR-75-2 tumour cell media resulted in a 45% and by 55% increase in α2(I) collagen mRNA, while no effect was observed with MCF-7 conditioned medium (figure 4.1A). Type III collagen was found to be affected differently, MDA-MB-231 conditioned medium caused a decrease of 16%, ZR-75-2

conditioned medium a 31% decrease whereas MCF-7 increased α1(III) collagen mRNA by 14% (figure 4.1B). The membranes were scanned densitometrically using β-actin as the internal standard and the results for type I and III collagen are shown in figure 4.1C and D respectively.

This increase in type I collagen mRNA in the conditioned media experiments is in contradiction to the decrease observed at the protein level (chapter three). A possible explanation for this observation include the existence of a positive feedback loop whereby the synthesis of collagen mRNA is stimulated in response to the decrease in protein. As to why type III collagen mRNA is not affected in the same way as type I collagen mRNA is not easily addressed. These two interstitial collagens are often regulated in the same way for many different diseases (eg breast cancer, specifically desmoplasia) although they are situated on two different genes (Vuorio and de Crombrugghe 1990).

4.2.1.2. The effect of tumour cells on types I and III collagen mRNA production in normal fibroblasts

Tumour cell conditioned medium was shown to increase or have no effect on collagen gene expression (section 4.2.1.1) whilst the level of collagen protein produced by the fibroblasts incubated with the tumour cell conditioned media was decreased (chapter three). It is possible that the fibroblasts require contact with the tumour cells for the levels of collagen mRNA to be decreased and therefore WI-38 fibroblasts were co-cultured with breast or colon tumour cells. After 48 hours the cells were harvested for RNA extraction as described in section 6.1.2 of Materials and Methods. 5μg of total RNA was electrophoresed on an 1% agarose gel containing 8% formaldehyde, transferred to a nylon membrane and hybridised with all three collagen probes as well as β-actin. WI-38 fibroblasts incubated on their own was used as a control and as can be seen the α2(I) collagen mRNA was decreased when co-cultured with MDA-MB-231, MCF-7, T47D, ZR-75-2, BE and HT-29 tumour cell lines (decreases of 80%, 52%, 66%, 75%, 78% and 60% respectively) whereas co-cultured with the normal breast epithelial cell line (MCF-

12F) did not change the levels of type I or type III collagen mRNA (figure 4.2A). Type III collagen mRNA was also found to be decreased; MDA-MB-231 (75%), MCF-7 (66%), T47D (63%), ZR-75-2 (75%), BE (75%) and HT-29 (33%) in line with the decreases observed for type I collagen mRNA (figure 4.2B). The levels of β-actin mRNA, remained unchanged, demonstrating that that the effect was specific for collagen. All northern blots were hybridised with an α1(I) collagen cDNA probe and the same results were found as that for α2(I) collagen mRNA (data not shown).

These results showed that the tumour cells either needed to be in direct contact with the fibroblasts for collagen mRNA to be decreased, or that the "factor" that affected collagen gene expression was lost during preparation of the conditioned media.

4.2.1.3. Search for a diffusible factor affecting types I and III collagen gene expression

In order to investigate whether tumour cells required specific contact with the fibroblasts to modulate collagen gene expression, Transwell dishes (Costar) were used. These dishes allowed the cells to share the same medium without actually being in contact with each other. 300 000 WI-38 fibroblasts were plated in the dish and 300 000 tumour cells were plated on top of the fibroblasts on a permeable membrane in the same well, so that the tumour cells were not in physical contact with the fibroblasts. After 48 hours, the fibroblasts were washed with PBS and the RNA extracted as described in section 6.1.2. WI-38 fibroblasts plated with WI-38 fibroblasts were used as a positive control. 5μg of total RNA was electrophoresed on a 1% agarose gel containing 8% formaldehyde, transferred to a nylon membrane and hybridised with a mixture of α1 (I), α2(I) or α1 (III) collagen probes and β-actin. α1(I) collagen mRNA results were the same as those for α2(I) collagen mRNA (data not shown). The breast tumour cell line T47D affected the fibroblasts the most, causing a 30% decrease on α2(I) collagen mRNA, MDA-MB-231 decreased α2(I) collagen mRNA by 8% (figure 4.3A). ZR-75-2 and the colon tumour cell line HT-29 had no effect on type I collagen mRNA. The breast tumour cell lines MDA-

MB-231 and ZR-75-2 increased type III collagen mRNA by 11% and 27% respectively, whereas T47D and HT-29 tumour cells decreased the collagen mRNA by 23% and 15% respectively (figure 4.3B). These results indicate that different tumour cell lines are capable of modulating collagen mRNA in different ways. A summary of these results is presented in Table IV.

TABLE IV: Summary of the interactions between tumour cells or tumour cell conditioned media and fibroblasts on collagen mRNA levels.

CELL LINE	CONDITIONED MEDIUM		CO-CULTURE		DIFFUSIBLE MEMBRANE	
	Type I	Type III	Type I	Type III	Type I	Type III
MDA-MB-231	↑ 45%	↓ 16%	↓ 80%	↓ 75%	↓ 8%	↑ 11%
MCF-7	no change	↑ 14%	↓ 52%	↓ 66%	ND	ND
T47D	ND	ND	↓ 66%	↓ 63%	↓ 30%	↓ 23%
ZR-75-2	↑ 55%	↓ 31%	↓ 75%	↓ 75%	no change	↑ 27%
BE	ND	ND	↓ 78%	↓ 75%	ND	ND
HT-29	ND	ND	↓ 60%	↓ 33%	no change	↓ 15%

Where ND = not done

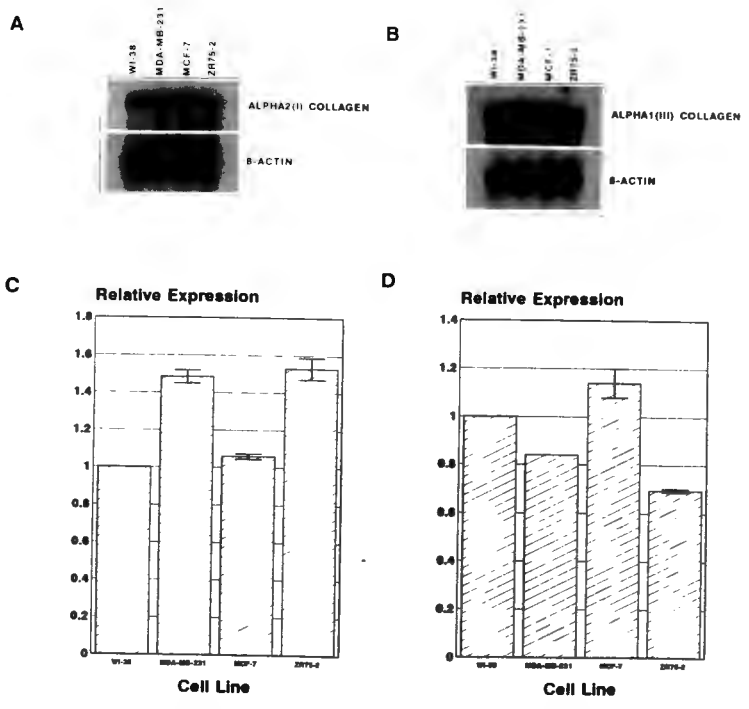


FIGURE 4.1.

Analysis of α2(I) and α1(III) collagen mRNA in fibroblasts incubated with tumour cell conditioned media. Total RNA was extracted from $2X10^6$ WI-38 fibroblasts incubated with tumour cell conditioned media for 48 hours (section 6.1.2). 5μg of total RNA was electrophoresed on a 1% agarose gel containing 8% formaldehyde, transferred to nylon membranes and hybridised with α2(I) collagen and β-actin cDNA probes **(A)**. Lane 1 is RNA from WI-38 conditioned medium, lane 2 MDA-MB-231, lane 3 MCF-7 and lane 4 ZR-75-2. The membrane was stripped and reprobed with β-actin and α1(III) collagen probes as shown in **(B)**. Both membranes were scanned densitometrically and these results are shown in **(C)** and **(D)**. All experiments were performed in triplicate.

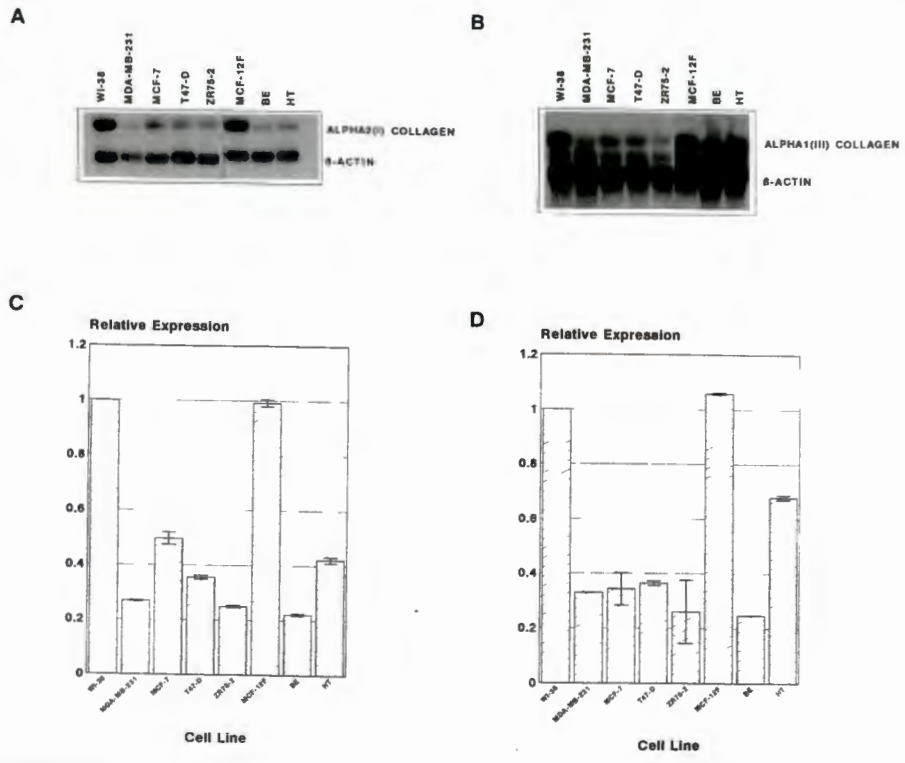


FIGURE 4.2

Analysis of α2(I) and α1(III) collagen mRNA in fibroblasts co-cultured with tumour cells. $2X10^6$ WI-38 fibroblasts were co-cultured with $1X10^6$ breast or colon cancer cells, total RNA was extracted and electrophoresed on a 1% agarose gel containing 8% formaldehyde, transferred to nylon membranes and hybridised with ^{32}P-labelled α2(I) or α1(III) collagen and β-actin cDNA probes as described in the legend to figure 4.1 and sections 6.1.2 and 6.2 [**(A)** α2(I) collagen mRNA and **(B)** α1(III)]. Lane 1; WI-38 fibroblasts co-cultured with WI-38 fibroblasts, lanes 2, 3, 4, 5 is RNA from WI-38 fibroblasts co-cultured with the breast tumour cell lines (MDA-MB-231, MCF-7, T47D and ZR-75-2 respectively); lane 6; RNA from fibroblasts co-cultured with the normal breast epithelial cell line MCF-12F while lanes 7 and 8 contain RNA from fibroblasts co-cultured with the colon cancer cell lines BE and HT-29. The gels were exposed to X-ray film for 8 hours after which they were scanned densitometrically [**(C)** and **(D)**]. All experiments were performed in triplicate.

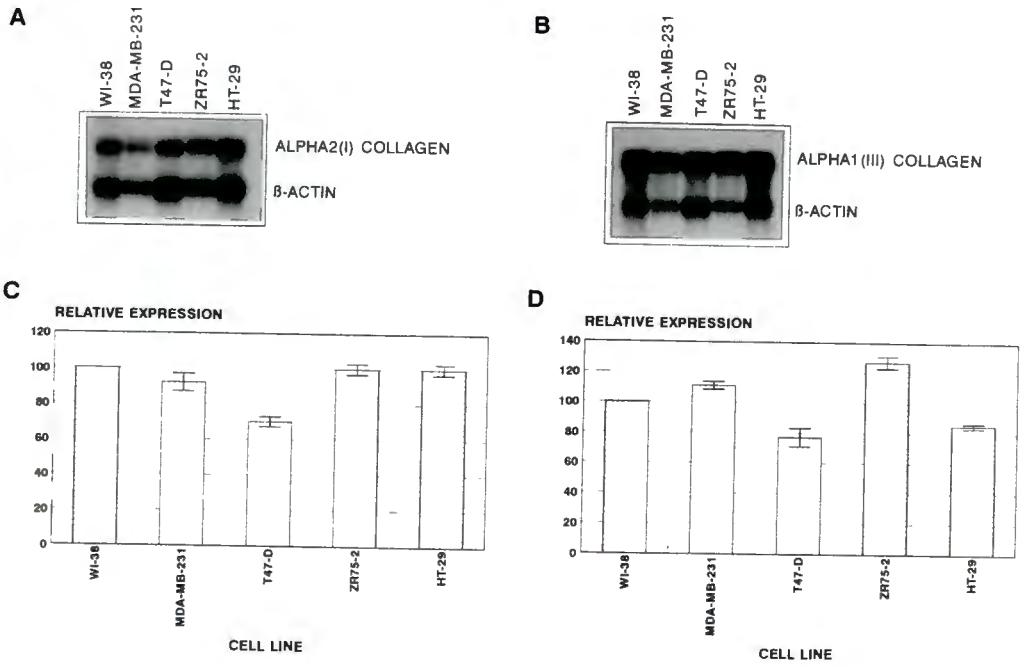


FIGURE 4.3.

Analysis of α2(I) and α1(III) collagen mRNA in fibroblasts co-cultured but physically separated from tumour cells. 300 000 WI-38 fibroblasts were plated in Transwell tissue culture dishes (Costar) and incubated with breast or colon tumour cells for 48 hours (as described in section). Total RNA was extracted (section 6.1.2), of which 5μg was electrophoresed on a 1% agarose gel containing 8% formaldehyde, transferred to nylon membranes and hybridised with a mixture of ^{32}P-labelled α2(I) or α1(III) collagen and β-actin cDNA probes; [**(A)** and **(B)**]. Lane 1; WI-38 fibroblasts cultured with WI-38 fibroblasts; lanes 2, 3, 4 and 5 is RNA from the tumour cell lines MDA-MB-231, T47D, ZR-75-2 and HT-29 incubated with WI-38 fibroblasts. The gels were exposed to X-ray film for 8 hours after which they were scanned densitometrically [**(C)** and **(D)**]. All experiments were performed in triplicate.

4.3 DISCUSSION

As mentioned previously, the decrease in collagen (chapter three), apart from degradation by collagenases, could also be due to a decrease in collagen mRNA levels. Addition of tumour cell conditioned media to fibroblasts did not cause the expected decrease in type I collagen mRNA levels, in fact an increase was detected with the breast tumour cell lines MDA-MB-231 and ZR-75-2. This effect was totally unexpected, not only because there had been a decrease in collagen protein (which admittedly could be explained by collagenases) but also from work done by Noel et al (1992) who found an increase in types I and III collagen in conditioned media when skin fibroblasts were co-cultured with the tumour cell lines MCF-7, SA52 and T47D with a concomitant increase in the levels of types I and III collagen mRNA. It is possible that the fibroblasts of breast and lung origin responded differently to skin fibroblasts. It could also be that the breast or lung fibroblasts require direct interaction with the breast tumour cells or that another factor was involved (other than EMMPRIN) that had been lost in the preparation of the conditioned media. Why collagen mRNA from fibroblasts of different origins were regulated in a different manner is not clear at this stage.

Type III collagen mRNA, on the other hand, was shown to be either decreased (by MDA-MB-231 and ZR-75-2) or increased (by MCF-7) which agrees with the work done by Noel et al, 1992. Both studies demonstrated that the different collagens were regulated differently, furthermore, other factors (such as the heterodimeric CCAAT factor) have also been shown to control the α1(III) gene but not the type I collagen gene (Vuorio and de Crombrugghe 1990). Studies have shown that there is often a co-ordinate decrease in the levels of α2(I) and α1(III) collagen RNA in transformed cells (Liau et al, 1985). However, in one mouse cell line, transformed by Rous sarcoma virus, α1(III) collagen mRNA showed a 4-fold increase while the α2(I) collagen mRNA was decreased 7-fold. These results demonstrated that, in most cases types I and III collagen mRNA is often co-ordinately expressed, but there are certain circumstances where this is not the case (Liau et al, 1985). Three mechanisms were suggested to explain this inhibition of type I collagen

gene transcription, they included the existence or presence of a repressor, an inactive activator and thirdly a change in the conformation of chromatin around the genes that are inhibited in transformed cells, preventing the activation of these genes (Liau et al, 1985).

When the fibroblasts were co-cultured with the breast or colon tumour cells, types I and III collagen mRNA were decreased by all the tumour cell lines. These results indicated, that the cells required either direct interaction or alternatively, the factor that affects collagen gene expression was lost during preparation of the tumour cell conditioned media. To answer this question, the cells were separated from each other by a membrane such that any diffusible factor(s) secreted by the tumour cells would be able to act on the fibroblasts. In this instance, type I collagen mRNA was decreased by tumour cell lines MDA-MB-231 (8%) and T47D (30%), compared to 80% and 66% respectively when the cells were co-cultured with the fibroblasts. ZR-75-2 and HT-29 had no effect on type I collagen mRNA, whereas decreases of 75% and 60% were obtained upon co-culture.

MDA-MB-231 and ZR-75-2 breast tumour cell lines, when cultured separately from the fibroblasts, increased type III collagen mRNA by 11% and 27% respectively, whereas decreases were obtained when the cells were cocultured (75% and 75%). T47D and HT-29 had no effect on type III collagen mRNA but decreases of 63% and 33% were detected in co-culture. These results demonstrate that, in most cases, a difusible factor was not involved but that the tumour cells required contact with the fibroblasts to decrease collagen mRNA.

It was therefore shown that a) types I and III collagen mRNAs were affected differently even though type I collagen and III was affected in the same way; b) different tumour cells (breast and colon) affect collagen mRNA differently and c) tumour cells modulate type I collagen using two different mechanisms; one causing the degradation of collagen and the other affecting collagen mRNA steady state levels, both of which would aid in tumour cell invasion and metastasis. The one mechanism involved secretion of factors which caused

the degradation of collagen whereas the other mechanism required direct interaction of fibroblasts and tumour cells.

It has been shown that skin fibroblasts, when cultured in a three-dimensional environment consisting mainly of collagen I fibrils, synthesize reduced levels of types I and III collagen as well as reduced levels of steady-state collagen mRNA (Mauch et al, 1988). The reduced steady-state levels were found to be partly due to reduced de novo transcription of the collagen I gene and in part to a reduction in pro α1(I) collagen mRNA stability (Eckes et al, 1993). There was also an increase in collagenase gene expression and all this data was found to be dependent on the contact of fibroblasts in a three-dimensional collagenous matrix (Mauch et al, 1989). This interaction between fibroblasts and the three-dimensional lattice has subsequently been shown to trigger tyrosine phosphorylation of pp120 which is identical to $pp125^{FAK}$, a novel tyrosine kinase localised in focal adhesions (Roeckel and Krieg 1994). This phosphorylation has been shown to be mediated by the integrin $\alpha_2\beta_1$ which presumably provides the structural link between the ECM and the cytoskeleton or in this case the fibroblasts and the collagen lattice (Klein et al, 1991; Schiro et al, 1991). It has been suggested that the phosphorylation event promotes alterations in cellular morphology, including formation and maintenance of focal contacts and cell spreading, all of which might influence gene expression (Roeckel and Krieg 1994). Recent data have shown that the integrin $\alpha_1\beta_1$ is involved in the down regulation of collagen gene expression, whereas the $\alpha_2\beta_1$ integrin mediates induction of collagenase (MMP-1). With the use of signal transduction inhibitors, it was shown that different signaling pathways are involved in the decrease in collagen mRNA and MMP-1. It was shown that induction of MMP-1 is mediated by tyrosine-specific protein kinases, the mechanism of down regulating type I collagen mRNA still needs to be elucidated (Langholz et al, 1995).

Data from this chapter, specifically the fact that tumour cells directly interact with fibroblasts to affect gene expression, implies that tumour cells may mimic the environment created by the three-dimensional collagen gel system and in binding to the fibroblasts via

specific receptors (possibly integrin(s)), certain signal transduction pathways are activated causing a reduction in collagen gene expression and an increase in collagen degradation.

CHAPTER FIVE

CONCLUSION

Tumour cell invasion and metastasis depend on the degradation of the collagen matrix which would normally maintain the integrity of the tissue or organ. The degradation of the extracellular matrix (ECM) in highly metastatic tumours is associated with high levels of collagenases (Stetler-Stevenson et al, 1993). Not much is known about collagen gene expression in fibroblasts present in the ECM surrounding the tumours. Type I collagen mRNA levels were therefore analysed in various stages of breast and colon cancer tissue since the disease is relatively common in the South African population and tissue is readily available.

Analysis of RNA levels in breast tumours and the adjacent normal tissue by Northern hybridisation indicated that stage I breast tumours had increased collagen mRNA compared to the adjacent normal tissue, while in stages II and III breast tumours it was decreased. These results were confirmed by non-radioactive in situ hybridisation which showed that stage I breast tumour cells had increased collagen mRNA levels, whereas stage II and III tumour cells had decreased expression (when compared to the adjacent normal cells). This stage-specific phenomenon was found not only to occur in breast cancer but also in colorectal cancer. These results showed high levels of collagen mRNA in the early stages of cancer and decreased or negligible expression as the tumours advanced to the later stages. This study showed that the stromal fibroblasts and not the tumour cells, were producing excess collagen mRNA (correlates with desmoplasia) in the early stages of the disease whereas fibroblasts in the later disease stages were synthesising collagen mRNA at reduced rates. One can hypothesize that excess collagen acts as a barrier against tumour cell invasion (in this way protecting the host) in the early stages of cancer but as the tumour progresses and becomes more aggressive, collagen mRNA production is switched off, enabling the tumour cells to migrate and metastasize. In situ hybridisation analysis also indicated that fibroblasts in the direct vicinity of the tumour cells were most affected, suggesting that tumour cells can modulate or control collagen

mRNA production by the fibroblasts to aid the process of tumour cell invasion and metastasis.

In order to analyse the relationship between normal fibroblasts and breast tumour cells, primary breast fibroblasts were co-cultured with MDA-MB-231, MCF-7, T47D or ZR-75-2 breast tumour cells, and as additional endeavor, normal cells were incubated with tumour cell conditioned media. The tumour cells and the tumour cell conditioned media caused a decrease in collagen protein levels and moreover type III collagen was found to be regulated in the same way as type I collagen (also decreased as demonstrated by interrupted gel electrophoresis). Fibroblasts of different origins responded differently to tumour cells; in this study skin fibroblasts when co-cultured with various tumour cell lines produced excess collagen, on the other hand, breast and lung fibroblasts produced less collagen. This indicated that collagen production in fibroblasts depends on the tissue of origin.

The decrease in collagen levels was analysed at two levels: degradation (by the production of collagenases) and decreased collagen mRNA production. Tumour cell conditioned media was found to be incapable of degrading type I collagen, demonstrating that the tumour cells themselves were not responsible for degrading type I collagen (ie that they did not secrete interstitial collagenases). When tumour cell conditioned media was incubated with normal fibroblasts, however, type I collagen was digested, indicating that the tumour cells secreted a factor(s) that stimulated the fibroblasts to produce collagenases. The collagenase could then degrade the collagen secreted by the fibroblasts. This factor was shown by Western blotting to be of similar molecular weight to the already known TCSF or EMMPRIN discovered by Biswas (1982, 1984), Ellis et al (1989) and Biswas et al (1995).

Collagen mRNA levels were also analysed in order to ascertain whether the decrease in collagen was also due to a decrease in mRNA. In these studies, normal fibroblasts were incubated with breast tumour cell conditioned media which resulted in an increase in $\alpha 2(I)$

collagen mRNA with MDA-MB-231 conditioned medium of 45% and ZR-75-2 conditioned medium of 55%, conditioned medium from MCF-7 cells had no effect. Similar results were obtained for α1(I) collagen mRNA. For type III collagen mRNA, however, MDA-MB-231 and ZR-75-2 conditioned media caused a decrease whilst MCF-7 conditioned medium had no effect. The reason for the difference in types I and III collagen mRNA levels is unknown, but it could be that the factor(s) in the tumour cell conditioned media also ablates co-ordinate regulation of these genes. Such lack of co-ordinate regulation of the types I and III collagen genes has been documented (Vuorio and de Crombrugghe 1990). It is also important to point out that even though types I and III collagen are often produced in the same tissue, they are not produced at the same levels for example, type III collagen is not present in bone whereas type I collagen is the most abundant protein.

The increase in type I collagen mRNA, especially when a decrease in type I collagen had already been demonstrated, was surprising. If EMMPRIN was involved in the down regulation of type I collagen, a decrease rather than an increase in collagen mRNA would have been detected. It is quite conceivable therefore that there was another factor involved in the down regulation of collagen mRNA and that it was lost during preparation of the conditioned media or that cell-cell contact was required. All the tumour cells were shown to decrease type I and III collagen mRNA production in co-culture experiments. Culture of the tumour cells with the fibroblasts in the medium separated by a membrane confirmed that the cells required some form of contact in order to decrease collagen gene expression. As to why the different tumour cells affected collagen mRNA levels in different ways is not quite clear, but this phenomenon has also been shown in other studies (Noel et al, 1992 and 1993). These tissue culture results indicated that fibroblasts require direct contact with tumour cells for there to be a substantial decrease in collagen gene expression, which would then explain the decrease in collagen detected by the collagenase assay. These results also support the in situ hybridisation data presented in this thesis which indicated that collagen gene expression was shut down only in the fibroblasts in the direct vicinity of the tumour cells.

How tumour cells interact with fibroblasts (ie via integrins or cadherins) and how they bring about a decrease in collagen gene expression is unknown at this stage. The increase in collagen, in the early stages of disease, possibly acts as a barrier for tumour progression and invasion and could be a host response to increased cell mass. The tumour cells then respond to this increase in two ways; first secretion of collagenases and secondly (or concurrently) by switching off collagen mRNA production in the fibroblasts. Being capable of modulating normal fibroblast functions, such as increasing the production of collagenases or switching off collagen gene expression, must surely aid in the process of tumour cell invasion and metastasis. This ability of tumour cells to shutdown type I collagen gene expression opens up a vast area of research which can be exploited from a potential therapy point of view. For instance, if tumour cells could be prevented from switching off collagen mRNA, the process of invasion and metastasis may be slowed down or inhibited. What now needs to be established is the signal transduction pathway which is utilised by the tumour cells in order to exert their effect on collagen production by the fibroblasts.

CHAPTER SIX

MATERIALS AND METHODS

6.1 ISOLATION OF RNA

6.1.1 Extraction of RNA from Tissue

RNA was extracted essentially as described by Chomczynski and Sacchi (1978). 1g of breast tissue was homogenised with 1ml of solution D (section 6.15) using an ultra turrex. The homogenate was transferred to a 10ml falcon tube, to which 100µl of 2M Na acetate pH 4, 1ml H_20 saturated phenol and 200µl chloroform:isoamyl alcohol (49:1) was added. This was followed by thorough mixing and placed on ice for 15 minutes. After centrifugation at 10 000 rpm in a JA 20.1 rotor for 20 minutes at 4°C, the aqueous phase was removed and transferred to a fresh tube. 1ml of isopropanol was added and the RNA was left to precipitate at -20°C overnight. The RNA was pelleted by centrifugation at 10 000 rpm for 20 minutes, the supernatant discarded and the pellet resuspended in 300µl of solution D. The RNA was again precipitated by addition of an equal volume of isopropanol and left at -20°C for 1 hour. The RNA was pelleted at 10 000 rpm for 10 minutes at 4°C, washed twice with 70% ethanol, dried under vacuum and resuspended in 50µl diethyl pyrocarbonate (DEPC) treated water (section 6.15).

The RNA concentration was determined spectrophotometerically and an aliquot of the sample was electrophoresed on a 1% agarose gel containing 8% formaldehyde to check the quality of the RNA.

6.1.2. Extraction of RNA from Cultured Cells

WI-38, FGo's or breast fibroblasts and MDA-MB-231, MCF-7, T47D, ZR-75-2, BE or HT-29's tumour epithelial cells were cultured in 6 well dishes in Minimal Essential Medium (MEM) supplemented with 10% heat inactivated foetal calf serum, 100 µg/ml penicillin and 100 units/ml streptomycin at 37°C. To harvest the cells, the culture medium was removed, the cell layer rinsed with Phosphate Buffered Saline Solution (section 6.15) and 1ml of solution D was added to the cells followed by mixing with

gentle circular movements for about 2 minutes. The solution became thick and viscous and was transferred to 10ml plastic falcon tubes to which was added 100µl of 2M Na acetate (pH 4.0), 1ml water saturated phenol and 200µl chloroform:isoamyl alcohol (49:1). This mixture was shaken for 10 seconds and placed on ice for 15 minutes. After centrifugation at 10 000 rpm in a JA 20.1 rotor for 20 minutes at 4°C, the aqueous layer was removed and transferred to a fresh tube, to which an equal volume of isopropanol was added. The RNA was precipitated at -20°C overnight and pelleted by centrifugation at 10 000 rpm for 20 minutes. The pellet was washed twice with 70% ethanol, dried under vacuum for 5 minutes and resuspended in 20-50µl DEPC treated water. The concentration and quality of the RNA was determined spectrophotometrically whereby $1A_{260} = 40\mu g$ RNA.

6.2. NORTHERN BLOTTING AND HYBRIDISATION

5-10µg of RNA was added to 16.5µl RNA loading buffer (section 6.15) and heated for 10 minutes at 55°C. The RNA was electrophoresed on a 1% agarose gel containing 8% formaldehyde at 30mA for 2-3 hours in RNA running buffer (section 6.15). The RNA was blotted onto Hybond N membranes (Amersham) for 16-18 hours. After blotting the gel was stained with ethidium bromide to check for transfer of RNA onto the membrane. The membrane was rinsed briefly in 6X SSC (section 6.15) and crosslinked using a UV crosslinker (Spectro-linker, Spectronics corporation) at optimal crosslinking conditions before placing it in a sterile glass bottle for prehybridisation.

The membrane was prehybridised for 4 hours at 42°C in prehybridisation buffer (section 6.15) in a Hybaid rotary oven. The probe was labelled by nick-translation as specified by the suppliers (Boehringer Mannheim). Prior to use, the probe was denatured at 95°C for 10 minutes and placed on ice for 5 minutes. 1-2 million cpm probe was added per ml of prehybridisation mixture and allowed to hybridise for 16-18 hours at 42°C. After hybridisation, the membrane was washed twice for 15 minutes at

room temperature in 2X SSC, 0.1% SDS followed by two washes for 15 minutes each at 65°C in 0.1X SSC, 0.1% SDS. The membrane was placed in a plastic bag, sealed and exposed to X-ray film at -70°C for 16-18 hours.

6.3. IN SITU HYBRIDISATION

Slides were soaked overnight in 250ml chromic acid (section 6.15), rinsed 5 times in distilled water, and then placed in distilled water for 30 minutes. The water was decanted and the slides left in 250ml acetone for 30 minutes. 5ml of 3-aminopropyltriethoxy-silane was added to 250ml fresh acetone which was then poured onto the slides and stirred for 30 minutes. The solution was decanted and the slides were placed at 80°C for 30 minutes. The slides were rinsed, left in DEPC treated water for 2 hours and placed at 80°C overnight to dry. The slides were then placed in a plastic packet with silicon crystals and sealed until further use.

Formalin fixed tissue sections were cut into 4 micron sections using a microtome, placed on slides and allowed to dry overnight at 65°C. The sections were stored in plastic packets until further use.

Fragments to be used as riboprobes, as well as the cloning vector pGEM3, were digested overnight with the appropriate restriction enzymes at 37°C. The digested samples were heated at 60°C for 10 minutes and subsequently electrophoresed on a 0.8-1% low melting agarose gel in TAE buffer (section 6.15) at 30mA for 3-4 hours. The desired fragment and pGEM3 were excised from the gel and and the agarose melted at 70°C for 10 minutes (Current Protocols in Molecular Biology 1995). Various amounts of insert and vector slices were combined in fresh microfuge tubes (total volume of 9µl) and placed at 37°C for an additional 10 minutes. 2 units T4 DNA ligase and 2µl of 10X ligase buffer (section 6.15) were added to each tube and incubated at 15°C for 16-18 hours. After the ligation reaction was completed the ligated products were heated at 73°C for 10 minutes, and 5µl was added to 200µl competent E. coli cells (prepared as described in section 6.14). Transformants were

selected by plating the cells on bacterial agar plates (section 6.15) containing 0.5 mg/ml ampicillin. The plasmid DNA from several selected transformants was purified using the rapid miniprep procedure (section 6.14.). Restriction enzyme analysis was used to confirm the presence of the insert DNA, which was then further purified using the Qiagen Maxi plasmid preparation procedure. As confirmation, the insert was sequenced using the Sanger dideoxy method (Sanger et al, 1977) as described by the manufacturers of Sequenase^{TM} (United States Biochemical Corporation, Cleveland, Ohio).

T7 RNA polymerase was used to produce digoxigenin-labelled (dig-labelled) RNA. Approximately 10µg of dig-labelled RNA was obtained from 1µg of linear template DNA. This reaction was carried out at 37°C for 2 hours, as recommended by the suppliers (Boehringer Mannheim). Briefly, 40 units of T7 RNA polymerase was added to 1µg of linearised plasmid DNA (pGEM3 containing ß-Actin, α1(I) or α2(I) collagen inserts or pGEM3 alone) in transcription buffer (section 6.15) in a final volume of 20µl containing 10mM each of ATP, CTP, GTP, 6.5mM UTP and 3.5mM DIG-UTP. 20 units RNasin was added to inhibit any possible contaminating RNases. The reaction mixture was incubated at 37°C for 2 hours after which 50mM EDTA (pH 8.0) was added to stop the reaction. The RNA was precipitated overnight at -20°C by the addition of 2µl of 5M LiCl and 75µl of 96% ethanol. The RNA was pelleted by centrifugation for 30 minutes at 10 000 rpm, washed twice with 70% ethanol, dried under vacuum and resuspended in 100µl DEPC treated water. To facilitate resuspension, the RNA was incubated at 37°C for 30 minutes prior to storage at -20°C. In order to check the quality of the labelled probe 5 to 10µl of the probe was electrophoresed on a 1% agarose gel containing 8% formaldehyde and transferred overnight to a Hybond N membrane. The membrane was washed for 5 minutes in buffer B (section 6.15) at room temperature and blocked with 2% normal sheep serum in buffer B at 37°C for 20 minutes. The membrane was then placed in buffer B containing 0.5% Tween for 5 minutes at room temperature, followed by the addition of

a 1:100 dilution of sheep anti-digoxigenin antibody conjugated with alkaline phosphatase. The membrane was placed in buffer C (section 6.15) for 5 minutes at room temperature after which the substrate was added to detect the blue-purple colour (the membrane was placed in the dark for this detection). Nitro-Blue-Tetrazolium Chloride (NBT) and 5-Bromo-4-Chloro-3-indolyl-phosphate (X-Phosphate) were used as substrates of which 4.5µl of NBT and 3.5µl of X-Phosphate were added to 1ml buffer C. Buffer C contains $MgCl_2$ which is required for alkaline phosphatase activity (as described by Boehringer Mannheim).

Paraffin embedded colon and breast tissue sections were dewaxed in 250ml xylene at 65°C for 30 minutes and washed twice in 250ml xylene for 10 minutes each. This was followed by rehydrating the sections in decreasing concentrations of 250ml ethanol (100%, 90% and 70%) for 2 minutes each. The slides were rinsed in 250ml DEPC treated water for 5 minutes, and transferred to 250ml PBS for 5 minutes.

Depending on the size of the section, 200-500µl proteinase K at a concentration of 1 to 10 µg/ml in buffer A (section 6.15) was placed onto each slide and incubated for between 15 and 45 minutes at 37°C in a humidified chamber. As a negative control RNase A (40 µg/µl) was added together with the proteinase K in some samples.

The slides were placed in 250ml PBS for 5 minutes. 500µl of 0.4% paraformaldehyde diluted in PBS (to refix the sections to the slides) was placed onto each section and the slides were left at room temperature for 5 minutes in a humidified chamber. The slides were transferred to a rack, and submerged in 250ml DEPC treated water for 2-3 minutes. 0.1M triethanolamine and 0.25% acetic anhydride (v/v) was added to the DEPC treated H_20, while stirring continuously at room temperature. The solution was stirred for 10 minutes, decanted, and the slides placed in 250ml PBS for 5 minutes. After acetylation the slides were dehydrated in increasing concentrations of 250ml

ethanol (50%, 70%, 90% and twice in 100%) for 2 minutes each, and allowed to air dry.

Before use both the prehybridisation and hybridisation mixtures (section 6.15) were diluted with an equal volume of deionised formamide. 50µl of the prehybridisation mixture was placed onto each section, covered with parafilm and incubated in a humidified container at 50°C for 1-2 hours. The parafilm was removed and 25µl of hybridisation mixture containing the appropriate dig-labelled probe was added to each section. The sections were covered with parafilm and incubated in a humidified chamber at 50°C overnight.

After hybridisation the slides were washed twice in 250ml of 2X SSC for 15 minutes, with constant stirring at room temperature. This was followed by two 15 minute washes at 43°C in 250ml of 0.1X SSC with gentle shaking. The slides were incubated at room temperature in 250ml of buffer B (section 6.15) for 5 minutes and blocked with 150µl blocking solution containing 2% normal sheep serum in buffer B. Each section was covered with parafilm and placed at 37°C for 20 minutes in a humidified chamber. The parafilm was removed and the slides were placed in 250ml of buffer B containing 0.5% Tween-20 for 5 minutes at room temperature. 100µl of a 1:100 dilution of anti-digoxigenin antibody were added to each slide and incubated at 37°C for between 30 minutes and 2 hours.

After incubation with the anti-digoxigenin antibody, the sections were washed twice in buffer B for 15 minutes each at room temperature, followed by 5 minutes in buffer C (section 6.15). The substrates (4.5µl NBT and 3.5µl X-P into 1ml of buffer C) were diluted prior to use and 100µl of this solution was placed onto each slide and the slides incubated in the dark until a blue-purple colour appeared (between 30 minutes and overnight). After the appearance of the blue-purple colour the reaction was stopped by placing the slides in 250ml of buffer D (section 6.15). The slides were finally

dehydrated in 250ml of increasing concentrations of ethanol (50%, 70%, 90% and twice in 100%) for 2 minutes each, followed by 2 or 3 washes in 250ml xylene (to remove any traces of water) after which they were mounted with entellen (Merck) and covered with a glass coverslip.

Each in situ hybridisation run included a negative control (pGEM-3), to control for non-specific binding and a positive control (ß-actin).

6.4 PREPARATION OF NORMAL PRIMARY BREAST FIBROBLASTS

Normal breast tissue was obtained from Groote Schuur Hospital (GSH) and cut into very fine pieces. These pieces were placed in 30mm sterile petri dishes and overlayered with a coverslip to ensure that the tissue remained firmly fixed to the dish. 1ml MEM containing 10% foetal calf serum, 100 µg/ml penicillin and 100 units/ml streptomycin was added to each dish and placed at 37°C. Every 3 days the medium was changed and replaced with fresh medium. After 10 days the fibroblasts from the tissue had anchored to the dish. The area under the coverslip was allowed to become confluent before the coverslip was removed. The fibroblasts were rinsed with PBS, trypsinised and placed into a 60mm sterile petri dish. The cells were allowed to become confluent after which an aliquot was frozen in MEM containing 10% foetal calf serum, 100 µg/ml penicillin and 100 units/ml strptomycin and 10% DMSO.

6.5. KARYOTYPE ANALYSIS

Breast fibroblasts were plated on a coverslip in a 30mm petri dish and left to grow at 37°C for 5-7 days in 2-3ml MEM supplemented with 10% foetal calf serum and antibiotics. Once the cells had become 80% confluent, 150µl of 10 µg/ml colcimid was added and incubated for 5 hours at 37°C. The medium was decanted, 2ml of 0.075M KCl was added to the dish and left for 1 hour at 37°C. 1ml of fixative solution (20ml acetic acid in 80ml cold methanol) was added to the cells and left for 45 minutes at 37°C. The fixative was decanted, the cells immersed in 2ml of fresh fixative and incubated at room temperature for a further 30 minutes. The coverslip was gently

removed from the dish and placed onto the lid of the petri dish and left overnight at 60°C to dry. The coverslip was stained with 2ml Giemsa for 2 minutes, washed with distilled water and placed at 60°C to dry for 30 minutes. The coverslip was fixed onto a glass slide and the chromosomes counted.

6.6. ANALYSIS OF TYPES I AND III COLLAGEN IN CULTURED CELLS

Approximately 150 000 to 200 000 fibroblasts or tumour epithelial cells were plated in 6-well plates in 2ml of MEM containing either 10% or 5% foetal calf serum respectively, 100 µg/ml penicillin and 100 units/ml streptomycin. When the cells were confluent (1-2 days) they were rinsed with PBS and 2ml of fresh serum free medium containing 20µl of 5 mg/ml ascorbate, 20µl of 5 mg/ml ß aminopropionitrile (ßAPN) and 20µl of ^{3}H-proline (44 Ci/ml) was added to the cells. The cells were incubated at 37°C for 16 hours, after which the medium was harvested. The medium was placed in 10ml falcon tubes (on ice), containing 200µl of 10X N-ethylmaleimide (NEM), 5M phenylmethylsulphonylfluoride (PMSF) and 50 µl rat tail collagen. The dishes were rinsed with 200µl of PBS which was subsequently combined with the medium fraction. 1.2ml of 96% ethanol was added to the medium fraction and incubated overnight at -20°C. 1ml of trypsin was added to the cell layer, followed by incubation at 37°C for 2-3 minutes to detach the cells. 100µl of the cell suspension was counted in 9.9ml isotone in a coulter counter to determine the number of cells per dish.

The precipitate was pelleted at 10 000 rpm in a JA20.1 rotor for 30 minutes at 4°C. The supernatant was decanted, the pellet washed twice in 70% ethanol, dried under vacuum and dissolved in 0.5M acetic acid by vortexing. The protein was incubated in 0.05 mg/ml pepsin at 12-15°C for 16 hours. 5µl of the sample was pipetted onto Whatmann # 1 paper discs which were pre-soaked in 10% trichloroacetic acid, (TCA) and allowed to air dry. The discs were rinsed twice in ice cold 10% TCA, boiled in 10% TCA for 5 minutes, followed by washing in 10% TCA then 70% ethanol and ether. The discs were dried and counted in a scintillation counter.

The calculated amount of labelled protein (either constant dpm or radioactivity per 10^5 cells) was dried, resuspended in 10-12µl of 2X treatment buffer (section 6.15) heated at 95°C for 5 minutes and electrophoresed on a 6% polyacrylamide SDS minigel (0.4M tris/HCl pH8.8, 0.1% SDS, 6% acrylamide:bisacrylamide (29:1), 0.05% ammonium persulphate and 1% TEMED) with a 3% stacking gel (0.125M tris/HCl pH6.8, 0.1% SDS, 3% acrylamide:bisacrylamide (29:1), 0.05% ammonium persulphate and 1% TEMED). The gels were electrophoresed in a Biorad minigel apparatus, at 100 volts for 1-2 hours in SDS running buffer (section 6.15) until the bromophenol-blue reached the bottom of the gel. The gels were incubated in Enhance^{TM} solution (Dupont) with gentle shaking for 1 hour, followed by another hour in distilled water, dried under vacuum at 70°C for 1 hour and exposed to X-ray film for 1-2 days.

Type III collagen was analysed essentially as described for Type I collagen. Labelled protein was calculated (as constant dpm), vacuum dried, resuspended in 12µl of 2X Treatment Buffer (section 6.15) without β-mercaptoethanol heated at 95°C for 5 minutes and electrophoresed on a 6% polyacrylamide SDS gel with a 3% stacking gel (as described above). Electrophoresis was for 1 hour at 100 volts in SDS running buffer (section 6.15) after which the current was switched off and the sample wells were filled with 10% v\v β-mercaptoethanol (in SDS running buffer). This was allowed to diffuse into the gel for 1 hour before the current was switched back on and the gel was electrophoresed for another hour (Sykes et al, 1976). The gel was incubated in Enhance^{TM} solution for 1 hour (with shaking), followed by another hour in distilled water, dried under vacuum at 70°C and exposed to X-ray fil for 2-3 days.

6.7. PREPARATION OF CONDITIONED MEDIA

10X conditioned media was prepared from MDA-MB-231, T47D, MCF-7 and ZR-75-2 breast cancer cell lines as well as from the colon cancer cell lines BE and HT-29.

The cancer and normal breast fibroblast cell lines were grown in Minimal Essential Medium (MEM) containing 5 or 10% foetal calf serum respectively, supplemented with 100 µg/ml penicillin and 100 units/ml streptomycin. After 2 days the medium was

removed, the cell layers rinsed twice with PBS and serum free MEM was added. After 2 days the medium was removed and centrifuged at 4000 rpm in a bench-top centrifuge (Beckman TJ-6) for 1 hour to remove any cellular debris. The medium was transferred to dialysis tubing (which had been previously boiled in 1mM EDTA, then boiled in distilled water and allowed to cool to 4°C) and dialysed overnight at 4°C against distilled H_20. The medium was lyophillised and reconstituted in 1/10th the original volume of sterile water, making the medium 10X concentrated. The medium was filter sterilised through 0.2µm millipore filters.

6.8. CONDITIONED MEDIUM AND CO-CULTURE EXPERIMENTS

6.8.1. Conditioned Medium Experiments

Breast fibroblasts were plated at a density of 40 000 cells per well in 24-well dishes in MEM containing 10% foetal calf serum and antibiotics (section 6.7) and allowed to grow until they were 80% confluent. The medium was removed and the cell layer rinsed with PBS. 10X conditioned medium (described in section 6.7) was diluted to the desired concentration in fresh serum free MEM containing antibiotics and added to the cells. The cells were incubated at 37°C for 32 hours and for a further 16 hours in the presence of 50 µg/ml ascorbate, 50 µg/ml ßAPN and 2 µCi/ml ^{3}H-proline (44 Ci/ml) to label the proteins.

After the labelling period, the medium and cell layers were harvested. The medium was added to a cocktail containing 50µl NEM and PMSF and 0.025 mg/ml bovine serum albumin (BSA) in 1.5ml microfuge tubes. Each well was washed with 50µl PBS which was added to the medium fraction. 500µl of 96% ethanol was added to the medium fraction and the proteins precipitated overnight at -20°C.

After removing the medium fraction, the cells were rinsed with 500µl PBS and harvested by adding 200µl trypsin to each well. After 2 minutes the cells were transferred to microfuge tubes and pelleted by centrifugation at 10 000 rpm for 2

minutes. The supernatant was discarded and the pellet dissolved in 500µl 0.05M tris/HCl pH 7.2 and 0.01M $CaCl_2$. The cells were lysed by 3 cycles of freeze-thawing and the cellular debris removed by centrifugation at 10 000 rpm for 2-5 minutes. The supernatant was transferred to fresh microfuge tubes containing a cocktail of 50µl NEM and PMSF and 0.025 mg/ml BSA, to which 500µl of 96% ethanol was added. The proteins were precipitated overnight at -20°C.

The precipitate was pelleted by centrifugation at 10 000 rpm for 30 minutes at 4°C followed by 2 washes with 70% ethanol. The pellets were dried under vacuum for 10 minutes and resuspended in 500µl of 0.1N NaOH and stored at 4°C until further use.

6.8.2 Co-Culture Experiments

A total of 40 000 cells were plated per dish (20 000 fibroblasts and 20 000 tumour cells) as described for the conditioned medium experiments (section 6.8.1). The cells were incubated in MEM containing 10% foetal calf serum and antibiotics. After 24 hours, the medium was removed, the cell layer rinsed with PBS and fresh medium was added. The cells were incubated for 8 hours at 37°C, prior to the addition of 50 µg/ml ascorbate, 50 µg/ml ßAPN and 2 µCi/ml ^{3}H-proline (44 Ci/ml). After 16 hours, the protein was harvested as described in section 6.8.1.

6.9. ESTIMATION OF COLLAGEN USING THE COLLAGENASE ASSAY

100µl of ^{3}H-labelled protein (sections 6.8.1 and 6.8.2) was added to 100µl of 0.08N HCl and 50µl collagenase digestion buffer (120mM tris/HCl pH 7.2, 0.5mM $CaCl_2$ containing 0.5 units bacterial collagenase) and incubated at 37°C for 2-3 hours (Hendriks et al, 1993). 100µl of the digest was removed for determination of total radioactivity **(T)**. 100µl of 50% TCA was added to the remaining solution which was incubated on ice for 1 hour to precipitate the undigested proteins. The precipitate was pelleted by centrifugation at 10 000 rpm for 5 minutes at room temperature and 400µl of the supernatant was removed for determining the collagenase sensitive digested

protein (S). Subtraction of the radioactivity in the collagenase sensitive protein fraction (S) from that in the total protein (T) yields the non-collagenous protein fraction (T-S). The % collagen synthesis was calculated using the formula of Peterofsky and Diegelmann (1971) which also takes into account the enrichment of proline in collagen compared with other proteins:

$$\%C = \frac{(S)}{5.4\,(T-S) + S} \quad X\ 100$$

Where S = Collagenase Sensitive Protein, T = Total Protein and %C = %Collagen Present

6.10. DETECTION OF COLLAGENASES

Labelled collagen from WI-38 cells was prepared as described (section 6.6). 10 000 dpm aliquots of protein were dried down in 1.5ml microfuge tubes and resuspended in 50µl of 0.1N acetic acid and neutralised with 50µl of 0.1M NaoH. 50µl collagenase digestion buffer (120mM tris/HCl pH 7.2 and 0.5mM $CaCl_2$) and either 50µl of tumour cell conditioned medium (section 6.7) or 50µl of tumour cell conditioned medium pre-incubated with breast fibroblasts was added to each tube. The digestion was allowed to occur at 25°C for 15 hours, after which the samples were dried under vacuum, resuspended in 20µl 2X treatment buffer (section 6.15), denatured at 95°C for 5 minutes and electrophoresed on 6% polyacrylamide SDS gels in SDS running buffer (section 6.15) for 2 hours at 100 volts, as described in section 6.7. The gels were dried and exposed to X-ray film for 1-2 days.

6.11 WESTERN BLOT ANALYSIS

20µl of 10X tumour cell conditioned media (prepared as described in section 6.7) was boiled in 2X Treatment Buffer (section 6.15) for 5 minutes before electrophoresis on 10% SDS polyacrylamide gels for 1 hour at 100 volts. The proteins on the gel were transferred to Hybond C in an electroblotting apparatus, after which the membrane was removed and blocked overnight in 10% fat free milk powder in PBS+0.1% Tween.

The membrane was washed and incubated with the monoclonal mouse EMMPRIN antibody for 1 hour with shaking at room temperature. The EMMPRIN antibody was removed and the membrane was washed for 25 minutes in PBS/Tween after which a 1:10 000 dilution of the secondary antibody (goat anti-mouse conjugated with horse radish peroxidase, Caltag) was added and left for 1 hour, with shaking at room temperature. The membrane was washed for 30 minutes with PBS/Tween and then incubated for 1 minute in ECL detection (Amersham) before overnight exposure to X-ray film.

6.12. SINGLE STRAND CONFORMATION POLYMORPHISM (SSCP) ANALYSIS

6.12.1 Extraction of DNA from paraffin embedded tissue sections

Paraffin embedded colon sections were placed into microfuge tubes containing 1ml of xylene. The tubes were inverted several times and the tissue pelleted by centrifugation at 10 000 rpm for 5-10 minutes. The xylene was removed with a pasteur pipette and the procedure repeated. 500µl of 100% ethanol was added to the tubes which were shaken well before centrifugation for 5-10 minutes at 10 000 rpm. The ethanol was removed with a pasteur pipette and the wash repeated with another 500µl of ethanol.

The samples were dried under vacuum and the pellet dissolved in 200µl digestion buffer (50mM tris/HCl, pH 8.5, 1mM EDTA and 0.5% Tween 20) containing 200 µg/ml proteinase K and incubated for 3 hours at 55°C, or overnight at 37°C. The proteinase K was inactivated by incubation at 95°C for 8-10 minutes. The samples were centrifuged briefly in order to remove any residual paraffin or remaining tissue. The supernatant containing the DNA was transferred to a clean, sterile tube and between 1 and 10µl was used in PCR reactions.

6.12.2. Polymerase Chain Reaction (PCR)

The DNA isolated in 6.12.1 was used in the amplification of specific regions of the ras genes by the polymerase chain reaction (PCR). 0.5-1 units Taq polymerase was added to 50-100ng DNA in Taq polymerase buffer (section 6.15) containing 0.2mM each of

dATP, dGTP, dCTP and dTTP and 5pmol of each primer specific for the various exons of the Ki-Ras and Ha-Ras genes in a final volume of 50µl. Amplifications were carried out in a Hybaid thermal cycler with the following PCR cycle: denaturation at 94°C for 1 minute, annealing at 54-68°C for 2 minutes (see various primer sets) and extension at 72°C for 2 minutes.

PRIMER SEQUENCES ARE AS FOLLOWS:

GENE	EXON	PRIMER	ANNEALING TEMPERATURE
Ha-Ras	1:	FORWARD 5'-GGGCCCTCCTTGGCAGGTGG-3'	68°C
	1:	REVERSE 5'-CACCTGGACGGCGGCGCTAG-3'	
Ha-Ras	2:	FORWARD 5'-GGAGAGGCTGGCTGTGTGAA-3'	56°C
	2:	REVERSE 5'-AAAAGACTTGGTGTTGTTGA-3'	
Ki-Ras 1:		FORWARD 5'-ACATGTTCTAATATAGTCAC-3'	59°C
1:		REVERSE 5'-CTATTGTTGGATCATATTCG-3'	
1:		REVERSE 5'-TCAAAGAATGGTCCTGCACC-3'	54°C
Ki-Ras 2:		FORWARD 5'-TTGAGTTGTATATAACACCT-3'	58°C
2:		REVERSE 5'-CATGGCATTAGCAAAGACTC-3'	

The PCR products were heated at 65°C for 5 minutes prior to electrophoresis on 8% polyacrylamide gels (acrylamide:bisacrylamide [39:1], 0.2% ammonium persulphate and 1% TEMED) to check for specificity of the PCR reaction. The gels were electrophoresed in 1X TBE for 1.5 hours at 150 volts or until the bromophenol blue reached the bottom. The gels were stained in 1mg/ml ethidium bromide and photographed.

6.12.3. Radioactive SSCP Analysis

The PCR was performed as described above, except with the addition of 2.5 µCi of ^{32}P-dCTP (3000 Ci/mmol) to each reaction mixture. 3µl of the PCR product was diluted with an equal volume of formamide loading buffer (section 6.15) containing 20mM NaOH. The samples were denatured at 95°C for 10 minutes, placed directly onto ice and loaded on a 6% polyacrylamide SSCP gel with or without 5% (v/v)

glycerol. The gels were electrophoresed at room temperature or 4°C in 1X TBE. The samples were electrophoresed at 70 watts for the first 10 minutes and then for 4-5 hours at 30 watts or until the bromophenol blue reached the bottom of the gel. The gel was dried and exposed to X-ray film. Samples with altered mobility compared to normal DNA were subjected to direct sequence analysis or cloned into pUC19 and subsequently sequenced.

6.12.4. Non-Radioactive SSCP Analysis

PCR samples were prepared as described in section 6.12.2. and 5µl was added to 0.4µl of 1M methylmercury hydroxide, 1µl 15% ficoll loading buffer (section 6.15) and made up to 20µl with 1X TBE. The samples were heated at 85°C for 5 minutes and placed onto ice prior to loading on a 20% polyacrylamide gel (1.5X TBE, 20% acrylamide:bisacrylamide (39:1), 0.05% ammonium persulphate and 1% TEMED) with or without 2% (v/v) glycerol. The gels were electrophoresed in 1.5X TBE at 200 volts for 1-2 hours at 4°C or room temperature. After electrophoresis the gels were stained in 0.5 µg/ml ethidium bromide and photographed.

Samples were also analysed on discontinous gels as follows: 10µl of PCR sample was added to 1µl formamide loading buffer (section 6.15), denatured at 95°C for 5 minutes, placed on ice and loaded onto a 2 phase discontinuous formamide gel. The lower phase or separating gel contained 6% acrylamide:bisacrylamide (49:1), 0.5X TBE, 0.05% ammonium persulphate, 1% TEMED, with or without 5% (v/v) glycerol. This was overlaid with isopropanol to allow polymerisation to proceed (30 minutes). The top phase or stacking gel contained 8% acrylamide:bisacrylamide (39:1), 0.2X TBE, 75% formamide, 0.25% ammonium persulphate, 1% TEMED, and was allowed to polymerise overnight. These gels were electrophoresed at 4°C or room temperature in 0.5X TBE at 200-300 volts for 1-2 hours, after which they were stained with 0.5 µg/ml ethidium bromide and photographed.

6.12.5. Direct Sequencing of PCR Products

PCR samples showing altered mobilities by SSCP analysis were sequenced directly using the USB PCR sequencing kit (Amersham) as recommended by the suppliers (United States Biochemical). Colon DNA was amplified as described in section 6.13.2. and an aliquot (15µl) was first checked on a 1% agarose gel prior to sequence analysis. After the sequencing reactions were completed, the samples were heated at 75°C for 2 minutes, loaded on a 6% polyacrylamide gel and electrophoresed in 1X TBE buffer.

6.13. CLONING OF PCR FRAGMENTS INTO pUC19

6.13.1. Crush Soak Method for Obtaining PCR Fragments

DNA fragments for cloning were amplified by PCR and electrophoresed on a 6% polyacrylamide gel. The bands of interest were excised from the gel and cut into small pieces which were transferred to a microfuge tube, to which an equal volume of DNA elution buffer (section 6.15) was added. The tube was incubated overnight at 37°C with gentle shaking, after which the buffer was removed and transferred to a fresh microfuge tube. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the DNA solution, mixed well and centrifuged at 10 000 rpm for 5 minutes. The aqueous phase was removed and extracted with an equal volume of chloroform/isoamyl alcohol (24:1). The solution was centrifuged at 10 000 rpm for 5 minutes after which the aqueous phase was removed and the DNA precipitated overnight at -20°C by the addition of 0.1 volume of 3M Na acetate pH 5.5 and 3 volumes of 96% ethanol. The DNA was pelleted by centrifugation at 10 000 rpm for 30 minutes at 4°C, washed twice with 70% ethanol, dried and resuspended in 10µl TE buffer (section 6.15).

6.13.2. Cloning of PCR Products

The gel-extracted PCR products were incubated at 37°C for 1 hour with T4 polynucleotide kinase (1 unit per µg of DNA) in buffer containing 1mM ATP (section 6.15) in a final volume of 20µl. The enzyme was heat inactivated at 65°C for 10 minutes.

20µl of phosphorylated DNA was incubated with 2 units Klenow fragment DNA polymerase in 1X Klenow buffer in the absence of deoxynucleotide triphosphates (dNTP's) for 5 minutes at room temperature. dNTP's were then added to a final concentration of 2.5mM and incubated at room temperature for a further 30 minutes. The enzyme was heat inactivated at 65°C for 10 minutes and the PCR product electrophoresed on a 1% low melting temperature agarose gel in 1X TAE buffer. The DNA bands were excised from the gel and an in-gel ligation was performed with SmaI digested and alkaline phosphatase treated pUC19 (section 6.3). The ligated products were transformed into E. coli DK-1 cells (section 6.14). Transformants were selected for ampicillin resistance and the plasmid DNA purified using the rapid plasmid prep procedure. Plasmid DNA was digested with restriction endonucleases to confirm the presence of the insert (section 6.3). Large scale plasmid DNA was prepared using the Qiagen Maxi Prep procedure. Inserts were confirmed by sequence analysis using the Pharmacia Sequencing Kit as per manufacturers instructions.

In order to prevent pUC19 from self-ligating (after enzymatic digestion with SmaI), calf intestinal alkaline phosphatase (CIAP) was used to remove the available phosphates at the 5' ends. 1 unit of CIAP was added per pmol of blunt-ended DNA in CIAP buffer (section 6.15) and incubated at 50°C for 1 hour. To terminate the reaction, 1/10th the volume of 500mM EGTA, pH 8.0 was added and the DNA was incubated at 65°C for 45 minutes. The enzyme was removed by phenol/chloroform/isoamyl alcohol extraction and the DNA precipitated at -70°C for 30 minutes by the addition of 3 volumes 96% ethanol. The pellet was washed twice with 70% ethanol, dried for 5-10 minutes and resuspended in 1X TE buffer.

6.14 PREPARATION OF COMPETENT DK-1 CELLS AND TRANSFORMATION

Competent E. Coli DK-1 cells were prepared by inoculating 10ml of Luria Broth (section 6.15) with 20µl of DK-1 cells from a glycerol stock. The cells were grown overnight at 37°C with vigorous shaking. 1ml of this overnight culture was used to

inoculate 300ml of Luria Broth in a sterile 2 litre flask. The cells were grown with vigorous shaking at 37°C until the OD_{650} was between 0.2-0.4 (it should not take longer than 3 hours). The cells were pelleted by centrifugation at 5000 rpm in a Beckman JA 10 rotor at 4°C. The cells were resuspended in 40ml of ice cold 60mM $CaCl_2$, 10mM Pipes, pH 7.2, left on ice for 20 minutes, centrifuged at 5000 rpm for 10 minutes at 4°C and resuspended in 4ml of 60mM $CaCl_2$, 10mM Pipes, pH 7.2 containing 15% glycerol. Cells were frozen in 200µl aliqouts in a dry ice-ethanol bath and stored at -70°C until further use. The transformation efficiency was usually between 10^6 and 10^7 transformants per microgram of DNA, using supercoiled pBR322 (Current Protocols in Molecular Biology).

Aliquots of competent cells were thawed on ice and 100µl were transferred to 10ml falcon tubes. 5µl of the ligation mixture was added to the cells, incubated for a further 30 minutes on ice and heat shocked at 42°C for 2-3 minutes. 1ml of prewarmed Luria Broth (37°C) was added and incubated for a further hour at 37°C. Aliquots of 20, 50 and 100µl were plated onto agar plates containing 0.5 mg/ml ampicillin and incubated at 37°C overnight.

The alkaline lysis procedure (Birnboim and Doly, 1979; Birnboim 1983) was used to prepare plasmid DNA for rapid analysis. 10ml of Luria Broth was inoculated with a single bacterial colony and grown overnight at 37°C with shaking. The cells were pelleted at 2000 rpm for 10 minutes in a Beckman TJ-6 centrifuge at 4°C. The pellet was resuspended in 200µl RAPS solution-1 (section 6.15), transferred to a microfuge tube and incubated for 5 minutes at room temperature. The bacteria were lysed by the addition of 400µl RAPS solution-2 (section 6.15) and placed on ice for 5 minutes. 300µl RAPS solution-3 (section 6.15) was added and the cells were incubated on ice for 10 minutes. The cellular debris was pelleted by centrifugation for 5 minutes at 4°C (to remove the chromosomal DNA and bacterial membranes). The supernatant was transferred to a fresh tube, re-centrifuged, and transferred to another fresh tube to

which was added 600µl of ice cold isopropanol to allow the plasmid DNA to precipitate at -20°C for 1 hour. The plasmid DNA was recovered by centrifugation, washed in 70% ethanol, dried under vacuum for 10 minutes and resuspended in 80µl of 1X TE buffer. The DNA was treated with Rnase A at a concentration of 1 mg/ml at 37°C for 10 minutes and an aliquot electrophoresed on a 1% agarose gel to ensure that the plasmid DNA was free of contaminating RNA. Restriction enzyme analysis confirmed the identity of the plasmid.

6.15. BUFFERS AND SOLUTIONS

30% ACRYLAMIDE STOCK SOLUTION (29:1)
29% Acrylamide
1% Bisacrylamide

40% ACRYLAMIDE STOCK SOLUTION (38:2)
38% Acrylamide
2% Bisacrylamide

40% ACRYLAMIDE STOCK SOLUTION (39:1)
39% Acrylamide
1% Bisacrylamide

50% ACRYLAMIDE STOCK SOLUTION (49:1)
49% Acrylamide
1% Bisacrylamide

CHROMIC ACID
100 g $K_2Cr_2O_7$
250 ml H_2SO_4 made up to 1 litre with H_2O

10X CIAP BUFFER
3 M NaCl
30 mM Triethanolamine (pH 7.6)
100 mM $MgCl_2$
100 mM $ZnCl_2$

100X DENHARDTS SOLUTION
2% (w/v) BSA
2% (w/v) Ficoll
2% (w/v) Polyvinylpyrolidone (PVP)

DEPC TREATED H_2O
Add DEPC to 0.1% (v/v), stir at room temperature and autoclave

IN SITU HYBRIDISATION BUFFER B

100 mM Tris-Cl (pH 7.5)
150 mM NaCl

IN SITU HYBRIDISATION BUFFER C

100 mM Tris-Cl (pH 9.5)
50 mM $MgCl_2$
100 mM NaCl

IN SITU HYBRIDISATION BUFFER D

10 mM Tris-Cl (pH 8.0)
1 mM EDTA

IN SITU HYBRIDISATION MIXTURE

0.25% Dextran Sulphate
12.5 mM Tris-Cl (pH 8.0)
12.5X Denhardts Solution
1.25 mM EDTA
12.5 mM DTT
2.5X SSC
0.005 mg/ml Herring Sperm DNA
0.005 mg/ml tRNA

IN SITU PRE-HYBRIDISATION MIXTURE

0.25% Dextran Sulphate
25mM Tris-Cl (pH 8.0)
25X Denhardts Solution
2.5 mM EDTA
25 mM DTT
1.25 mg/ml Herring Sperm DNA
0.125 mg/ml tRNA

10X KLENOW BUFFER

100 mM Tris-Cl (pH 7.5)
100 mM $MgCl_2$
500 mM NaCl
10 mM DTE

LURIA AGAR

10 g/l Tryptone
5 g/l Yeast Extract
5 g/l NaCl
15 g/l Agar

LURIA BROTH

10 g/l Tryptone
5 g/l Yeast Extract
5 g/l NaCl

NORTHERN BLOT PRE-HYBRIDISATION MIXTURE

10% Dextran Sulphate
5X SSC
50 mM Sodium Pyrophosphate
5X Denhardts Solution
50% Formamide
0.1 mg/ml Herring Sperm DNA
0.1% SDS

PHOSPHATE BUFFERED SALINE

137 mM NaCl
2.7 mM KCL
4.3 mM $Na_2HPO_4.7H_20$ (pH 7.4)
1.4 mM KH_2PO_4

RAPS SOLUTION 1

25 mM Tris-Cl (pH 8.0)
10 mM EDTA
50 mM Glucose

RAPS SOLUTION 2

200 mM NaOH
1% SDS

RAPS SOLUTION 3

3 M Potassium Acetate (pH 4.8)

RNA LOADING BUFFER

1X RNA Running Buffer
45% Deionised Formamide
6% Formaldehyde
1X Formaldehyde loading buffer

10X RNA RUNNING BUFFER

41.8g MOPS to 800ml water (pH 7.0)
16.6ml 3 M Sodium Acetate (pH 5.5)
20ml 0.5 M EDTA (pH 8.0)
Make up to 1 litre with water

5X SDS RUNNING BUFFER

250 mM Tris-Cl (pH 8.3)
1.92 M Glycine
0.5% SDS

SOLUTION D

4.2 M Guanidine (Thiocyanate Salt)
0.03 M Sodium Citrate (pH 7.0)
0.5% Sarcosyl
360µl 2-Mercaptoethanol (14.1M) is added per 50ml solution D

20X SSC

3 M NaCl
300 mM Sodium Citrate

10X TAE BUFFER

40 mM Tris-Acetate (pH 7.8)
5 mM Sodium Acetate
1 mM EDTA

10X TBE BUFFER

90 mM Tris-Borate (pH 8.0)
90 mM Boric Acid
2.5 mM EDTA

10X TE BUFFER
100 mM Tris-Cl (pH 8.0)
10 mM EDTA

10X TRANSCRIPTION BUFFER
400 mM Tris-Cl (pH 8.0)
60 mM $MgCl_2$
100 mM DTT
20 mM Spermidine
100 mM NaCl

2X TREATMENT BUFFER
0.125 M Tris-Cl (pH 6.8)
4% SDS
20% Glycerol
5 mM EDTA
10% 2-Mercaptoethanol

10X T4 POLYNUCLEOTIDE KINASE BUFFER
500 mM Tris-Cl (pH 7.5)
100 mM $MgCl_2$
50 mM DTT
0.5 mg/ml BSA

REFERENCES

Al-Adnani M. S., Kirrane J. A., and McGee J. O'D (1975) Br. J. Cancer **31**: 653-660

Alvarez O. A., Carmichael D. F. and DeClerck Y. A. (1990) J. Natl. Cancer. Inst. **82**: 589-595

Aycock R. S., Raghow R., Stricklin G. P., Seyer J. M. and Kang A. H. (1986) J. Biol. Chem. **261**: 14355-14360

Barbacid M. (1987) Ann. Rev. Biochem. **56**: 779-827

Barsky S. H., Rao C. N. , Grotendorst G. R. and Liotta L. A. (1982) Am. J. Pathology **108**: 276-283

Barsky S. H. and Gopalakrishna R. (1987) Cancer Res. **47:** 1663-1667

Basic Pathology (1992) (Kumar V., Cotran R. S. and Robbins S. L. eds) , W. B. Saunders Company, Philadelphia, 473-522

Bauer E. A., Uitto J., Walters R. C. and Eisen A. Z. (1979) Cancer Res. **39**: 4594-4599

Bevilacqua G., Sobel M. E., Liotta L. A. and Steeg P. S. (1989) Cancer Res. **49**: 5185-5190

Birnboim and Doly (1979) Nucleic Acids Res. **7**: 1513-1523

Birnboim (1983) Methods Enzymology **100**: 243-255

Biwas C. (1982) Biochem. Biophys. Res. Communications **109**: 1026-1034

Biswas C. (1984) Cancer Letters **24**: 201-207

Biswas C. , Zhang Y. , DeCastro R. , Guo H. , Nakamura T. , Kataoka H. and Nabeshima K. (1995) Cancer Res. **55:** 434-439

Boguski M. S. and McCormick F. (1993) Nature **366**: 643-654

Bornstein P. and Sage H. (1989) Prog. Nucleic Acids. Res and Mol. Biol. **37**: 67-106

Bos J. L., Fearon E. R., Hamilton S. R., Verlaan-de Vries M., van Boom J. H., van der Eb A. J. and Vogelstein B. (1987) Nature **327**: 293-297

Bronner C. E. et al, (1994) Nature **368**: 258-261

Cai H., Szeberenyi J. and Cooper G. M. (1990) Mol. Cell. Biol. **10**: 5314-5323

Cailleau R., Young R., Olive M. and Reeves W. J. Jr. (1974) J. Natl. Cancer Inst. **53**: 661-673

Chen Y., Chen C-F., Riley D. J., Allred D. C., Chen P-L., Von Hoff D., Osborne C. K. and Lee W-H (1995) Science **220**: 789-791

Chomczynski P. and Sacchi N. (1987) Analytical Biochem. **162**: 156-159

Chu M-L., Myers J. C., Bernard M. P., Ding J-F. and Ramiriz F. (1982) Nucleic Acids. Res. **10**: 5925-5933

Chu M-L. , Weil D. , de Wet W. , Bernard M. , Sippola M. and Ramirez F. (1985) J. Biol. Chem. **260:** 4357-4363

Coles C., Londie A., Chetty U., Steel C. M., Evans H. J. and Prosser J. (1992) Cancer Res. **52**: 5291-5298

Collins M. R. (1994) PhD Thesis, University of Cape Town

Cornil I. , Theodorescu D. , Man S. , Herlyn M. , Jambrosic J. and Kerbel R. S. (1991) Proc. Natl. Acad. Sci. **88:** 6028-6032

Crissman J. D. and Barwick K. W. (1993) in Colorectal Cancer (Wanebo H. J. ed) Mosby Publishers 57-74

Current Protocols in Molecular Biology (1995) Volume 1, (Ausubel F. M., Brent R., Kingston R. E., Moore D. D., Scidman J. G. , Smith J. A. and Struhl K. eds) John Wiley and Sons, U. S. A. , 3.16.3.

Current Protocols in Molecular Biology (1995) Volume 1, (Ausubel F. M., Brent R., Kingston R. E., Moore D. D., Scidman J. G., Smith J. A. and Struhl K. eds) John Wiley and Sons, U. S. A. , 1.8.1.-1.8.3.

Davis B. H. ,Chen A. and Beno D. W. A. (1996) J. Biol. Chem. **271:** 11039-11042

Eckes B. , Mauch C. , Huppe G. and Krieg T. (1993) FEBS Letters **318:** 129-133

Elias J. A., Freundlich B., Adams S. and Rosenbloom J. (1990) Annal N. Y. Acad. Sci. **580**: 233-244

Ellis L. M. and Fidler I. J. (1995) The Lancet **346:** 388-390

Ellis S. M., Nabeshima K. and Biswas C. (1989) Cancer Res. **49**: 3385-3391

Eng C. and Ponder B. A. J. (1993) FASEB J. 7: 910-919

Engel L. W., Young N. A., Traika T. S., Lippman M. E., O'Brien S. J. and Joyce M. J. (1978) Cancer Res. **38**: 3352-3364

Fearon E. R. and Vogelstein B. (1990) Cell **61**: 759-767

Fidler I. J. and Kripe M. L. (1977) Science **197**: 893-895

Fidler I. J. (1978) Cancer Res. **38**, 2651-2660

Fidler I. J. and Hart I. R. (1982) Science **217**: 998-1003

Fidler I. J. and Poste G. (1985) Seminars Oncol. **12**: 207-221

Fidler I. J. and Balch (1987) Current Problems. Surgery. **24**: 129-209

Fidler I. J. (1990) Cancer Res. **50**: 6130-6138

Finkelstein S. D., Say Egh R., Christensen S. and Swalsky P. A. (1993) Cancer **71**: 3827-3838

Fishel R. et al, (1993) Cell **75**: 1027-1038

Folkman J. (1974a) Adv. Cancer Res. **19**:331-358

Folkman J. (1989) J. Natl. Cancer Inst. **82**: 4-6

Folkman J. (1995) Seminars in Med. **333:** 1757-1763

Folkman J. (1995) Nat. Med. **1:** 27-31

Forrester K., Almognera C., Han K., Grizzle W. E. and Perucho M. (1987) Nature **327**: 298-303

Going J. J. , Anderson T. J. and Wyllie A. H. (1992) Br. J. Cancer **65:** 45-50

Gross J. and Lapiere C. M. (1962) Proc. Natl. Acad. Sci. **48:** 1014-1022

Guo H. , Zucker S. , Gordon M. K. , Toole B. P. and Biswas C. (1997) Journal Biol. Chem. **272:** 24-27

Gutman M. , Singh R. K. , Xie K. , Bucana C. D. and Fidler I. J. (1995) Cancer Res. **55:** 2470-2475

Hall J. M. , Lee M. K. , Newman B. , Morrow J. E. , Anderson C. A. , Huey B. and King M-C (1990) Science **250:** 1684-1689

Harris E. D. and Krane S. M. (1974) New Engl. J. Med. **291** : 557-563

Harris E. D. and Krane S. M. (1974) New Engl. J. Med. **291**: 605-609

Harris J. R., Lippman M. E., Veronesi U. and Willett W. (1992) New Engl. Med. **327**: 319-328

Harris J. R., Lippman M. E., Veronesi U. and Willett W. (1992) New Engl. J. Med **327**: 390-398

Harris J. R., Lippman M. E., Veronesi U. and Willett W. (1992) New Engl. J. Med **327**: 473-480

Haslam J. M. (1991) From the book: Regulatory Mechanisms in Breast Cancer, Lippman M. and Dickson R. (eds) 401-420

Hendriks T., Martens M. F. W. C., Huyben C. M. L. C. and Wobbes T. (1993) Br. J. Cancer **67**: 545-550

Hewitt R. E., Powe D. G., Carter G. I. and Turner D. R. (1993) Int. J. Cancer **53**: 62-69

Hewitt R. E. , Keeble W. , Powe D. G. , Williamson R. J. and Turner D. R. (1994) Int. J. Cancer **56:** 675-680

Hewitt R. E. , Linton V. , Powe D. G. , Sam W. , Stevens A. and Turner D. R. (1996) Int. J. Cancer **66:** 24-28

Holmgren L. , O'Reilly M. S. and Folkman J. (1995) Nat. Medicine **1:** 149-153

Hongyo T., Buzard G. S., Calvert R. J. and Weghorst C. (1993) Nucleic Acids Res. **21**: 3637-3642

Horlein D., Mcpherson J., Swee Han Goh and Bornstein P. (1981) Proc. Natl. Acad. Sci. USA. **78**, 6163-6167

Ignotz R.A., Endo T. and Massagne (1987) J. Biol. Chem. **262**: 6443-6446

Imai K., Yokohama Y., Nakanishi I., Ohuchi E., Fujii Y., Nakai N. and Okado Y. (1995) J. Biol. Chem **220**: 6691-6697

Inagaki Y. , Truter S. and Ramirez F. (1994) J. Biol Chem. **269:** 14828-14834

Inagaki Y. , Truter S. , Tanaka S. , Di Liberto M. and Ramirez F. (1995) J. Biol. Chem. **270:** 3353-3358

Isaacs J. T. (1986) in Breast Cancer Treatment and Prognosis, (Stoll B. A. ed), Blackwell Scientific Publications, 45-56

Itano N. , Oguri K. , Nagayasu Y. , Kusano Y. , Nakanishi H. , David G. and Okayama M. (1996) Biochem. J. **315:** 925-930

Judware R. and Culp L. A. (1996) Encyclopedia of Cancer, Volume 1, Academic Press Incorp., San Diego, California

Kataoka H. , DeCastro R. , Zucker S. and Biswas C. (1993) Cancer Res. **53:** 3154-3158

Keydar I., Chen L., Karby S., Weiss F. R., Delarea J., Radu M., Chaitcik S. and Brenner H. J. (1979) Eur. J. Cancer **15**: 659-670

Klein C. E. , Dressel D. , Steinmayer T. , Mauch C. , Eckes B. , Krieg T. , Bankert R. B. and Weber L. (1991) J. Cell Biol. **115:** 1427-1436

Kohn E. C. and Liotta L. A. (1995) Cancer Res. **55:** 1856-1862

Langholz O. , Rockel D. , Mauch C. , Kozlowska E. , Bank I. ,Krieg T. and Eckes B. (1995) J. Cell Biol. **131:** 1903-1915

Liau G. , Yamada Y. and de Crombrugghe B. (1985) J. Biol. Chem. **260:** 531-536

Liotta L. A. , Abe S. , Gehron P. R. and Martin G. R. (1979) Proc. Natl. Acad. Sci. **76:** 2268-2272

Liotta L. A., Thorgeisson U. P. and Garbisa S. (1982) Cancer Metastasis Rev. **1**: 277-288

Liotta L. A., Rao C. N. and Barsky S. H. (1983) Lab. Invest. **49**: 636-649

Liotta L. A. (1986) Cancer Res. **46**: 1-7

Liotta L. A. , Steeg P. S. and Stetler-Stevenson W. G. Cell **64:** 327-336

Liotta L. A. (1992) Scientific American **266**: 34-41

Luparello C. , Sheterline P. , Pucci-Minafra I. and Minafra S. (1991) J. Cell Sci. **100:** 179-185

Lynch H. T., Marcus J. N., Watson P. and Lynch J (1991) in The Breast, Comprehensive Management of Benign and Malignant Diseases, (Bland K. I. and Copeland E. M. eds.), Philadelphia. PA, W. B. Sanders Company, 262-209

Mauch C. , Adelmann-Grill B. , Hatamochi A. and Krieg T. (1989) FEBS Letters **250:** 301-305

Mauviel A., Daireaux M., Redini F., Galera P., Loyan G. and Pujol J.-P. (1988) FEBS Letters **1**: 47-52

Mauviel A , Teyton L. , Bhatnagar R. , Penfornis H. , Laurent M. , Hartmann D. , Bonaventure J. , Loyau G. , Saklatvala J. , Pujol J. P. et al, (1988) Biochem. J. **252**: 247-255

Miki Y. , Swensen J. , Shuttuck-Eidens D. , Futreal P. A. , Harshman K. , Tavtigian S. , Liu Q. , Cochran C. , Bennett L. M. et al (1994) Science **266**: 66-71

Minafra S. , Luparello C. , Pucci-Minafra I. , Sobel M. E. and Garbisa S. (1992) J. Cell Sci. **102:** 323-328

Minafra S. , Giambelluca C. , Andriolo M. and Pucci-Minafra I. (1995) Int. J. Can. **62:** 777-783

Muraoka K. , Nabeshima K. , Murayama T. , Biswas C. and Koono M. (1993) Int. J. Can. **55:** 19-26

Myers J.C., Chu M-L., Faro S. H., Clark W. J., Prockop D. J. and Ramirez F. (1981) Proc. Natl. Acad. Sci. USA. **28**: 3516-3520

Nakanishi H. , Okayama M. , Oguri K. , Hayashi K. , Tateno H. and Hosoda S. (1991) Virchows. Archiv. A. Pathol. Anat. **418:** 531-538

Nakanishi H. , Oguri K. , Yoshida K. , Itano N. , Takenaga K. , Kazama T. , Yoshida A. and Okayama M. (1992) Biochem. J. **288:** 215-224

Nakanishi H. , Takenaga K. , Oguri K. , Yoshida A. and Okayama M. (1992) Virchows. Archiv. A. Pathol. Anat. **420:** 163-170

Nakanishi H. , Oguri K. , Takenaga K. , Hosoda S. and Okayama M. (1994) Lab. Inves. **70:** 324-332

Nicolaides N. C. , Papadopoulos N. , Liu B. , Wei Y. F. , Carter K. C. , Ruben S. M. Rosen C. A. , Haseltine W. A. , Fleischmann R. D. , Fraser C. M. et al, (1994) Nature **371**: 75-80

Nicolson G. L. and Custead S. E. (1982) Science **215**: 176-178

Nicolson G. L. (1987) Cancer Res. **47**: 1473-1487

Nicolson G. L. (1988) Biochim. Biophys. Acta **948**: 175-224

Niitsu Y. , Ito N. , Kohda K. , Owada M. , Morita K. , Sato S. , Watanabe N. , Kohgo Y. and Urushizaki I. (1988) Br. J. Cancer **57:** 79-82

Noel A., Manaut C., Boulvain A., Calberg-Bacq C.M., Lambert C.A., Nusgens B. , Lapiere C.M. and Foidart J.M. (1992) J. Cell. Biochem. **48**: 150-161

Noel A., Munant C., Nusgens B., Lapiere Ch. M. and Foidart J. M. (1993) Invasion Metastasis **13**: 72-81

Nori M., Vogel U. S., Gibbs J. B. and Weber M. J. (1991) Mol. Cell. Biol. **11**: 2812-2818

Ohtani H., Kuroiwa A., Obinata M., Ooshima A. and Nagura H. (1992) J. Histochem. Cytochem. **40**: 1139-1146

O'Reilly M. S. , Holmgren L. , Chen C. and Folkman J. (1996) Nat. Med. **2:** 689-692

Osborne R. J. et al, (1991) Cancer Res. **51**: 6194-6198

Paglia L., Wilczek J. Lino Diaz de Leon , Martin G. R , Horlein D. and Muller P. K. (1979) Biochemistry **18**: 5030-5034

Pauli B. U., Schwartz D. E., Thonar E. J. M. and Knettner K. E. (1983) Cancer Metastasis Rev. **2**: 129-152

Peltomaki P. et al, (1993) Science **260**: 810-812

Peterkofsky B and Diegelmann R. (1971) Biochemistry **10**: 988-994

Pierson K. K. and Wilkinson E. J. (1991) in Comprehensive Management of Benign and Malignant Diseases (Bland K. I. and Copeland E. M. eds.), Philadephia. PA, W. B. Sanders Company, 193-209

Polakis P. and McCormick F. (1993) J. Biol. Chem. **268**: 9157-9160

Prescott J. , Troccoli N. and Biswas C. (1989) Biochem. Int. **19:** 257-266

Prockop D. J., Kivirikko K. I. Tuderman L. and Guzman N. A. (1979) New Engl. J. Med **301**: 13-23

Prockop D. J., Kivirikko K. I., Tuderman L. and Guzman N. A. (1979) New Engl. J. Med. **301**: 77-85

Prockop D. J. and Kivirikko K. I. (1995) Ann. Rev. Biochem. **64**: 403-434

Pucci-Minafra I., Minafra S., Sciarrino S., Tomasino R. M. and Tinervia R. (1986) J. Submicrosc. Cytol. **18**: 795-805

Pucci-Minafra I. , Luparello C. , Andriolo M. , Basirico L. , Aquino A. and Minafra S. (1993) Biochem. **32:** 7421-7427

Pucci-Minafra I. , Andriolo M. , Basirico L. , Aquino A. , Minafra S. , Boutillon M. M. and van der Rest M. (1995) Biochem. Biophys. Res. Comm. **207:** 852-859

Radford D. M. , Fair K. , Thompson A. M. , Ritter J. H. , Holt M. , Steinbrueck T. , Wallace M. , Wells S. A. Jr. and Donis-Keller H. R. (1993) Cancer Res. **53**: 2947-2950

Ravazzolo R. , Karsenty G. and de Crombrugghe B. (1991) J. Biol. Chem. **266:** 7382-7387

Rennie J (1996) (editor in chief) Scientific American 126-132

Rochlitz C. F., Scott G. K., Dodson J. M., Liu E., Dollbaum C., Smith H. S. and Benz C. C. (1989) Cancer Res. **49**: 357-360

Roeckel D. and Krieg T. (1994) Exp. Cell Res. **211:** 42-48

Roesel R. A. , Cutroneo K. R. , Scott D. F. and Howard E. F. (1978) Cancer Res. **38:** 3269-3275

Rossi P., Karsenty G., Roberts A. B., Roche N. S., Sporn M. B. and de Crombrugghe B. (1988) Cell **52**: 405-414

Sakakibara K. , Suzuki T. , Motoyama T. , Watanabe H. and Nagai Y. (1982) Cancer Res. **42:** 2019-2027

Sakakura T. (1991) Int. Rev. Cytol. **125:** 165-202

Sanger F., Nicklen S. and Coulson A. R. (1977) Proc. Natl. Acad. Sci. USA. **74**: 5463-5467

Schiro J. A. , Chan B. M. C. , Roswit W. T. , Kassner P. D. , Pentland A. P. , Hemler M. E. , Eisen A. Z. and Kupper T. S. (1991) Cell **67:** 403-410

Schuster V. L. , Lu R. , Kanai N. , Bao Y. , Rosenberg S. , Prie D. , Ronco P. and Jennings M. L. (1996) Biochim. Biophys. Acta **1311:** 13-19

Setoyama C. , Liau G. and de Crombrugghe B. (1985) Cell **41:** 201-209

Shirasawa S., Furuse M., Yokoyama N. and Sasazuki T. (1993) Science **260**: 85-88

Singh R. K. , Gutman M. , Bucana C. D. , Sanchez R. , Llansa N. and Fidler I. J. (1995) Proc. Natl. Acad. Sci. **92:** 4562-4566

Sitas F. , Terblanche M. and Madhoo J. (1990 and 1991) Natl. Cancer Registry of South Africa, Annual Statistic Report

Slack J. L., Parker M. I., Robinson V. R. and Bornstein P. (1992) Mol. Cel. Biol. **12**: 4714-4723

Slack J. L., Liska D. J. and Bornstein P. (1993) Amer. J. Med. Genet. **45**: 140-151

Slack J. L., Parker M. I. and Bornstein P. (1995) J. Cell. Biochem. **58**: 380-392

Smith M. R., DeGudicibus S. J. and Stacey D. W. (1986) Nature **320**: 540-543

Soule H. D., Vazquez J., Long A., Albert S. and Brennan M. (1973) J. Natl. Cancer Inst. **51**: 1409-1413

Spandidos D. A. (1987) Anticancer Res. **1**: 991-996

Stetler-Stevenson W. G. , Aznavoorian S. and Liotta L. A. (1993) Ann. Rev. Cell Biol. **9:** 541-573

Stracke M. L. , Aznavoorian S. , Mulvaney P. T. and Liotta L. A. (1996) From the book Encyclopedia of Cancer, Volume III, Academic Press Incorp. , San Diego, California

Strauli P. and Haemmerli G. (1984) Cancer Metastasis Rev. **3**: 127-141

Swanson G. M., Ragheb N.E, Lin C-S., Hankey B.F., Miller B., Horn-Ross P., White E., Liff J.M., Harlan L.C., McWhorter W.P., Mullan P.B. and Key C.R. (1993) Cancer **72**: 788-798

Sykes B. , Puddle B. , Francis M. and Smith R. (1976) Biochem. Biophys. Res. Comm. **72:** 1472-1480

Tandon A. K., Clark G. N., Charmers G. C., Ullrich A. and Mc Guire W. L. (1989) J. Clinc. Oncol. 7: 1120-1128

Thompson A. M., Anderson T. J., Condie A., Prosser J., Chetty U., Carter D. C., Evans H. J. and Steel C. M. (1992) Int. J. Cancer **50**: 528-532

Thorgeirsson U. P., Turpeenniemi-Hujanen T., Williams J. E., Westin E. H. , Heilman C. A., Talmadge J. E. and Liotta L. A. (1985) Mol. Cell. Biol. 5: 259-262

Van Den Hooff A. (1988) Adv. Cancer Res. **50:** 159-196

Varley J. M., Brammar W .J., Lane D. P., Swallow J. E., Dolan C. and Walker R. A. (1991) Oncogene **6**: 413-421

Vezeridis M. P. (1993) in Colorectal Cancer (Wanebo H. J. ed) Mosby Publishers, 86-105

Vogelstein B. , Fearon E. R. , Hamilton S. R. , Kern S. E. , Preisinger A. C. , Leppert M. , Nakamura Y. , White R. , Smits A. M. and Bos J. L. (1988) N. Engl. J. Med. **319:** 525-532

Vogelstein B. and Kinzler K. W. (1994) Cell **79**: 1-3

Vuorio E. and de Crombrugghe B. (1990) Ann. Rev. Biochem. **59:** 837-872

Weinberg R. A. (1989) Cancer Res. **49**: 3713-3721

Weiss J. B. and Ayad S. (1982) From the book: Collagen in Health and Disease (Weiss and Jayson eds) Churchill Livingstone, Medical Division of Longman Group Limited

Weis L. and Ward P. M. (1983) Cancer Metastasis Rev **2**: 111-127

Wiestner M., Krieg T., Horlein D., Glanville R. W., Fietzek P. and Muller P. K. (1979) J. Biol. Chem. **254**: 7016-7023

Wilson C. L., Heppner K. J., Rudolph I. A. and Matrisian L. M. (1995) Mol. Cell. Biol. **6**: 851-869

Wooster R. et al, (1994) Science **265**: 2088-2090

Yoshida K. , Yokozaki H. , Niimoto M. , Ito H. , Ito M. and Tahara E. (1989) Int. J. Cancer **44:** 394-398

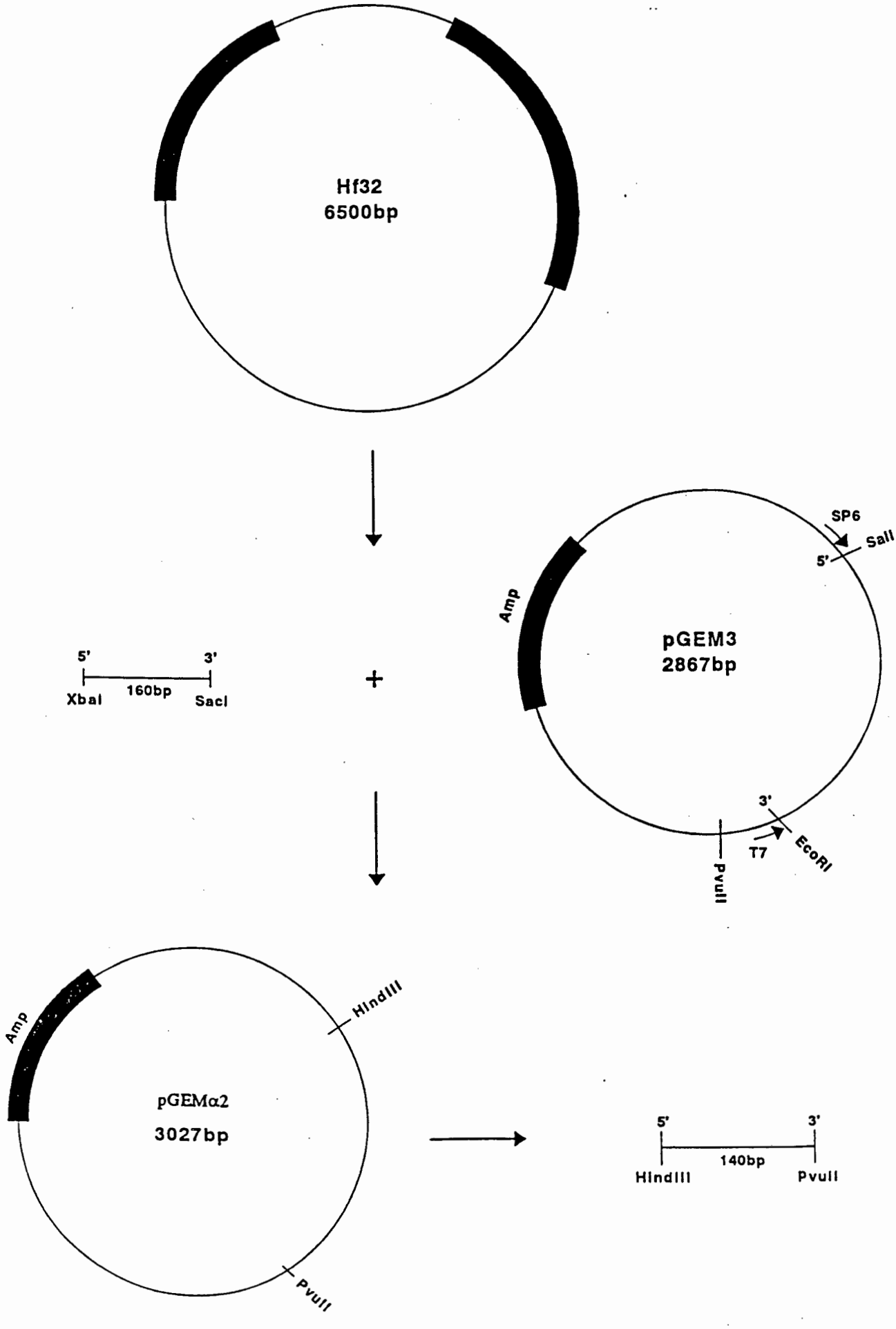
Zhu G-G. , Stenback F. , Risteli L. , Risteli J. and Kauppila A. (1993) Cancer **72:** 1679-1684

APPENDIX A

CLONING FRAGMENTS OF THE α1(I) AND α2(I) COLLAGEN GENES INTO THE VECTOR pGEM3 AS RIBOPROBES FOR IN SITU HYBRIDISATION

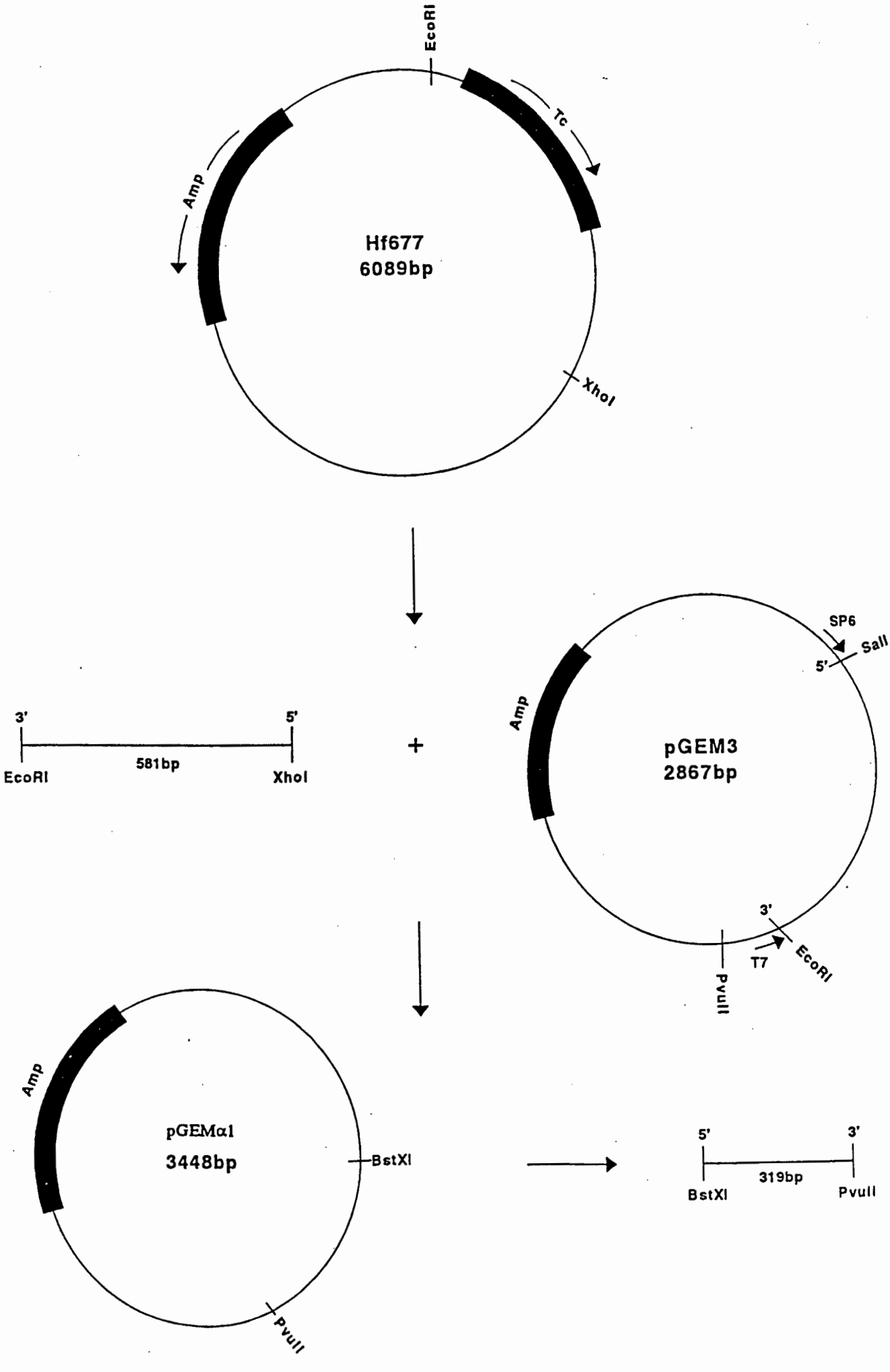
THE α1(I) COLLAGEN FRAGMENT

The vector pGEM3 was digested with restriction enzymes EcoRI and SalI and the plasmid Hf677, which contains the full length α1(I) collagen cDNA, was digested with EcoRI and XhoI (producing a fragment size of 581 base pairs). This fragment was eluted from a 1% low melting agarose gel, electrophoresed in 1X TAE, along with pGEM3, and cloned by means of in gel ligation. pGEM3 containing the α1(I) collagen fragment was termed pGEMα1. A schematic diagram of the cloning is illustrated on page . For the purpose of in situ hybridisation, pGEMα1 was digested with the restriction enzymes PvuII and BstXI, producing a 319 base pair fragment. This fragment was electrophoresed on a 1% low melting agarose gel and eluted from the gel using Qiaex as per the manufacturers instructions, 40 units T7 RNA polymerase was then used to transcribe antisense digoxigenin-labelled RNA.


Hf32
6500bp
5'
3'
XbaI
160bp
SacI
+
SP6
SalI
5'
Amp
pGEM3
2867bp
3'
EcoRI
T7
PvuII
Amp
HindIII
pGEMα2
3027bp
PvuII
5'
3'
HindIII
140bp
PvuII

THE α2(I) COLLAGEN FRAGMENT

The vector pGEM3 as well as the plasmid Hf322 (which contains the full length α2(I) collagen cDNA) were subjected to restriction analysis using the enzymes Xbal and Sacl. Upon digestion, these enzymes produced a 160 base pair fragment from Hf322 which was cloned into pGEM3 and termed pGEMα2. A schematic diagram of the cloning strategy is shown on page . For the purposes of in situ hybridisation, pGEMα2 was digested with restriction enzymes Pvull and Hindlll, producing a 140 base pair fragment. This fragment after elution and Qiaex was used to transcribe antisense digoxigenin-labelled RNA with T7 RNA polymerase.


EcoRI
Tc
Amp
Hf677
6089bp
XhoI
3'
5'
581bp
EcoRI
XhoI
+
SP6
SalI
5'
Amp
pGEM3
2867bp
3'
EcoRI
T7
PvuII
Amp
pGEMα1
3448bp
BstXI
PvuII
5'
3'
319bp
BstXI
PvuII