THE UNIVERSITY OF NOTTINGHAM
QUEEN'S MEDICAL CENTRE
DEPARTMENT OF BIOCHEMISTRY

EXTRACELLULAR MATRIX GLYCOPROTEINS OF SKIN FIBROBLASTS IN TUBEROUS SCLEROSIS

by

Hamdi UYSAL, D.V.M

Thesis submitted to the University of Nottingham
for the degree of Doctor of Philosophy

December 1995

This thesis is dedicated to the memory of my mother,

Zeliha UYSAL

who died shortly after I began primary school.
Her greatest wish was to see me become a doctor.

Table of Contents

Abstract

The first main objective of this project was to isolate cellular fibronectin from cultures of skin fibroblasts derived from tuberous sclerosis (TS) patients and normals and to compare their structures, paying particular attention to the content and pattern of carbohydrate (glycosylation). The second main objective of this project was to establish the expression and localisation of glycoproteins fibronectin, laminin and tenascin of the extracellular matrix (ECM) in cultures of skin fibroblasts derived from patients with TS and normal individuals. In order to achieve these objectives, fibroblasts were established from primary cultures of skin explants of patients with TS. Control cells were cultured from skin explants donated by people not known to be suffering from any disorder. The purification of cellular fibronectin was achieved from conditioned medium of skin fibroblasts of TS patients and control fibroblasts using Prosep-gelatin affinity chromatography and gel filtration chromatography techniques.

Analysis of purified cellular fibronectin by high pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) revealed that the carbohydrate portion of the fibronectin molecule was made up of galactose, mannose, glucosamine, galactosamine, sialic acid and fucose. An increased concentration of sialic acid, galactosamine, glucosamine, galactose and mannose was observed in purified fibronectin derived from neck and ungual fibromas of patients with TS. To provide a total increase of carbohydrates more than two fold in comparison to normal fibroblasts-derived fibronectin. Purified cellular fibronectin from conditioned medium of fibroblasts grown from skin lesions of different TS patients and from normal skin fibroblasts did not express HNK-1 (anti-leu 7) carbohydrate epitope. Normal skin fibroblasts showed an altered morphology and less confluence when grown on cell culture plates coated with cellular fibronectin derived from TS fibroblasts compared with control fibronectin. This may be a consequence of an altered glycosylation of this protein. The amino acid composition of the purified fibronectin from TS fibroblasts was very similar to that purified fibronectins from normal fibroblasts and to standard commercial plasma and cellular fibronectins.

Laminin and tenascin were partially purified from conditioned cell culture medium demonstrating their synthesis and secretion into the cell culture

medium by dermal skin fibroblasts. Expression and distribution of fibronectin, tenascin and laminin by established TS and normal skin fibroblasts using immunofluorescence, ELISA, and flow cytometry techniques were analysed and presented qualitative and quantitatively in this thesis. Increased expression and altered distribution of fibronectin and tenascin were observed in the fibroblasts derived from ungual fibroma lesion of a TS patient, but not in fibroblasts of neck fibroma, forehead plaque lesion or unaffacted skin of TS patients in comparison to control fibroblasts. However, increased expression and altered distribution of laminin were observed in neck fibroma-derived fibroblasts in contrast to fibronectin and tenascin. Laminin expression was not changed in ungual fibroma and forehead plaque lesion-derived fibroblasts in comparison to control fibroblasts. Altered distribution of fibronectin was well observed by immunofluorescence particularly in large cells of ungual fibroma. Similar differences were observed with laminin of cells from neck fibroma of TS patients.

These results suggest the abnormal assembly of ECM in different TS skin lesions. Abnormal migration of cells during early embryonic development and the hardening of tissues associated with TS may result from abnormal assembly of the ECM. Alterations in distribution and structure of these adhesive glycoproteins may cause functional disruption in their binding and interactions with cells and ECM macromolecules. Studies of these changes in the ECM components may contribute to the understanding of the mechanisms involved in the aetiology of hardened tissues of TS.

Acknowledgements

I would firstly like to thank my supervisor, Professor Frank Hemming, for his constant support, encouragement, patience and wisdom during this project. I was fortunate to have the opportunity to work with Professor Hemming and to have his kind supervision in this interesting field of study.

I would also like to thank past and present deans of the Faculty of Veterinary Medicine, and chancellors of the University of Yüzüncü Yıl in Turkey for providing a scholarship to study Ph.D.

I would particularly like to thank Biochemistry Professors Nihat Baysu, Hayati Çamas, Hilal Karagül, Arif Altıntas and Ethem Ersoy who supported and played significant roles in my education in Turkey.

I must thank Professor J.N. Hawthorne for providing the opportunity to study in the department and his friendly welcoming and ongoing support. A special thank you to Miss Janice Saxton, always my first port of call in the lab. for her expert knowledge of 'Dionex'. I am ever so grateful to her for supporting and helping to me. Many thanks to Miss Jackie Bostock, and Miss Nicola Conran for creating a cheerful and friendly atmosphere around the lab. and giving priority in delivering my letters personally. Many thanks also go to Dr. Zhila Ellis and Mrs Maureen Mee who introduced me to cell culture work.

I also wish to acknowledge the following people; Dr. Adrian Robins and Mrs Alison Galvin for their help in providing the flow cytometer for use in the Department of Immunology. Dr. Stephen Fleming for providing a confocal microscope for use in the Department of Obs. & Gyn. Mr. Alan Pyper for providing an immunofluorescence microscope for use in the Department of Human Morphology, and Mr Kevin Bailey for his excellent technical assistance.

I am most grateful to my friends, Dr. Robert Layfield and Dr. Washington Y. Ochieng who made many helpful suggestions during the completion of this thesis. I am also most grateful to my other friends Ulvi, Nihal, Talat, Öner, Kezi, Ilknur and Donald whose friendship has helped to relieve the pressure.

Last but not least, my biggest and most special thanks go to my parents, brother and sisters for all their care and support throughout my education, and Nermin for her love, support, patience and friendship.

List of Figures

List of Tables

Abbreviations

ABTS	: 2,2'-azinobis(3-ethylbenzthiazoline sulphonic acid)
APS	: ammonium persulphate
BSA	: bovine serum albumin
DMEM	: Dulbecco's modified eagle's medium
DMSO	: dimethyl sulfoxide
DSA	: *Datura stramonium agglutinin*
ECM	: extracellular matrix
EDTA	: ethylenediamine tetraacetic acid
EGF	: epidermal growth factor
ELISA	: enzyme-linked immunosorbent assay
FCS	: foetal calf serum
FITC	: fluorescein isothiocyanate
FN	: fibronectin
FPLC	: fast performance liquid chromatography
FSC (or FALS)	: forward scatter light (forward angle light scatter)
Fuc	: fucose
GAGs	: galactose
GalN	: galactosamine
GFAP	: glial fibrillary acidic protein
Glc	: glucose
GlcN	: glucosamine
GNA	: *Galanthus nivalis agglutini*
GPI	: glycosylphosphatidyl inositol
H_2O_2	: hydrogen peroxide
HPAEC	: high performance anion exchange chromatography
LM	: laminin
mA	: milliamps
MAA	: *Maackia amurensis agglutinin*
Man	: mannose
NaCl	: sodium chloride
NaOH	: sodium hydroxide
NC	: nitrocellulose
Neu5Ac	: *N*-acetylneuraminic acid
PAD	: pulsed amperometric detection
PBS	: phosphate-buffered saline
PMSF	: phenylmethylsulphonyl fluoride
PNA	: *Peanut agglutinin*
PNGase	: peptide-N-glycosylhydrolase
PVDF	: polyvinylidene fluoride
RGD	: Arginine-Glycine-Aspartic acid, cell binding sequence
SDS	: sodium dodecyl sulfate
SDS-PAGE	: sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNA	: *Sambucus nigra agglutinin*
SSC (or RALS)	: side scatter light (right angle light scatter)
TBS	: tris-buffered saline
TEMED	: N,N,N',N'-tetramethylenediamine
TFA	: trifluoroacetic acid
TN	: tenascin
Tris	: tris (hydroxymethyl)-aminomethane

Chapter 1

Chapter 1

Introduction

This chapter will review some of the general aspects of tuberous sclerosis, and also structure function relationships of the major adhesive glycoproteins fibronectin, laminin and tenascin of the extracellular matrix synthesised in cultures of human skin fibroblasts.

1.1 What is Tuberous Sclerosis ?

Tuberous sclerosis (TS) is a genetic disorder that causes a variety of neurological and physical symptoms including seizures, mental retardation, tumours and skin lesions. The name derives from a description of its cerebral lesions by the French neurologist Bourneville in 1880. The brain, skin, heart, eyes, lungs and kidneys are the most frequently affected organs. Therefore, the disease is often referred to as tuberous sclerosis complex (TSC), emphasising the multiplicity of organs involved. In 1890, Pringle succeeded in bringing about the first explanation of the significance of the neurocutaneous relationship of adenoma sebaceum, epilepsy and mental retardation. Berg reported the first hereditary nature of TS in 1913 (Gomez, 1979). Recently, linkage studies have established locus heterogeneity with disease-determining genes on chromosomes 9 (Fryer *et al.*, 1987) and more recently 16 (Kandt *et al.*, 1992; Nellist *et al.*, 1993). It has been shown histopathologically that TSC is a disorder of cellular migration, proliferation and differentiation (Johnson and Gomez, 1991). Recent data suggest that TS has a birth incidence of up to one in 5800 (Osborne *et al.*, 1991).

1.1.1 Clinical Signs of Tuberous Sclerosis

The complete syndrome of TS exhibits a wide range of clinical symptoms with degrees of severity. Gomez (1988) suggested that the clinical features of TS may be classified according to their diagnostic importance as pathognomonic (definitively diagnostic), or presumptive (provisionally diagnostic).

(i) Definitive Diagnosis of TS

The following symptoms are considered pathognomonic:

a) Cortical tubers, which can be recognised by direct inspection, histopathological examination or computed tomography (CT) scan and magnetic resonance (MR).

b) Subependymal glial nodules, which are demonstrated by pathologic examination or neuroimaging (CT or MR).

c) A lesion in the retina with the typical appearance of a hamartoma.

d) Skin lesions such as facial angiofibroma, periungual or subungual fibromas and scalp/ forehead fibrous plaques.

e) Multiple renal angiomyolipomas (unilateral or bilateral) as demonstrated by renal imaging methods (CT, MR or ultrasound).

(ii) Presumptive Diagnosis of TS

The common diagnostic features of TS include:

a) infantile spasms.
b) seizures.
c) hypomelanotic macules.
d) shagreen patches.
e) gingival fibromas.
f) multiple renal tumours.
g) renal cysts.
h) cardiac rhabdomyoma.
i) pulmonary lymphangiomyomatosis.

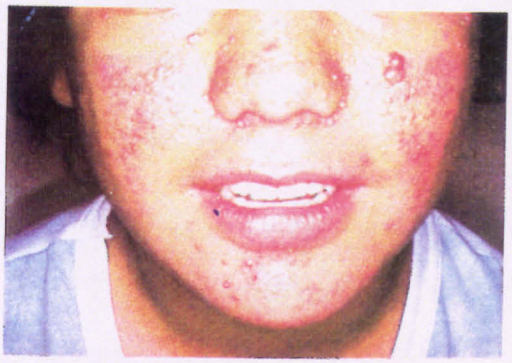
j) dental enamel pits.
k) immediate relative with TSC.

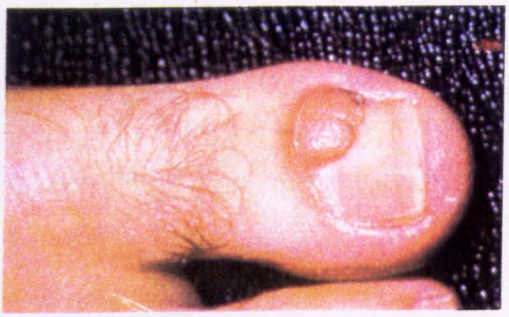
The diagnosis of TS should be suspected if only one feature is present, or considered presumptive if two or more features are present. These diagnostic features imply that what constitutes TS is not a particular symptom or sign but specific lesions in certain organs (Gomez, 1988). In one study involving 160 patients (88 male, 72 female), the most common presenting symptom (88% of the cases) was seizures. However, this symptom was less frequent than the most common abnormal finding on examination, namely the skin lesions observed in 91% of the patients. Mental retardation (60%) and retinal hamartoma (50%) were the third and fourth most common clinical features of TS (Gomez, 1979).

1.1.1.1 Dermatologic Manifestations

As the most common abnormal finding, dermatologic manifestations are critical to the diagnosis of TS. The typical skin lesions of TS are described briefly below.

The most characteristic of the skin lesions are facial angiofibromas (improperly called adenoma sebaceum) which occur in pink red papules or nodules with a smooth shiny surface. These pink or flesh coloured papules occur in nasolabial folds and on the cheeks (see *Fig. 1.1-a*). Facial lesions start to appear in the first year of life and fully developed angiofibromas occur by the age of 5 years. When the patient reaches puberty the lesions may become more various and prominent, and can require repeated dermal abrasion or other procedures for cosmesis (Gomez, 1988; Kwiatkowski and Short, 1994).





(a) Angiofibroma **(b)** Ungual fibroma

Figure 1.1 Skin lesions of tuberous sclerosis (Photographs kindly provided by TS association of Great Britain).

The main findings in facial lesions, dermal fibrosis and vasodilatation, make the term "angiofibroma". Elastic tissue is absent from these lesions. Irregularly, large stellate fibroblasts give the tissue a "glial appearance" (Gomez, 1988). Angiofibromas also develop under and around the nails (ungual fibromas) as seen in *Figure 1.1-b.*

Multiple ungual fibromas are diagnostic of TS and usually appear late in life, age 15 through 60 years (Kwiatkowski and Short, 1994). The ungual fibromas are flesh or red coloured papules or nodules appearing on the toes, but can sometimes occur on fingers. Histopathologically, ungual fibromas are similar to facial angiofibromas or forehead plaques. The older lesions of ungual fibromas may also accommodate large stellate fibroblasts with a "glial appearance".

Shagreen patch (skin with the appearance of untanned leather) is a connective tissue hamartoma. Such patches are usually organised in the

lumbosacral area. The size of a shagreen patch may change from a few millimetres to 10 or more centimetres. They are slightly raised above the surrounding skin surface with a pink or yellowish-brown colour and the appearance of pig skin. These shagreen patches are not absolutely pathognomonic signs but still a very helpful finding in the diagnosis of TS (Gomez, 1988).

1.1.2 Pathogenesis

The name tuberous sclerosis (TS) is derived from the descriptive designation originally based on the particular anatomic abnormalities in the brain that are distinctive for the disease. For instance, tubers (cerebral hamartias) and subependymal nodules or subependymal giant cell astrocytomas are the characteristic features of central nervous system in this disease (Edward and Richardson, 1991; Kwiatkowski and Short, 1994). The other organs most frequently involved in TS are the retina, skin, kidneys, heart and lungs. Many speculations have been made concerning the pathogenesis of TS. Bournville and Brissaud (1881) postulated an inflammatory aetiology (fetal encephalitis) in one of the first papers on this disease. Hartdegen proposed a neoplastic aetiology for TS in 1881. Others supported this idea based on the presence of "monster cells, benign astrocytic nodules and occasional malignant tumours in the brain of patients with TS, all indicating a neoplastic tendency" (Ferraro and Doolittle, 1936). Moolten (1942) reported the hamartomatous nature of the tumours in the brain and other organs emphasising their lack of malignant characteristics such as mitotic cells or metastatic spread.

Inglis (1950) indicated initially that an intrinsic neural factor influenced cells in various organs at an early multipotential stage of organogenesis, causing them to develop along the lines of immature nerve sheath cells. He also observed that the renal lesions contain elongated cells that are histologically

more similar to immature nerve sheath cells than to smooth muscle fibres. He proposed that the same neuronal intrinsic factor underlies the angiomatous and lipomatous elements of extracerebral TS tumours.

Rundle *et al.* (1975) searched serum levels of four main immunoglobulins, IgA, IgM, IgG and IgD, in 54 patients with TS. They found normal levels of IgA and IgD in the TS patients, but increased levels of IgM, and a significant decrease in IgG levels.

Fabing (1934) studied TS in twins, identical in appearance and both with mental dullness, seizures, and adenoma sebaceum. He suggested that the mirror-image nature of their central nervous system and skin lesions revealed the defect had developed early in embryogenesis. His cases, however, lacked pathological demonstration of the cerebral involvement and the mirror-image skin lesions were not those of TS. Recently Gomez *et al.* (1982) declared two sets of monozygous twins who were discordant in seizures and intelligence. The mentally normal twin of each set had few or no seizures whereas the mentally abnormal twins had been subjected to frequent infantile spasms or seizures.

It has been reported that TS is a disorder of collagen metabolism, which results in the absence of elastic tissue from the lesions of adenoma sebaceum. As the angiofibromas mature, the collagen becomes sclerotic and layered. Occasionally, large stellate fibroblasts occur with a "glial appearance". Histologically, the forehead and scalp plaques are fibromata without the vascular components. Hyalinisation and sclerosis of tissue due to changes in the collagen fibres are common findings in older adenoma sebaceum lesions (Gomez, 1988).

1.1.3 Genetics

Tuberous sclerosis (TS) is a multisystem disorder that is inherited as an autosomal dominant trait. The hereditary nature of TS was first recognised by Berg in 1913 (Wiederhalt *et al.*, 1985). Furthermore, Gunther and Penrose (1935), and Nevin and Pearce (1935) explained the dominant inheritance of TS and its high mutation rate.

Recognition of genetic heterogeneity is essential in trying to locate a disease gene and also for prenatal and presymptomatic diagnosis. It has been demonstrated that locus heterogeneity, in which the same phenotype can result from genes at two or more loci, occurs in tuberous sclerosis (Conneally, 1991). Linkage studies have confirmed locus heterogeneity for TS with disease-determining genes on chromosomes 9 (Fryer *et al.*, 1987) and 16 (Kandt *et al.*, 1992). Recent studies have suggested that the gene for TS is linked to the loci for the ABO blood group, which are also on the long arm of chromosome 9 (q34). More recently, a TS gene on chromosome 16 has been identified and characterised as a result of the co-operation between three different research groups collectively known as the European Chromosome 16 Tuberous Sclerosis Consortium (Nellist *et al.*, 1993)

1.1.4 Biochemistry

There is no basic defect described in TS or any known specific biochemical marker for the disease in spite of previous intensive studies. Biochemical investigators, however, continue to search for a clue to identify the main defect of TS.

Collagen synthesis, measured by the activities of prolylhydroxylase, was clearly increased in angiofibromas of three patients and in one soft tumour of the face, but it was unchanged in shagreen patches (Oikarinen *et al.*, 1982). Tanaka *et al.*, (1983) found increased levels of free serum proline in TS

patients. Rundle (1976) found quantitative differences in haptoglobulin α-1 acid glycoprotein, ceruloplasmin, β-1 E-globulin and transferrin in comparison with TS. According to this study, there were no changes in α_1-antitripsin and fibrinogen levels. Ceruloplasmin, α_1-acid glycoprotein and β_1-globulin levels were increased in affected subjects. Transferrin and haptoglobulin showed decreased concentration in affected subjects. Subsequently, none of these protein markers have proven to be consistently different in patients with TS. Fischer *et al.,* (1977) previously reported that amino acid analyses on the acid hydrolysates of an angiofibroma and skin established that the former tissue contained less collagen than skin based on the reduced content of glycine, proline, alanine and hydroxyproline in the tumour. Lysosomal enzyme measurements have shown that the specific activities of the hexosaminidases, β-galactosidase and β-glucuronidase were raised. It was also demonstrated, by uronic acid and hexosamine measurements, that the tumour contained more glycosaminoglycan than skin. The most outstanding finding of their study was the seven fold increase in the total carbohydrate in the glycosaminoglycan fraction of the tumour. The sulphate content was higher in tumour tissue than foetal skin.

Henderson (1991) studied the glycosaminoglycan composition of the fibroblasts, and showed that there was no change in the glycosaminoglycan composition of TS and normal cells. Fibroblasts produced heparan sulphate with markedly less N-sulphation than did normal fibroblasts. Previous metabolic studies of Ellis and Hemming (1990) at the University of Nottingham revealed that fibroblasts from skin lesions of TS patients contained higher levels of carbohydrates associated with glycoproteins. The most important increases were observed in glycoproteins with molecular masses in the region of 200-240, 135-160 and 80-90 kDa. These were further investigated using antibody raised against human fibronectin. TS and normal fibroblasts cell extracts were applied to SDS/PAGE followed by

blotting on to nitrocellulose. Treatment of the "blot" with rabbit anti-fibronectin and peroxidase confirmed that the 220 kDa protein was fibronectin. Their work also showed an elevated production of fibronectin synthesized from fibroblasts of TS patients. One of the aims of this thesis is to extend this preliminary work of Ellis and Hemming in this laboratory.

1.1.5 Biology of the Cell

Histopathologically, TS is a disorder of cellular migration, proliferation and differentiation. Large, slowly dividing dendritic cells in cultures of skin lesions have been described by Johnson *et al.* (1991). The large slowly dividing dendritic cells in cultures of TS lesions are referred to as N-cells. These N-cells could represent a normal cell type in an unexpected location or a pathologic cell in TS lesions. N-cells differ from astrocytes because they stain negatively for glial fibrillary acidic protein (GFAP). GFAP occurs in astrocytes, some ependymal cells, and tumours of these cells (Johnson *et al.*, 1991).

However, Ishibashi *et al.* (1990) reported that fibroblasts of adenoma sebacea in patients with TS synthesized a GFAP in addition to tubulin, actin, fibronectin and vimentin. Lallier (1991) proposed a model for TS mutation effects on neural crest cell migration. In early embryonic development, normal neural crest cells may migrate through a series of extracellular matrix molecules, causing them to differentiate into a specific cell type. TS mutant cells may lack the ability to interact properly with the matrix molecules they encounter, leading to misplaced cells or cells that differentiate into abnormal cell types as seen in *Figure 1.2.*

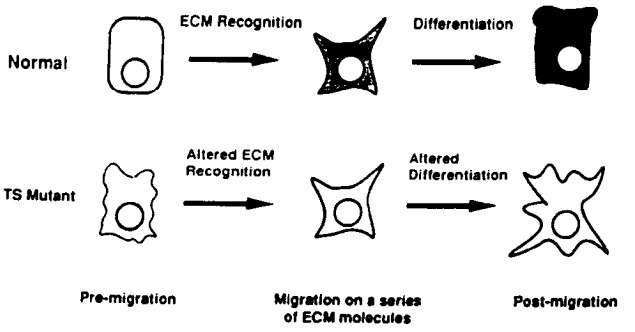


Figure 1.2. A model for tuberous sclerosis mutation effects on neural crest cell migration (After Lallier, 1991).

1.1.6 Prevalence and Treatment of the Disease

The prevalence rate of tuberous sclerosis at birth is now estimated to be perhaps as high as 1 person in 5800 (Osborne *et al.*, 1991). The disorder affects both sexes and all races. Unfortunately there is no cure for TS, but there is treatment available for its symptoms. For instance, anticonvulsant drugs (Nitrazepam and Clonazepam) have been used to control seizures more effectively. Children with learning disabilities and speech and language difficulties are being helped through more sophisticated educational techniques. Orthopaedists, neurologists, physical therapists and other professionals help handicapped patients learn daily living skills and ways to improve mobility.

Argon laser treatment is quite effective in removing facial angiofibromas. Fibromas of nails can also be treated with lasers, and can be removed by surgery. Renal disease is most troublesome when angiomyolipomas bleed. Angiomyolipomas and renal cysts are the most characteristic renal abnormalities of TS. In patients with severe bleeding, the only life-saving

procedure may be the removal of the whole kidney. A person can live with only one healthy kidney, but in patients with tuberous sclerosis both kidneys are already affected by angiomyolipomas, and the removal of one kidney may leave one that is already impaired. For this reason, in cases of emergency operations partial removal of the kidney or removal of the bleeding angiomyolipoma will be preferable, leaving the functioning part of the kidney (Gomez, 1983).

1.2 Extracellular Matrix (ECM) and Tuberous Sclerosis

The extracellular matrix, the substratum that cells adhere to *in vivo*, consists of glycoproteins and proteoglycans that are secreted by cells. The extracellular matrix contains several adhesive glycoproteins that bind to both cells and other matrix macromolecules. Connective tissue cells, such as dermal fibroblasts and chondrocytes, are completely surrounded by their extracellular matrix. Matrix constituents can modulate the shape of the cell in culture. Fibronectin promotes spreading of many cell types such as fibroblasts. Laminin promotes attachment of cells. Both fibronectin and laminin promote adhesion of cells to collagen (Watt, 1986). Fibronectin, laminin, tenascin, vitronectin, the collagens, von Willebrand factor and thrombospondin are examples of adhesive ECM glycoproteins. Fibronectin is widely distributed in connective tissues, whereas laminin is found mainly in basal laminae. Several ECM proteins such as tenascin, laminin and thrombospondin contain domains with homology to epidermal growth factor (EGF), providing growth-promoting activity. As a result, they play an important role in the development and maintenance of cellular organisation and in tissue repair (Engel, 1989). Many of the adhesive glycoproteins contain the common tripeptide sequence (RGD) which is recognised by the cell surface integrins and helps bind cells to the ECM (see *Fig. 1.3*).

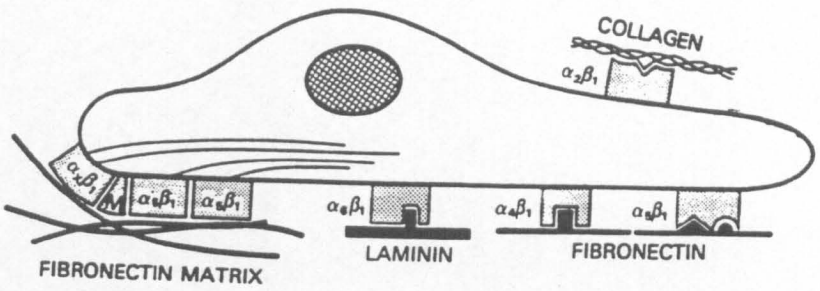


Figure 1.3. Representation of several cell interactions with ECM glycoproteins via specific recognition sequences (After Yamada, 1991).

Cells can use integrin receptors, for example $\alpha 5\beta 1$ to bind fibronectin, and $\alpha 6\beta 1$ to bind laminin. These receptors can participate in a number of biological processes, including adhesion, migration and assembly of a matrix. Fibroblasts are a class of cells that adhere, migrate and grow readily in tissue culture, adopting a flattened and/or elongated morphology. They are responsible for the production and maintenance of the connective tissue matrix. Fibroblasts in culture produce fibronectin. The fibronectin synthesised by fibroblasts has two fates; it can be incorporated into fibrillary matrices at the cell surface or it can be secreted to the culture medium (Hynes, 1990).

Fibroblasts also produce thrombospondin (Jaffe *et al.,* 1983) and tenascin (Erickson and Taylor, 1987) into the ECM but usually fail to synthesize significant amounts of laminin (Timpl *et al.*, 1982). As indicated previously, components of ECM such as fibronectin play an important role in the migration of cells during early embryonic development.

One of the various features of tuberous sclerosis (TS) is the hardening of affected tissue. The interrelations between cells and the surrounding extracellular matrix is an important parameter in the degree of hardening (Ellis *et al.,* 1991). Alterations in the structure and distribution of these

glycoproteins may change their binding and interactions with cells and ECM macromolecules. Studies of these changes could contribute to the understanding of the mechanisms involved in the aetiology of hardened tissues of TS.

1.3 Structure and Biosynthesis of Glycoproteins

Glycoproteins are defined as proteins which contain glycan chains linked glycosidically to selected amino acid residues. Glycosylation is an example of the post-translational modification of proteins. Monosaccharides commonly found in the glycans of the glycoproteins include mannose, galactose, glucose, fucose, xylose, N-acetylglucosamine, N-acetylgalactosamine, glucuronic acid, iduronic acid and N-acetylneuraminic acid (Hemming, 1991).

1.3.1 Carbohydrate-Protein Linkages

The oligosaccharide chains are attached to the polypeptide backbone of glycoproteins at one of five amino acid residues: asparagine (Asn), serine (Ser), threonine (Thr), hydroxylysine (Hyl) or hydroxyproline (Hyp). Oligosaccharides are covalently linked to the side chains of these amino acids along the polypeptide backbone by glycosidic links. The carbohydrate chains are classified according to the linkage between sugar and amino acid. Carbohydrates are attached to proteins at different positions by either an N-linkage, an O-linkage, or both, or a phosphoethanolamine-linkage (via glycosyl-phosphatidylinositol, GPI, anchor) (Lis and Sharon, 1993). Many glycoproteins such as human fibronectin, plasminogen, and calf fetuin contain both N- and O- linked glycans on the same polypeptide chain (Schachter and Brockhausen, 1992).

i) N-glycosidic link:

The most common type of linkage occurs between the anomeric carbon of N-acetylglucosamine monosaccharide and the nitrogen of the amide group of asparagine within the polypeptide chain (see *Fig. 1.4-b*).

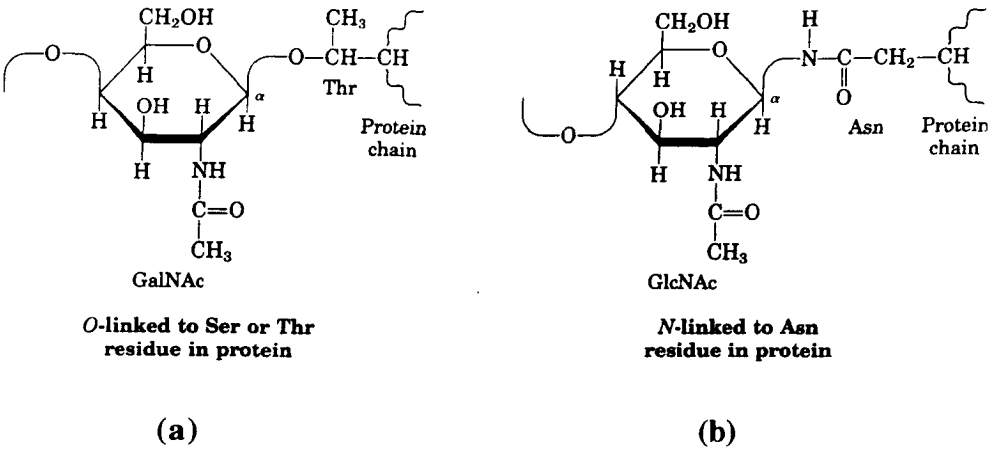


Figure 1.4. Two ways to link oligosaccharide chains to proteins (a) An O-glycosidic linkage, (b) An N-glycosidic linkage (Lehninger *et. al.*, 1993).

The many N-linked glycans of known structure can be categorised in terms of three basic oligosaccharide structures: high mannose, complex and hybrid as summarised in *Figure 1.5*. All of the known N-linked oligosaccharides have a common core pentasaccharide structure: Man α1-3(Man α1-6) Manβ1-4GlcNAcβ1-4GlcNAc-Asp (the boxed areas in *Fig. 1.5*).

a) *High Mannose (simple)*:

High-mannose oligosaccharides typically have 2-6 additional mannose residues linked to the pentasaccharide core.

b) *Complex (lactosaminyl)*:

Glycoproteins of complex type generally contain branches of a terminal sialic acid residue and underlying galactose and N-acetylglucosamine, the last two sugars often constituting the disaccharide N- acetyllactosamine

linked to the branched mannose residues of the core. The oligosaccharide branches are commonly referred to as antennae, so that bi-, tri-, tetra-, and penta-antennary structures may all be found. These structures sometimes contain a further N-acetylglucosamine linked to the β-mannose of the core. Some also contain fucose linked to the first N-acetylglucosamine of the core.

c) *Hybrid (mixed)*:

This form is the result of a combination of the above two cases.

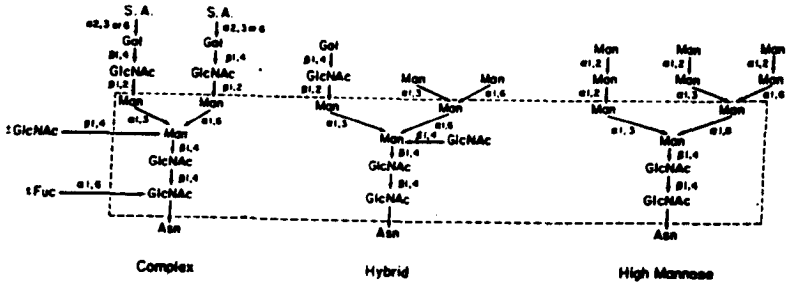


Figure 1.5. Major types of asparagine-linked oligosaccharide structures. The boxed area represents the pentasaccharide core common to all N-linked structures (from Kornfeld and Kornfeld, 1985).

All of the asparagine-linked oligosaccharide structures have the common pentasaccharide core structure (*Fig. 1.5*), because they all arise from the same biosynthetic precursor lipid-linked oligosaccharide. This is transferred to nascent peptide chains and then processed to form these various structures (Kornfeld and Kornfeld, 1985). Lis and Sharon (1993) reviewed the more recent advances on the structure and function of glycoproteins. *Figure 1.6* illustrates the protein-carbohydrate linkages known in 1982 and in 1992. Several new asparagine-linked monosaccharides have been discovered during the last decade. These include glucose (both in α- and β-linkages), β-N-acetylgalactosamine and L-rhamnose (in bacterial glycoproteins). More recently, a novel N-glycosylation (laminin contains the linkage unit β-glucosylasparagine) has also been reported in eukaryotes (Schrainer *et al.*, 1994).

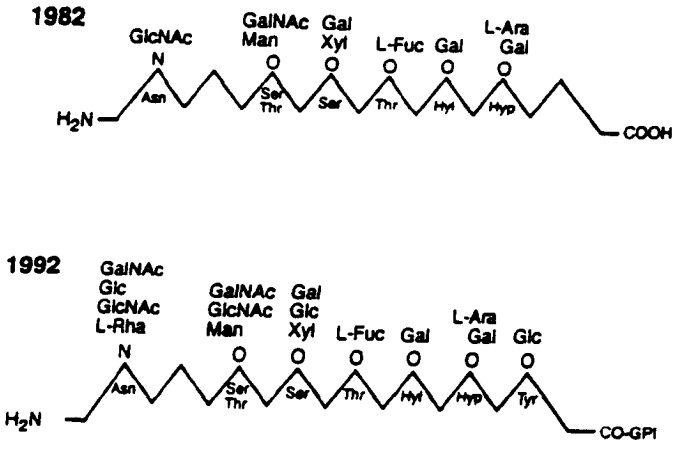


Figure 1.6. Protein-carbohydrate linkages known in 1982 and in 1992 (Lis and Sharon, 1993).

ii) The O-glycosidic link:

Many glycoproteins contain oligosaccharides linked through an *O*-glycosidic bond to the hydroxyl groups of serine, threonine, hydroxylysine or hydroxyproline amino acid residues. The most common *O*-glycosidic linkage found is that between N-acetylgalactosamine and serine or threonine (*Fig. 1.4-a*). This protein-carbohydrate linkage is usually referred to as the "mucin" type of linkage. *O*-glycosidic linkages may also occur between xylose and serine. Glycosaminoglycans (GAGs) are attached to proteins via this type of linkage. An *O*-glycosidic linkage is also found between galactose and hydroxylysine in collagen. The novel *O*-glycosidic linkages can be seen in *Figure 1.6*. Tyrosine has been added to the list of *O*-linked amino acids with the presence of Glcα-Tyr in glycogenin (Lis and Sharon, 1993). *Figure 1.7* shows some of the peripheral sugar sequences that may occur in glycoproteins.

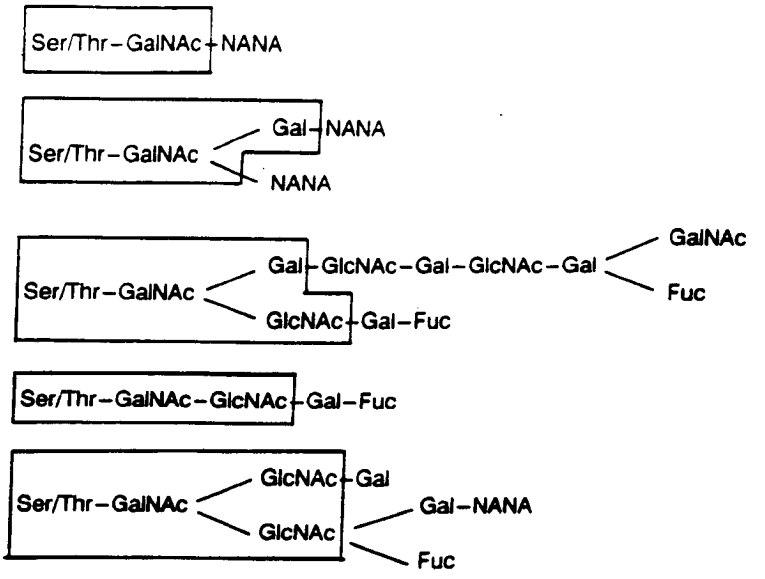


Figure 1.7. *O*-linked oligosaccharide sequences. Examples of peripheral sequences that may be linked to one of five core structures. Ser, serine; Thr, threonine; Gal, galactose; GlcNAc, N-acetylglucosamine; GalNAc, N-acetylgalactosamine; Fuc, fucose; NANA, N-acetylneuraminic acid (sialic acid). The core oligosaccharide is boxed (From Graham, 1992).

1.3.2 Synthesis of Oligosaccharides

The understanding of the *N*-glycosylation of proteins is well developed, unlike *O*-glycosylation or GPI assembly. Particular attention is therefore given to *N*-glycosylation here. As indicated in *Figure 1.8*, the endoplasmic reticulum (ER) and Golgi apparatus are the major sites involved in glycosylation processes. The oligosaccharide portion of glycoproteins is assembled in the endoplasmic reticulum on the lipid carrier dolichol phosphate (reviewed in Kornfeld and Kornfeld, 1985). The sugars are added by stepwise donation, with the first seven sugars (two GlcNAc and five Man) derived from the nucleotide sugars, UDP-GlcNAc and GDP-Man. The next seven sugars (four Man and three Glc residues) are derived from the lipid intermediates, Dol-P-Man and Dol-P-Glc residues. These initial stages of N-linked oligosaccharide synthesis result in formation of the precursor oligosaccharide $Glc_3Man_9GlcNAc_2$ on a pyrophosphoryl-dolichol backbone. This oligosaccharide is then transferred en block to a suitable

peptide asparagine residue. While the nucleotide sugar substrates are cytoplasmic, newly glycosylated proteins are found in the lumen of the rough endoplasmic reticulum. Moreover, dolichol (polyisoprenoid compound) is present mainly in endoplasmic reticulum membranes.

After the precursor $Glc_3Man_9GlcNAc_2$ is first transferred to the polypeptide chain (step 1, *Fig. 1.8*), processing is initiated by the removal of the terminal glucose residue from oligosaccharide by α-glucosidase-I (step 2). The two inner glucose residues are then removed by α-glucosidase-II (step 3). A specific α mannosidase which catalyzes the removal of at least one α1,2-linked mannose residue (step 4), acts next. The non-lysosomal glycoproteins (lysosomal glycoproteins undergo phosphorylation in the Golgi) can be further trimmed by the Golgi α1,2 mannosidase (Golgi Mannosidase 1) to yield a $Man_5GlcNAc_2$ structure (step 5).

In medial cisternae of the Golgi, a *N*-acetylglucosamine residue is added catalyzed by *N*-acetylglucosaminyl transferase 1, to become complex type structures (step 6). This is followed by the removal of two mannose residues by Golgi α-mannosidase II (step 7) and subsequent addition of another outer chain N-acetylglucosamine residue catalyzed by *N*-acetylglucosaminyl transferase II (step 8). At this stage, the addition of fucose to the asparagine-linked *N*-acetylglucosamine is catalyzed by fucosyltransferase (step 9).

In the final steps of the complex oligosaccharide synthesis, the galactosyl and sialyl transferase enzymes that then transfer galactose and sialic acid, respectively, to the glycoprotein are located mainly in the trans region of the Golgi apparatus (steps 10 and 11). The completed glycoproteins then exit Golgi and are transported to their final destinations. The final stage of biosynthesis in the Golgi apparatus is called "terminal glycosylation" to distinguish it from "core glycosylation", which takes place in the ER. More detailed description can be found in Kornfeld and Kornfeld (1985), and Hirschberg and Snider (1987).

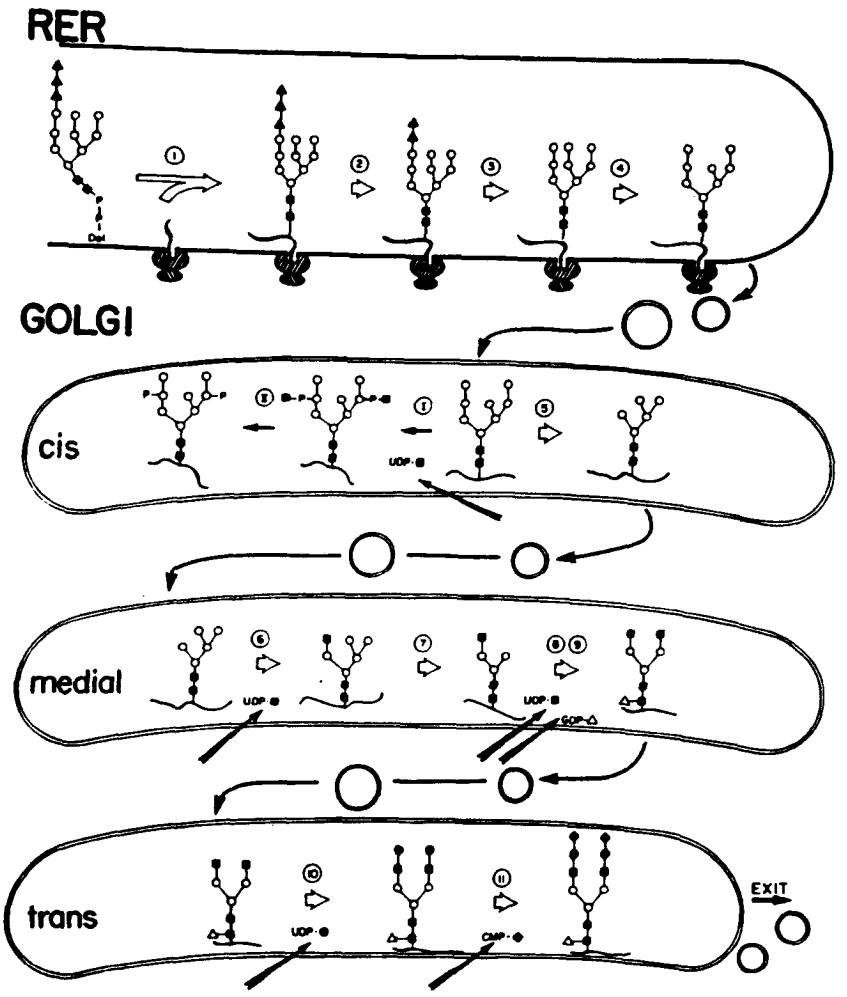


Figure 1.8. Schematic pathway of oligosaccharide processing on newly synthesized glycoproteins (from Kornfeld and Kornfeld, 1985)

Glycoproteins in the secretory pathway receive GalNAc in *O*-glycosydic linkage from-UDP-GalNAc via a transferase that acts directly on serine or threonine residues of proteins. No dolichol-linked intermediates are involved. Such *O*-linked glycosylation occurs only after the protein has been transported to the Golgi apparatus. The sugars of the oligosaccharide chains of the *O*-glycosidic type of glycoprotein are built up by the stepwise donation of sugars from nucleotide sugars (such as UDP-GalNAc, UDP-Gal, and CMP-NeuAc) catalyzed by membrane-bound specific glycosyltransferases.

1.4 Adhesive Glycoproteins of ECM Synthesized in Cultures of Human Skin Fibroblasts

1.4.1 Fibronectins

Fibronectins (FNs) are a group of large, dimeric adhesive glycoproteins possesing two subunits of 220-250 kDa which are joined by disulphide bonds. Fibronectin is the "grandfather" of extracellular matrix proteins and is currently one of the most widely studied and best known matrix proteins, although much still remains to be learned about its structure and function. Fibronectins are adhesive glycoproteins present on cell surfaces and in extracellular matrices, as well as in blood, amniotic fluid, and cerebrospinal fluid. All fibronectins contain approximately 4-8% carbohydrate and have an acidic p*I* (5.5-6.3) (Hynes, 1990).

1.4.1.1 Synthesis

There are at least two major classes of fibronectins which are termed "cellular fibronectin" (cFN) and "plasma fibronectin" (pFN). Fibronectin is found in blood as a soluble plasma glycoprotein at around 0.3 g/litre; the form found in plasma is termed "plasma fibronectin" (Hay, 1991). Tamkun and Hynes (1983) demonstrated that the liver is the major source of pFN which is

secreted by hepatocytes. Cellular FNs are produced by many different cell types which secrete them and often organize them into extensive extracellular matrices. Cellular FN was first detected on fibroblasts using both *in vivo* and *in vitro* techniques. The fibronectin synthesised by fibroblasts has two destinations; it can be incorporated into fibrillar matrices at the cell surface or it can be secreted to the culture medium (Hynes, 1990).

1.4.1.2 Structure of Fibronectins

Fibronectin (FN) polypeptide is composed of three types of homologous repeating units, termed types I, II, and III. The general structure of fibronectin polypeptide is shown in *Figure 1.9* (Potts and Campbell, 1994). Twelve type I repeats are about 45 amino acid residues long and make up the NH_2 and COOH-terminal ends of the polypeptide. Two type II repeats (each 60 amino acids long) interrupt a row of nine type I repeats at the NH_2-terminus. Moreover, 15-17 type III repeats, each about 90 amino acids long, make up the middle of the polypeptide. The FN molecule is composed of two polypeptides that associate through two disulfide bonds near the COOH- terminus to form a dimer.

At a genetic level the beginning and end of most of the repeat sequences coincides with intron and exon boundaries within the single FN gene. This suggests that the FN gene has evolved by acquiring pre-existing domain motifs from other genes by "exon shuffling" and subsequent duplications of such domains (Ruoslahti, 1988). Hence, domains homologous to the type I repeats in fibronectin are present also in blood clotting factor XII and tissue plasminogen activator. Type II homologies are also found in factor XII as well as in prothrombin, plasminogen and urokinase (Patel *et al.*, 1987). Like the type I and II repeats of FN, type III repeats are also present in other proteins. Tenascin (TN), another extracellular matrix protein, contains 11 type III repeats of FN (Jones *et al.*, 1988). A neuronal cell-cell adhesion

molecule (L1) also contains three type III repeats of FN (Moos *et al.*, 1988), whereas the other neuronal cell surface protein, contactin, contains two type III repeats of FN (Ranscht, 1988) and N-CAM contains one type III repeat of FN (Cunningham *et al.*,1987).

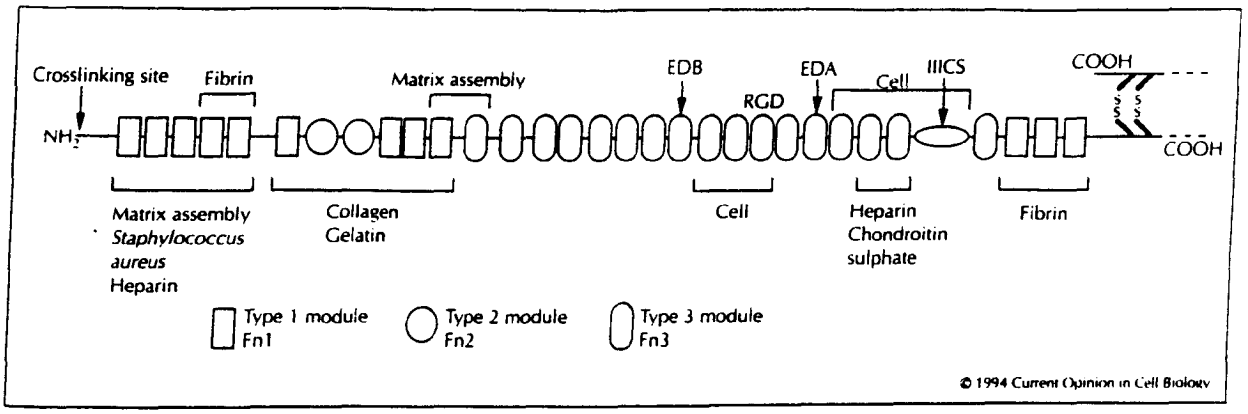


Figure 1.9. The general structure of the Fibronectin polypeptide showing repeating homologous units. The positions of the EDA, EDB and IIICS alternatively spliced regions are shown. FN3 contains the Arg-Gly-Asp (RGD) sequence involved in integrin binding (After Potts and Campbell, 1994).

The differences in FNs from different tissue sources of the same species are due to post-translational modifications and alternative splicing mRNA transcript which give rise to variations in the mature protein. Comparison of FNs from different sources reveals slight differences in the molecular weights of the FN polypeptides when studied by SDS-PAGE. Fibronectin isolated from plasma is smaller in molecular weight than that produced by most types of cultured cells, such as fibroblast cell cultures, and also runs a doublet unlike the other types (Hynes, 1990).

Three potential sites of alternative splicing have been demonstrated in FN polypeptide as shown in *Figure 1.9*. Two of these involve the addition or deletion of complete type III repeats, ED-A and ED-B. The third one,

IIICS=V (the variable region), is more complex. This IIICS (V) region of FN is a 120-amino acid stretch of polypeptide that can either be included completely or partially in the polypeptide, or completely left out. The patterns of alternative splicing of FN can produce the insertion or deletion of cell-type specific adhesion sites. The different isoforms of FN result from alternative splicing of the EDA and EDB modules and of the type III connecting segment, IIICS (Pott and Campbell, 1994). Although alternatively spliced sequences (ED-A, ED-B, and IIICS) tend to be retained in "cellular" fibronectins, especially by certain embryonic and malignant cells, plasma FN produced by liver generally lacks ED-A and ED-B sequences. It can contain 50% (or less) of the IIICS type of alternatively spliced region of FN. Thus, inclusion of ED-A and/or ED-B is characteristic of cFN and is a major distinction between cFN and pFN. cFN and pFN differ in solubility when isolated. *In vivo*, pFN is a soluble plasma protein whereas the characteristic form of cFN is a fibrillar matrix around cells. Earlier reports indicated that cFN is more effective in restoring normal morphology to transformed cells and is a better hemagglutinin than pFN (Yamada *et al*, 1975; Yamada and Kennedy, 1979). The apparent binding of cFN to hyaluronic acid is known to be due to a contaminating proteoglycan in the hyaluronic acid preparation. Apart from these studies, few researchers have analyzed the functions of cFN because it is more difficult to obtain in quantity than is pFN. Therefore, there are no differences between cFN and pFN that have been well defined, although they probably exist (Hynes, 1990).

The functions of the variable regions (IIICS=V) within FN and the two distinct forms (cellular and plasma) are not clear. However, using fragments isolated from intact human pFN and synthetic peptides based on the V region sequence, Humphries *et al.* (1986 and 1987) showed clearly that the V region can act as cell adhesion sites for B16 melanoma cells. Moreover,

the V region of rat (Schwarzbauer *et al.*, 1983) and bovine pFN (Skorstengaard *et al.*, 1986) contains an RGDV sequence which promote cell adhesion. Furthermore, the IIICS region possesses a cell-attachment site that interacts with the $\alpha 4\beta 1$ integrin and is distinct from the major tripeptide (Arg-Gly-Asp;RGD) cell-binding site recognized by $\alpha 5\beta 1$ integrin (Hughes, 1992).

There is no definitive evidence on the potential roles of ED-A and ED-B, the two segments that show most distinction between cFN and pFN. But they seem to be more readily expressed in embryonic tissues compared to adult tissues (Norton and Hynes, 1987). Wounding of rat skin can induce the re-expression of these domains in localized areas (Ffrench-Constant *et al.*, 1989). Other studies have shown that the expression of ED-A and ED-B increase on oncogenic transformation (Borsi *et al.*, 1987, Zardi *et al.*, 1987).

1.4.1.3 The Binding Domains of Fibronectin

Fibronectin (FN) polypeptide is extremely sensitive to proteolysis. Each FN polypeptide chain consists of a series of structural and functional domains. These domains have been defined by proteolytic fragmentation studies to locate interchain disulphide bonds, sulfhydryl groups, and carbohydrate residues. The study of proteolytic fragments using biochemical methods, immunological methods (using antibodies), biological assays (cell adhesion and migration) and affinity chromatography has led to a reasonably complete picture of the structure of FN. Many of these proteolytic fragments are globular, relatively resistant to further proteolysis and retain their functional and binding properties (Hynes, 1990).

1.4.1.3.1 The N-terminal domain of Fibronectin

The N-terminal domain of FN (domain a, *Fig. 1.10*) can be isolated as a 24-32 kDa fragment using proteases (Site 1, *Fig. 1.10*) such as trypsin, thrombin, plasmin, and thermolysin on intact FN (Kurkinen *et al.*, 1980; Sekiguchi and

Hakomori, 1983). This domain is basic with a pI of 8.2-8.6 (Mosher and Proctor, 1980) and contain no carbohydrate (Wagner and Hynes, 1979).

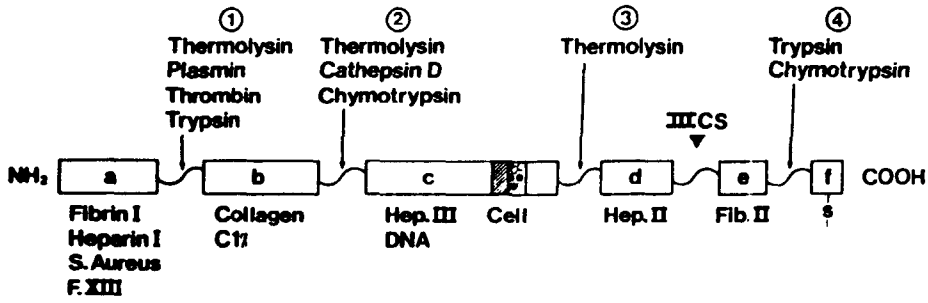


Figure 1.10. Domain model of fibronectin subunit.
Lower case letters (a to f) identify discrete domains proceeding from amino- to carboxy terminal. Ligand affinities are listed beneath each domain. The open and solid circles indicate the approximate location of the Arg- Gly-Asp (RGD) cell-binding sequence. *The cross-hatched region indicates a potential area of sequence variability due to* insertion of a domain via differential mRNA splicing. IIICS (arrowhead) is a differentially spliced region connecting domains d and e. S is the location of the interchain disulfide link. Arrows indicate protease-sensitive sites (1to 4) where limited proteolysis results in the release of major domains (From Carsons, 1989).

It also contains a five-fold repeat of disulfide-bonded type I homologies and comprises various binding sites. Cathepsin D digestion gives a 70kDa fragment which includes N-terminal and gelatin-binding domains. This 70kDa fragment of FN can be further cleaved with chymotrypsin, plasmin or thrombin to separate these two domains (Balian *et al.*, 1979; Hormann and Seidl, 1980).

The N-terminal domain of FN binds to:

(i) heparin (HEP I) (Sekiguchi and Hakomori, 1980),
(ii) fibrin (Hormann and Seidl, 1980)
(iii) *Staphylococcus aureus* (Mosher and Proctor, 1980)
(iv) gangliosides (Thompson *et al.*, 1986).

(v) a self association site in the C-terminal region of FN (Homandberg and Erickson, 1986).

(vi) thrombospondin (Homandberg and Kramer-Bjerke, 1987).

The N-terminal domains of FN also comprises a glutamine residue reactive with factor XIIIa transglutaminase (Mosher and Proctor, 1980). It is also able to participate in matrix assembly (Quade and McDonald, 1988). This domain may bind to other glycosaminoglycans (GAGs) at low salt concentration (50mM). However at physiological salt concentrations it is specific to heparin. In addition, it also participates in folding of FN, in fibrillogenesis and in the binding of fibrin to macrophages (Hynes, 1990).

1.4.1.3.2 The Gelatin-Binding Domain of Fibronectin

The gelatin-binding domain of FN (domain b, *Fig. 1.10*) is adjacent to the N-terminal domain. Various proteases, including thermolysin and chymotrypsin (site 2, *Fig. 1.10*) cleave FN so as to produce gelatin binding fragments ranging in size from 40-60 kDa (Sekiguchi and Hakomori, 1983). Cathepsin D treatment results in the generation of a slightly larger 70 kDa collagen-binding domain joined to the ~30 kDa N-terminal domain (domains a and b, *Fig. 1.9*) (Richter *et al.*, 1981). The gelatin-binding domain identified on FN as the first functional domain binds to collagen. The gelatin-binding domain includes a single type I repeat, followed by two type II repeats and then three more type I homologies of FN polypeptide as seen in *Figure 1.9*. This domain is quite resistant to proteolysis due to intrachain disulfide bonding and attached carbohydrates. Many carbohydrate side chains are attached to the gelatin-binding domain and they may cause small differences in the size of this domain in pFN, cFN, and aFN (Balian *et al.*, 1979). Olden *et al.* (1978, 1979) reported that FN synthesized in the presence of tunicamycin lacks the carbohydrates and is much more sensitive to proteolysis.

The central region of the FN following the gelatin-binding site comprises type III repeating units towards the C-terminal, and the first three of these are labile to proteolysis. The next three type III repeats (4-6) can be isolated as a 30 kDa DNA binding domain and a heparin binding domain (HEPIII) (Skorstengaard *et al.*, 1986a).

1.4.1.3.3 The Cell-Binding Domain

Most cells such as fibroblasts can adhere to fibronectin by binding to the centrally located cell-binding domain (domain c, *Fig. 1.10*). The large central domain of fibronectin is composed of type III repeating units, each 90 amino acids long. The active cell attachment peptide is located on the tenth type III segment (Kornblihtt *et al.*, 1985). Three important variable areas have been identified in the cell-binding domain. One type of variation occurs as a result of the insertion or deletion of an entire type III repeat following the seventh and/or the eleventh type III repeat (Kornblihtt *et al.*, 1984). As mentioned before, the presence of these "extra domains" referred to as ED(A-B) is a feature of cellular fibronectins whereas plasma lack the ED segments (*Fig. 1.9*).

A significant sequence in the cell-binding domain is Arginine-Glycine-Aspartic acid (abbreviated RGD using the single-letter amino acid code). Many other adhesive proteins present in extracellular matrices and in the blood also contain the tripeptide RGD as their cell recognition site. In addition to FN these include vitronectin, osteopontin, collagens, thrombospondin, fibrinogen, and von Willebrand factor, tenascin, laminin and entactin/ nidogen (Ruoslahti and Pierschbacher, 1987; Yamada and Kleinman, 1992). The RGD sequences of each of the adhesive proteins are recognised by integrins.

1.4.1.3.4 The High-Affinity Heparin-Binding Domain

There are at least three heparin-binding sites on each monomer subunit of fibronectin. One heparin-binding site is located in the amino terminal 29- or 30-kDa domain of the molecule (domain a, *Fig. 1.10*) and this binding is inhibited by 0.25 M salt or divalent cations. Fibronectin contains a second and stronger heparin-binding site (domain d, *Fig. 1.10*) that is located near the carboxyl-terminus and is unaffected by divalent cations. Higher salt concentrations (0.5 M NaCl) are necessary to disrupt binding of the carboxyl terminal domains to heparin than to distrupt binding of the amino terminal domain. A third heparin-binding site (domain c, Fig. *1.10*) with lower affinity is located in the region between the gelatin- and cell-binding domains. Fibronectin interacts with cell surface heparan sulfate proteoglycans and mediates cell adhesion in this way (Hynes, 1990).

1.4.1.3.5 The C-Terminal Fibrin Binding Domain

The C-Terminal fragment (21-37 kDa) binds to fibrin but is different from the N-terminal (25-30 kDa) fragment, which also binds to fibrin. This fibrin binding domain (domain e, *Fig. 1.10*) is relatively sensitive to proteases and does not bind to gelatin or heparin or act as a substrate for factor XIIIa (Sekiguchi *et al.*, 1981). It has an acidic p*I* (5.1-5.8) (Richter and Horman, 1982) whereas the N-terminal domain is basic. The complete amino acid sequence of this domain consists of three type I repeats. There are no carbohydrates in this C-terminal fibrin binding domain.

1.4.1.3.6 The C-Terminal Interchain Disulfide Bonds

A small (3-6 kDa) domain located at the carboxyterminal (domain f, *Fig 1.10*) has been found to contain the interchain disulfide bond that joins the subunits of the fibronectin dimer (Hayashi and Yamada, 1983). Both pFN and cFN are disulfide-bonded dimers. The region containing these

interchain bonds has been sequenced and shown, by digestion with plasmin, to contain the interchain disulfide bonds in the last 20 residues (Skorstengaard *et al.*, 1982) and cathepsin D (Richter and Hormann, 1982).

1.4.1.4 Glycosylation of Fibronectin

Fibronectins are glycoproteins that contain 4-9% sugar, depending on the tissue or cell origin (Petersen *et al.*, 1989). Most of the carbohydrate is present in complex-type asparagine-linked oligosaccharide chains (Fisher and Laine, 1979; Fucuda, Levery and Hakomori, 1982). The presence of O-glycosidically linked carbohydrate on fibronectin has also been described (Krusius, Fukuda, Dell, and Ruoslahti, 1985). Fibronectin from tumour cells has a higher content than that from adult human skin fibroblasts (Ruoslahti, 1988). As the bovine sequence has been determined on the protein level, the putative attachment sites of the carbohydrate groups are known for this species (Skorstengaard *et al.*, 1986b). In fact, each polypeptide chain contains seven glycans N-linked to asparagines at positions 399, 497, 511, 846, 976, 1213, and 2168. The IIICS (alternatively spliced) region carries one or two groups which are O-linked to threonines at positions 2124 and/or 2125 (*Fig. 1.11*).

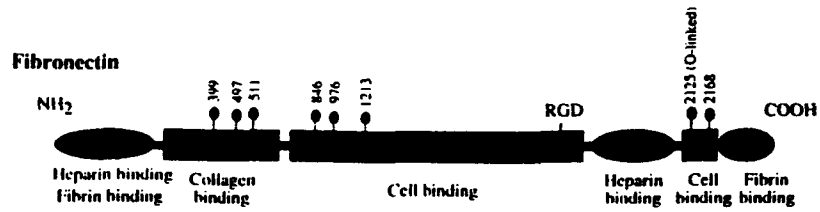


Figure 1.11. Domain structure and potential sites of N-linked carbohydrate addition for fibronectin. Various potential N-glycosylation sites are marked as lollipops and the numbers above them indicate the amino acid position for each site (From Tanzer *et al.*, 1993). The basic fibronectin structure was derived from the longest polypeptide chain of human plasma fibronectin (Skorstengaard *et al.*, 1986b).

The major type of side chain is the biantennary complex asparagine-linked type. Consistent differences in the sialylation pattern of plasma and cellular fibronectins have been reported. Cellular fibronectins contain less sialic acid, which is linked to α 2-3 galactose in contrast to the α 2-6 linkage found in plasma fibronectin. Cellular fibronectin also contains fucose linked to the proximal N-acetylglucosamine units. Fucose is not detected in plasma fibronectin (Fukuda and Hakomori, 1979; Fukuda *et al.*, 1982). Human plasma fibronectin contains mainly biantennary complex types, whereas amniotic fluid fibronectin contains triantennary groups with fucose linked to the innermost N-acetylglucosamine residue. Human placenta fibronectin contains highly branched carbohydrates with a large percentage of polylactosamine (Petersen *et al.*, 1989).

1.4.1.4.1 Functions of Fibronectin Glycosylation

Although the physiological functions of the carbohydrates of fibronectin are unresolved, given the organization of functional domains on the fibronectin molecule, modification of glycan structure at critical locations is likely to result in functional alterations. Various oligosaccharide-dependent functional alterations have been described. A significant function of the carbohydrate moiety of a glycoprotein is protection of the protein from proteolytic attack. It has been demonstrated that the heavily glycosylated collagen-binding domain of chicken embryo fibroblast fibronectin was selectively resistant to a broad variety of proteases. When N-linked glycosylation was inhibited by tunicamycin treatment, the collagen domain became increasingly susceptible to proteolysis (Bernard *et al.*, 1982). Zhu *et al.* (1984) demonstrated that the gelatin-binding domain from placental (embryonal) fibronectin was more resistant to proteolysis than the corresponding domain from plasma fibronectin. Although attention has focused on the role of the RGD sequence in fibronectin-mediated cell

adhesion, Jones *et al.* (1986) have presented data that suggest that the oligosaccharides of fibronectin may also be important, acting as modulators of fibroblast adhesion and spreading. Zhu and Laine (1985, 1987) have reported that fibronectin-carrying polylactosamine (PLA) type chains show weaker binding to denatured collagen than plasma fibronectin. This suggests that PLA or possibly increased branching may modulate fibronectin-mediated adhesions of cells to collagen subtrate. Interestingly, glycosylation of amniotic fluid and placental fibronectin appears to vary during gestation and the appearance of PLA in amniotic and placental fibronectins may have biological significance (Hughes, 1992).

1.4.1.5 The HNK-1 (Leu-7) Carbohydrate Epitope

Significant advances in understanding of the functional roles of oligosaccharides have been made using carbohydrate-specific monoclonal antibodies. The neural cell adhesion molecules such as L1 and N-CAM share a common carbohydrate epitope that is recognised by monoclonal antibodies L2 and HNK-1. The L2/HNK-1 carbohydrate epitope is also present on tenascin, myelin-associated glycoprotein (MAG) and the intercellular cell adhesion molecule (I-CAM) (Schachner, 1989). This epitope has been identified as a 3'-SO_4-glucuronic acid that is found on glycolipids (Chou *et al.*, 1986), proteoglycans (Gowda *et al.*, 1989) and glycoproteins (Kruse *et al.*, 1985). Harper *et al.* (1990) previously reported that human tumours of neuroectodermal origin secrete fibronectin that contains the HNK-1 carbohydrate epitope. Thus, fibronectin joined the growing the list of RGD-containing extracellular matrix glycoproteins that possess the HNK-1 epitope, strengthening the correlation between these two structures.

1.4.2 Tenascin-C

The tenascins are a family of structurally related, large oligomeric glycoproteins of extracellular matrix. The first member of the family to be discovered, previously known by the names tenascin, hexabrachion protein, myotendinous antigen, glioma mesenchymal extracellular matrix (GMEM) protein, cytotactin, and J1-200-220, is now referred to as tenascin-C (TN-C) (Erickson and Bourdon, 1989; Erickson, 1993). The original version of tenascin is denoted by TN-C, where the "C" represents cytotactin. The TN-C of human, pig, mouse, chicken and newt show a high degree of homology (75-90 %) at the domain level of structure. The more recent members of the tenascin family are tenascin-R (TN-R) and tenascin-X (TN-X). TN-R was originally found as restrictin in chicken (Norenberg *et al.*,1992) and J1-160-180 in rats (Fuss *et al.*, 1993). These proteins are as closely related to each other as are the TN-Cs from chicken and rat, but more distantly related to all species of TN-C. Tenascin-X (human) was originally reported as a sequence encoded by gene X. TN-X is structurally most different than the others (Bristow *et al.*, 1993). Tenascin C was the first discovered glycoprotein of the tenascin family and therefore its structure and expression are better established than those of TN-R and TN-X. In this research, TN-C expression has also been investigated in cultured fibroblasts derived from skin tissues of tuberous sclerosis patients. Hence, the expression and structure of TN-C are briefly described below. For simplicity the name tenascin, without a letter, will be used for TN-C in this thesis.

1.4.2.1 Structure

The native tenascin molecule exists as a six armed, star-shaped structure consisting of similar subunits linked by interchain disulfide bonds. The amino terminal region possesses oligomerization sites where the subunits are joined to form hexamers. The known tenascin family members share a very

distinctive pattern of three types of domains: epidermal growth factor (EGF) domains, fibronectin (FN) III domains and fibrinogen-like domains (F) (*Fig. 1.12*) (Hay, 1991).

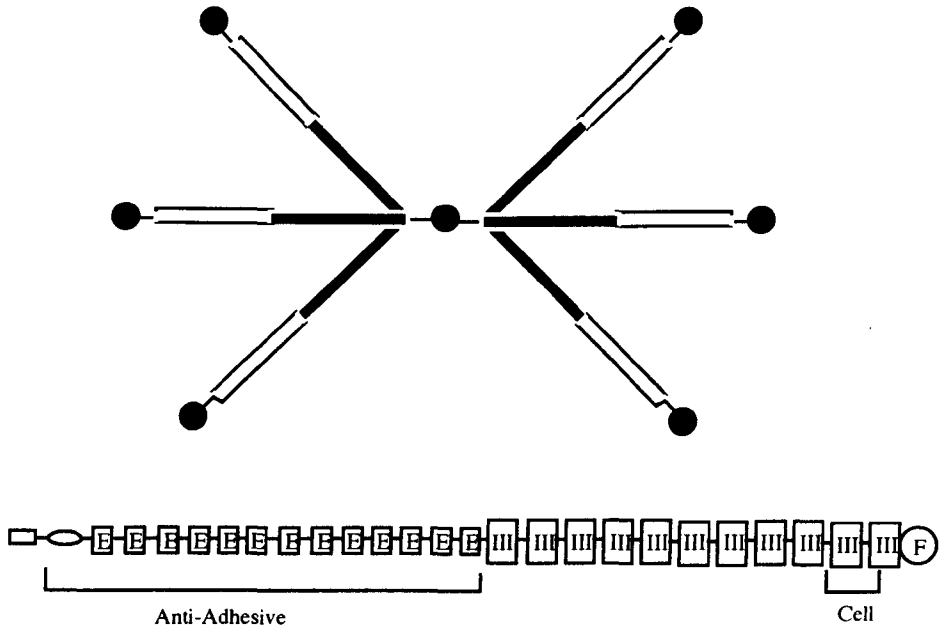


Figure 1.12. The domain structure of tenascin (After Hay, 1991).

Tenascin contains a single cystein amino-terminal to the trimer-forming segment, which is able to connect two trimers into the characteristic hexabrachion structure. The next segment consists of EGF-like domains, each 31 amino acids long. The next segment comprises a series of FN-III domains, each about 91 amino acids long. Approximately two-thirds of potential N-linked glycosylation sites in tenascin are found within type III repeats. FN-III repeats of the tenascin arms promote cell adhesion via RGD-integrin receptor interaction (Bourdon and Ruoslahti, 1989), whereas the fragments containing the cysteine-rich EGF-like repeats are anti-adhesive (Spring *et al.*, 1989). The alternatively spliced domains are also present within the fibronectin type III repeats (Jones *et al.*, 1989). At the COOH

terminus a fibrinogen-like sequence is found, and is abbreviated in *Figure 1.12* as F.

1.4.2.2 Expression of Tenascin

Tenascin is secreted by fibroblasts, myoblasts, and glial cells in tissue culture, and can be purified from spent medium of tissue culture. The human glioma cell line (U-251 MG) is the champion producer of tenascin (5-10 ug/ml). Chicken embryo fibroblasts are also a good source of tenascin (2-5 ug/ml) while human foreskin fibroblasts produce tenascin in smaller amounts (0.2-0.5 μg/ml) (Erickson and Lightner, 1988). Bourdon *et al.* (1983) reported that tumours of glial cells (glioblastomas) produce large quantities of tenascin. They suggested that tenascin may be a useful marker of glioblastomas *in vivo* and *in vitro*. Tenascin synthesis can be stimulated in fibroblasts by serum and transforming growth factor β (Pearson *et al.*, 1988).

In contrast to most other major proteins of the extracellular matrix, tenascin expression is confined to a few tissues and often occurs only in a transient manner. During embryogenesis, tenascin is transiently expressed in mammary gland, tooth, or kidney. It is present in embryonic cartillage and bone. In the adult, tenascin becomes confined to the perichondrium and periosteum, ligaments, tendons, and myotendinous junctions. Smooth muscle also contains tenascin, whereas many other organs or tissues, such as the heart, skeletal muscle and epithelial organs, contain little or no tenascin.

Transient expression of tenascin is also reported in healing wounds (Chiquet-Ehrismann, 1990). In normal human skin, tenascin is detected in a zone beneath the dermal-epidermal junction (Lightner *et al.*, 1990). Expression of tenascin in a wide variety of tumours was one of the most remarkable early findings. Tenascin expressed abundantly in malignant breast carcinomas (Koukoulis *et al.*, 1991). In general it appears that

tenascin expression is often low in adult tissues and is drastically increased during many pathological conditions.

1.4.2.3 Biological Functions of Tenascin

Culture studies have shown that tenascin has multiple functions including cell attachment and detachment, promotion and inhibition of neural crest cell migration, cell growth stimulation and hemagglutination (Sakakura and Kusano, 1991). In contrast to fibronectin, tenascin was found to be a very poor adhesion molecule and the addition of tenascin to the cell culture medium inhibited fibroblast adhesion to fibronectin (Chiquet-Ehrismann *et al.*, 1988). Although cells were able to adhere to tenascin at 4°C, this adhesion became weaker at 37°C (Lotz *et al.*, 1989). Because of these studies tenascin has been classified as an antiadhesive or adhesion-modulating extracellular matrix protein (Chiquet-Ehrismann, 1991; Sage and Bornstein, 1991). EGF-like repeats are adjacent to the amino-terminal globular domain and are involved in the anti-adhesive activity of tenascin. Major cell-binding sites of tenascin are present in the tenth and eleventh fibronectin type III repeating units toward the carboxyl-terminus of the protein (Spring *et al.*, 1989). Tenascin binds heparin by its fibronectin type III domain five (Weber *et al.*, 1995). Tenascin also binds to chondroitin sulfate proteoglycan. It binds weakly to fibronectin, but it does not bind to laminin (Hay, 1991). Crossin (1991) reported that tenascin inhibited cell growth. In contrast to this report, tenascin stimulates cell growth by acting as a mitogen. This mitogenic activity, however, appears to be associated with the region in the fibronectin type III domains (End *et al.*, 1992). Tenascin has contrary effects, stimulation or inhibition, on neurite outgrowth (Lochter *et al.*, 1991). All these observed functions support the idea that tenascin is a multifunctional extracellular matrix molecule. A summary of

proposed functions for tenascin based on experimental data (Chiquet-Ehrismann, 1990) is presented in *Table 1.1.*

Table 1.1 A summary of proposed activities for tenascin.

*	Hemagglutination (Erickson and Iglesias, 1984)
*	Neuron-glia adhesion (Kruse *et al.*, 1985)
*	Growth promotion (Chiquet-Ehrismann *et al.*, 1986)
*	Inhibition of cell attachment/ spreading on fibronection (Chiquet-Ehrismann *et al.*, 1988)
*	Promotion of chondrogenesis (Mackie *et al.*, 1987)
*	Necessary for cerebellar granule cell migration (Chuong *et al.*, 1987)
*	Guidance of neural crest cell migration (Chiquet, 1989)
*	Promotion of neurite outgrowth (Chiquet, 1989)
*	Inhibition of epithelial cell-cell contacts (Chiquet-Ehrismann *et al.*, 1989)
*	Promotion of cell attachment, not spreading (Spring *et al.*, 1989; Chiquet Ehrismann *et al.*, 1988).
*	Anti-adhesive activity (Lotz *et al.*, 1989; Spring *et al.*, 1989)
*	Immunosuppression of T cells (Ruegg, *et al.*, 1989)

1.4.3 Laminin

Laminin is a large extracellular glycoprotein found in the matrix, known as the basement membranes to which epithelial tissues, muscle, nerves and fat cells are bound. Laminin can serve various biological activities, including promotion of cell adhesion, growth, migration, differentiation, neurite outgrowth and tumour metastases. It can also take part in the structural assembly of basement membranes. Many of these properties are related to specific interactions of laminin with connective tissue components and cell surface receptors. For example, laminin binds to itself and other components in the matrix such as type 4 collagen, heparan sulfate proteoglycan and entactin to create an integrated structure within the basement membrane

(Kleinman and Weeks, 1989). This introduction will focus on the structure, occurrence, sources, active sites and biological activities of this molecule.

1.4.3.1 Occurrence and sources of laminin

Most of what is known about the biosynthesis of laminin is derived from studies using tumour-derived cell lines. Laminin was originally purified in a native form from the Englebreth-Holm-Swarm (EHS) tumour of a mouse (Timpl *et al.*, 1979). Engel (1993) indicated that the preparation of laminin from nontumor sources is generally difficult and usually only small amounts can be obtained for analytical characterizations. Paulsson and Saladin (1989) reported a successful isolation of native laminin in small quantities from heart muscle tissues using the EDTA extraction method. It has been reported earlier that a variety of cultured cells including various epithelial cells, smooth muscle cells, schwann cells, endodermal and several tumour cell lines were found to produce and secrete laminin (Timpl *et al.*, 1982; Martin and Timpl, 1987). Fibroblasts from human skin were found not to produce significant laminin (Foidart *et al.*, 1980; Timpl *et al.*, 1979, 1982).

1.4.3.2 Structure

Since laminin was first identified as a large glycoprotein from a mouse EHS tumour, most biochemical and structural work has been performed with the EHS laminin. In electron microscopy it is visualised as a cross-shaped structure with three short arms and one long arm. Laminin consists of three polypeptide chains, a 400-kDa heavy (A) chain and two light (B) chains of about 200-kDa as shown in *Figure 1.13.* The A chain has three globular domains separated by three epidermal growth factor (EGF)-like repeats at the amino terminus. The B chains (B1 and B2) are structurally similar but contain only two amino terminal globules (in the short arm) and EGF-like repeats and no globule at the carboxyl end (in the long arm). Laminin has

multiple structures in tissues due to variations in the number and type of chains. Mouse and human laminin have approximately 90% sequence homology, whereas *Drosophila* is only about 45% homologous to mouse protein (Kleinman and Weeks, 1989).

Laminin was originally thought to be a single protein; however, investigations have shown that there are different forms of laminin that are separate gene products. Merosin is one member of the laminin gene family. The deduced amino acid sequence of merosin (380 kDa) is present in placenta, striated muscle, and peripheral nerve. Merosin is homologous to the carboxy-terminal part of the laminin A chain. It is associated with laminin B1 and B2 chains (Ehrig *et al.*, 1990). S-laminin, another form of laminin, is homologous to the B1 chain of traditional laminin and is found in synaptic basement membranes, as well as in glomeruli and blood vessels (Hunter *et al.*, 1990). The existence of different forms and tissue distributions of laminin implies that there may be different specific functions for each type of laminin.

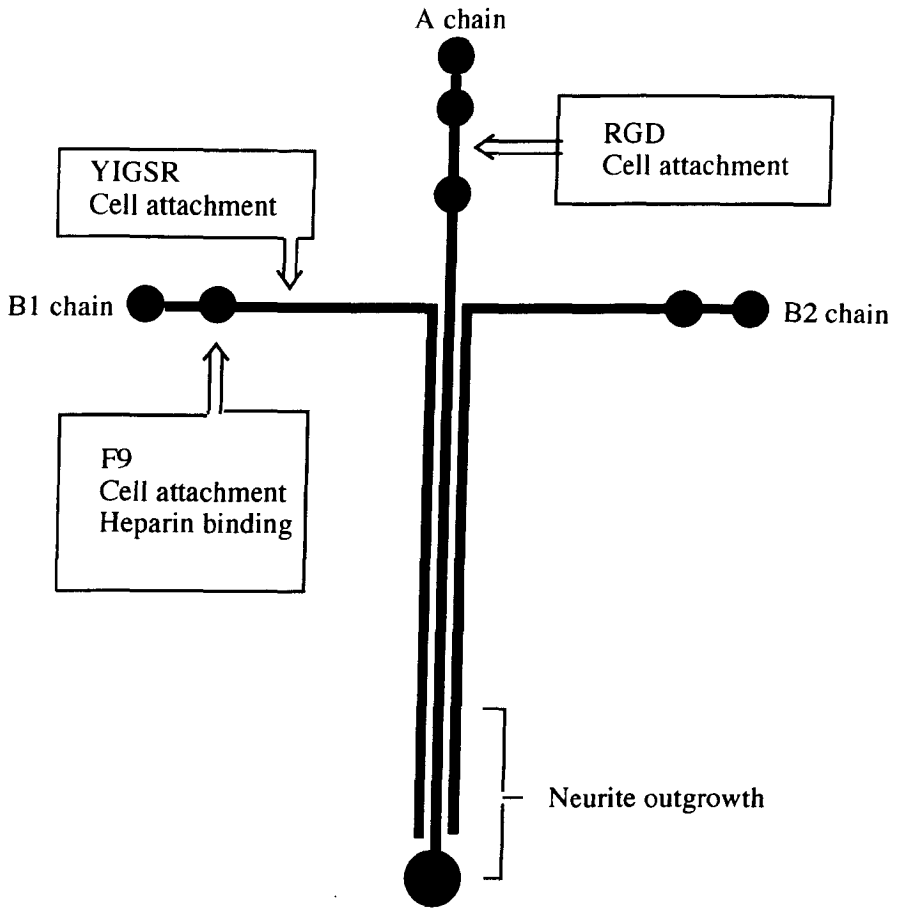


Figure 1.13. Schematic model of laminin showing approximate locations of biologically active sites (After Kleinman and Weeks, 1989).

1.4.3.3 Active sites and biological activities of laminin

Numerous studies indicate that laminin possesses diverse biological activities which include promotion of cell adhesion, migration, differentation, growth, neurite outgrowth, collagenase IV activity, tyrosine hydroxylase activity and tumor metastases (Yamada and Kleinman, 1992; Engel, 1993). Laminin can interact with cells via a variety of adhesive recognition sequences and receptors. According to Kleinman and Weeks (1989), the center of the cross region was found to have cell adhesion activity while the end of long arm had both cell attachment and neurite outgrowth activity. Growth-promoting functions have been reported for laminin and some other ECM proteins containing EGF-like domains (Engel, 1989). A unique sequence of five

amino acids, Tyr-Ile-Gly-Ser-Arg (YIGSR), from one of the EGF-like repeats is located in the B1 chain of laminin (*Fig. 1.13*). YIGSR peptide was identified as promoting cell adhesion and migration (Graf *et al.*, 1987). Likewise YIGSR, PDSGR and F9 (RYVVLPR) are located on the B1 chain and inhibit tumour metastases (Yamada, 1991; Iwamoto *et al.*, 1987; Murata *et al.*, 1989; Skubitz *et al.*, 1990). In contrast, IKVAV containing peptide increases tumor metastases, growth, angiogenesis, plasminogen activation and collagenase IV activity (Kanemoto *et al.*, 1990; Stack *et al.*, 1990; Kubota *et al.*, 1992). The YIGSR peptide has also been reported to interact with normal cells and to influence cell differentiation. From these data, it is clear that YIGSR is one of the major functional sites in laminin (Yamada and Kleinman, 1992).

Sasaki *et al.* (1988) reported that there is an Arg-Gly-Asp (RGD) sequence in one of the cystein-rich domains of the A chain. They indicated that this potential cell binding sequence could be active as another adhesion signal, in addition to the previously identified cell binding sequence YIGSR of the B1 chain. Several receptors proposed for laminin on mammalian cells have been reviewed by Mecham (1991) and Engel (1992). At least 8 members of the integrin family have been reported to bind to EHS laminin: $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 6\beta 1$, $\alpha 7\beta 1$, $\alpha v\beta 3$, $\alpha 6\beta 4$ and $\alpha IIb\beta 3$. Among these receptors, $\alpha 6\beta 1$ is considered a specific laminin receptor as EHS laminin is its only known ligand (Tryggvason, 1993).

Laminin is a heavily glycosylated molecule. In addition to earlier studies that reported 12-15% (w/w) glycosylation (Arumughan *et al.*, 1986 ; Fujiwara *et al.*, 1988), a value of 25-30% (w/w) glycosylation has also recently been reported (Chandrasekaran *et al.*, 1991). A functional role of glycosylation of laminin was indicated for tumor cell adhesion, cell spreading and neurite outgrowth by Dean *et al.* (1990).

1.5 Aims of the project

The principal aims of this study are outlined below:

(i) To characterise the amino acid composition and compare the glycosylation of purified cellular fibronectin from the conditioned medium of cultured skin fibroblasts derived from patients with TS and from normal individuals. Preliminary work by Ellis and Hemming (1990), involving metabolic studies of such cells, showed a general increase in glycosylation and secretion of proteins, in particular fibronectin, in the TS fibroblasts compared to normal cells. Detailed in this thesis is a comparative study of purified fibronectin from TS and normal skin fibroblasts to ascertain variations in the structure of the protein in the diseased state.

(ii) To establish the expression, distribution, and localisation of extracellular matrix (ECM) glycoproteins fibronectin, laminin and tenascin in cultures of skin fibroblasts derived from patients with tuberous sclerosis (TS) and normal individuals.

Fibronectin, laminin and tenascin play an important role in the migration of cells during early embryonic development, and are involved in the assembly of the ECM. These glycoproteins have also been noted to affect the cell morphology, differentiation, proliferation, and attachment of the cells during embryonic development. Alterations in distribution and structure of these glycoproteins may cause functional disruption in their binding and interactions with cells and ECM macromolecules. This may result in abnormal assembly of the ECM, a consequence of which could be the hardening of tissues associated with TS. Variation in the assembly of the ECM may cause an abnormal migration of cells during early embryonic development. Therefore, studies of these changes may contribute to the understanding of the mechanisms involved in the aetiology of the hardened tissues of TS.

Chapter 2

Chapter 2

Materials and Methods

2.1 Introduction

The process of analysing carbohydrates of purified fibronectin and looking at the expression of major extracellular matrix glycoproteins of skin fibroblasts from patients with tuberous sclerosis and normal persons requires extensive equipment and numerous materials. The following is a list of the equipment and materials. Details of their use in the project can be found in the next sections.

2.2 Materials and Equipment

The plate reader was a Dynatech MR 5000 (Dynatech Laboratories inc.). The electrophoresis unit (SE-250) was purchased from Hoeffer Scientific Instruments, San Francisco, CA. The BIO-RAD Western-blotting equipment was driven by a LKB power supply unit. Nitrocellulose membrane (Hybond-C or Hybond-C super) was purchased from Amersham. PVDF membranes were obtained from Millipore. The DIG glycan differentiation kit and DIG glycan detection kit were purchased from Boehringer Manheim Biochemica. 0.22μm filters were from Millipore.

2.2.1 Buffers and Reagents

All chemicals used for the work presented in this thesis were analytical grade and obtained from the following companies:

Sigma Chemical Company, Poole, Dorset, UK :

bovine serum albumin (BSA), 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS), polyoxyethylene-sorbitan monolaurate (Tween 20), gelatin-agarose, mercaptoethanol, silver nitrate, Coomassie Brilliant Blue R-250, sodium dodecyl sulphate (SDS), Folin's and Ciocalteu's phenol reagent, diaminobenzidine tetra-hydrochloride (DAB), sodium citrate, phenylmethyl-sulphonyl fluoride (PMSF).

Fisons Scientific Apparatus Ltd., Loughborough, Leicester, UK.:

hydrogen peroxide, copper sulphate, sodium potassium tartrate, formaldehyde, acetone, methanol, magnesium chloride, potassium chloride, disodium hydrogen orthophosphate, urea, bromophenol blue, ammonium sulphate, calcium chloride, diaminoethanetetraacetic-acid (EDTA).

BDH Chemicals Ltd., Poole, Dorset UK:

acrylamide-bisacrylamide, potassium dihydrogen orthophosphate, N,N,N',N'-tetramethylethylene-diamine (TEMED), tris (hydroxymethyl) methylamine (Tris), sodium carbonate (anhydrous).

FSH Laboratory Supplies:

methanol, ammonium persulphate.

2.2.2 Cell Culture Materials

Dulbecco's Modification Eagle's Medium (DMEM), trypsin and L-glutamine were purchased from Gibco BRL, Paisley, Scotland. Foetal calf serum (FCS) was from Advanced Protein Products (APP). 100mm cell culture dishes, 6 and 96 well plates were obtained from Costar (Northumbria Biologicals Ltd., Cranlington, Northumberland, UK.). Kanamycin was purchased from Sigma.

2.2.3 Chromatography equipment

Prosep-gelatin was purchased from Bioprocessing Ltd., Medomsley Road Consett, CoDurham. The HPAEC-PAD chromatography system was supplied by Dionex (UK) Ltd., Camberly, Surrey. The Superose-12 column, Mono Q HR 5/5 column, and fast protein liquid chromatography system (FPLC) was from Pharmacia.

2.2.4 Enzymes

Pepsin was purchased from Sigma. Chymotrypsin was obtained from Calbiochem.

2.2.5 Antibodies

HNK-1 (anti-Leu-7) monoclonal antibody was obtained from Becton Dickinson. Horse radish peroxidase conjugated swine-derived antisera against rabbit antibodies was purchased from Dako. Rabbit-derived polyclonal antisera against human fibronectin, human laminin, and human tenascin were puchased from Telios Company, USA.

2.3 Methods

2.3.1 Cell Culture

2.3.1.1 Primary Culture : Growing Fibroblasts from Skin Explants

In this study, skin samples were obtained from patients undergoing surgery. The following cell lines, established from primary cultures of skin samples, were available for our experimental studies :

TSJH : Skin tissue was obtained from a forehead plaque (lesion) of a 14 year old female tuberous sclerosis (TS) patient.

TSHS : Skin tissue was obtained from an ungual fibroma of a 31 year old female TS patient.

TSFC : Skin sample was obtained by biopsy from the upper forearm of the left second phalanx of a 10 year old male.

TSFM : Skin tissue was derived from an ungual fibroma of the second phalanx of a 10 year old male.

TSFL : Skin tissue was obtained from a neck fibroma of a 13 year old male TS patient.

TSSC : Skin tissue was from a toe lesion (ungual fibroma) of a 43 year old female TS patient.

TSAH : Unaffacted skin was obtained from the lumbo-sacral area of a 65 year old male TS patient.

NSFS : Normal (control) skin tissue was obtained during operation on a congenital hair lip malformation of a 4 month old male infant.

DAKO (12 year old female), JPA (8 year old male), and RICA (6 year old female) control fibroblast cell lines were kindly provided by Dr Bruce Middleton of Nottingham University.

The primary cell culture work mentioned above was performed in a laminar flow cabinet to avoid contamination of the samples. Fibroblast cultures were established from the above mentioned skin explants as described below. The method used was a modified version of that of Sly and Grubb (1979).

The Establishment of Fibroblast Cultures from Skin Tissue :

The subcutaneous fat was scraped carefully from the skin samples with a sterile scalpel in 100mm plates. The tissue was then cut into approximately 1mm^2 pieces in the presence of saline and Dulbecco's Modification Eagle's Medium (DMEM) to prevent the tissue from drying. Following this, tissue pieces (6-10) were placed with the dermal side in contact with the surface of the sterile 60mm tissue culture dishes. Tissue pieces were fixed to the petri dishes in the incubator (5-6 minutes) using a serum clot on each explant.

When the explants settled on the cell culture dishes, 5ml of DMEM containing 15% foetal calf serum and kanamycin (100ug/ml) was added gently to avoid any disturbance. The culture dishes were incubated at 37°C in a humidified incubator with 5% CO_2 in air, without moving for at least 10 days. After 10 days the dishes were examined with a light microscope.

2.3.1.2 Maintenance of Cultured Skin Fibroblasts

When enough fibroblasts had grown from the explant, the explant was removed from the plates. The plates were rinsed with phosphate buffered saline (PBS) and enough trypsin (1X, Gibco) was added to cover the surface of the dishes before being incubated at 37°C for 10-15 minutes. At this stage medium (8ml) containing 10% foetal calf serum (FCS) was added. The trypsin was quenched by the presence of natural protease inhibitors in the serum. The procedure for the preparation of the single cell suspension was as follows. The cells were sedimented in a sterile universal tube by centrifuging at 185g, and the medium was aspirated from the tubes. The cells were then resuspended in 10ml fresh medium (DMEM) containing 10% FCS, 2mM L-glutamine and kanamycin (50μg/ml). Finally, the cells were transferred to 100mm cell culture dishes, and incubated at 37°C in a humidified incubator with 5% CO_2 in air. The cells were fed by replacing the used medium by fresh 10% FCS in DMEM (10ml) every 3 days.

2.3.1.3 Subculture of Fibroblast Cells and Collection of the Spent Medium

When cell culture dishes were confluent, the conditioned medium was removed by aspiration and the cell layer washed twice with PBS. The cell layer was covered with trypsin solution and incubated at 37°C for 10-15 minutes. The activity of the trypsin was stopped by the addition of medium containing 10% foetal calf serum (FCS). The cell suspension was transferred

to a sterile universal tube and centrifuged at 185g for 7-8 minutes in a bench centrifuge (Sartorius). The supernatant was removed by aspiration, and the cell pellet resuspended in 10% FCS and 2mM L-glutamine. Resuspended cells from each 100mm dish were distributed into four 100mm dishes and incubated at 37°C in humidified incubator with 5% CO_2 in air until they reached confluence. At confluence, the standard medium was replaced by fibronectin-depleted medium to avoid any plasma fibronectin contamination with cellular fibronectin which is synthesised by fibroblasts. After 2-3 days, the conditioned (spent) medium was harvested from the cell culture dishes in the presence of protease inhibitors (2mM PMSF, 5mM EDTA). Harvested medium of TS and normal fibroblasts was stored at -80°C until sufficient medium was available for fibronectin purification.

2.3.1.4 Freezing and Storage of Cells in Liquid Nitrogen

For long term storage, cells were harvested when the plates were nearly confluent and then frozen. For this purpose, cells were washed twice with PBS, and trypsin was added before being kept at 37°C for 10-15 minutes as described above. Trypsin activity was stopped by the addition of growth medium containing foetal calf serum (FCS). The cell suspension was centrifuged and the supernatant removed as before. The cell pellet was resuspended in 1ml of growth medium containing 20% (v/v) FCS and 10% (v/v) dimethyl sulphoxide (DMSO), and then transferred to Costar 2ml freezing vials. Finally, the vials were frozen at -80°C overnight before being stored at -196 °C under liquid nitrogen.

2.3.1.5 Cell Defrosting

The vials containing cells were removed from liquid nitrogen storage when required. Firstly, they were warmed to 37°C to defrost completely. 5ml of medium was then added and the cells were immediately centrifuged at 185g for 5 minutes, after which the supernatant was removed by aspiration. This process was repeated to remove any remaining DMSO. The cells were resuspended in DMEM containing 15% (v/v) FCS, then plated out and grown in small 60mm cell culture dishes initially. All pipettes, flasks and media used in the above procedures were either purchased sterile or sterilised by autoclaving or filtering through 0.22 μm filter.

2.3.1.6 Cell Quantification and Cell Viability

The cell viability and counting was achieved on a haemocytometer by the trypan blue exclusion method. The dye exclusion test is based on the concept that viable cells do not take up certain dyes, whereas dead cells are permeable to these dyes (Freshney, 1992). The cell viability and counting was performed as follows ; 700 μl of phophate buffered saline (pH 7.4) and 200 μl of 0.8% Trypan blue (w/v) were added to 100 μl of cell suspension. The suspension was mixed and cell numbers determined by using an improved haemocytometer (Neubauer) on a light microscope at 100x magnification. The haemocytometer plate contains 16 equal large squares each subdivided into 25 equal small squares. The average number of cells in each large square was determined (all 16 squares were counted). The total cells per ml were calculated by multiplying this number of cells by 10 (the dilution factor) and then by 10^4. Trypan blue stains the the dead cells dark blue, whilst healthy cells remain clear.

2.3.2 Fibronectin Purification Methods

In this work, fibronectin was purified from culture medium of fibroblasts derived from the skin of tuberous sclerosis patients and normal individuals. Serum previously depleted of fibronectin was used in preparation of the medium to ensure that the product was pure cellular fibronectin of the fibroblasts. Optimised purification of fibronectin was achieved by gelatin-affinity and size column chromatography (gel filtration) techniques.

2.3.2.1 Gelatin-affinity Gel Chromatography

The affinity of fibronectin (FN) for gelatin is used routinely for chromatographic purification of fibronectin. The use of gelatin columns for this purpose was initially developed and popularised by Engvall and Ruoslahti (1977). A Prosep-gelatin column purchased from Bioprocessing Ltd was used for FN purification in this study. First step purification of fibronectin is described below.

Purification of Cellular Fibronectin using Prosep-Gelatin :

1) When the fibroblasts reached confluence, cell culture medium was removed, cells were rinsed three times with PBS, and fresh culture medium lacking plasma FN (plasma FN was depleted from foetal calf serum by prosep-gelatin) was added into each cell culture dishes.

2) Fibroblasts isolated from TS patients and normal individuals were cultured for 3-4 days.

3) The medium of the cells was harvested and filtered with Whatman No. 50 filter paper to remove debris.

4) PMSF (2mM) and EDTA (10mM) were added into the medium, which was stored at -80 ^{o}C in plastic roller bottles.

5) 500-1000 ml medium was added to Prosep-Gelatin prewashed with PBS containing 2mM EDTA and 2mM PMSF.

6) The mixture was shaken for 2 hr at room temperature in 1 liter plastic roller bottles.

7) Prosep-Gelatin was removed, and transferred into a polypropylene econopac column.

8) Culture medium once again was passed through the Prosep Gelatin.

9) The prosep column was washed with 10 column volumes of PBS containing 2mM PMFS and 2mM EDTA.

10) The column was washed with 1M NaCl and 1M urea in PBS to remove other contaminants of FN.

11) Bound fibronectin was eluted from Prosep Gelatin with 4M urea in 20 mM CAPS, pH 11, containing 0.15 NaCl, 2mM PMFS and 2mM EDTA.

12) Eluted fibronectin solution was dialysed, freeze dried and concentrated in vacuum dryer.

13) The pellet was redissolved in 20mM CAPS, pH11, and stored at -80 ^{o}C until further purification on Superose 12 (size column) by FPLC was performed.

2.3.2.2 Final Purification of Fibronectin By Gel-filtration Column by Fast Performance Liquid Chromatography (FPLC) System

An FPLC system was used in conjuction with a gel filtration column (Superose 12) for the second step of fibronectin purification . All column buffers were made with nanopure water and filtered through 0.22μm micropore filters prior to use. Partially-pure fibronectin of Prosep-Gelatin column was applied onto the size column using 200μl loop.

The superose 12 column (24 ml bed volume) was first equilibrated with 20mM CAPS buffer, pH 11. 5 bed volumes of buffer was enough to equlibrate the column. When a flat baseline occurred on the chart recorder, 200μl of fibronectin solution was injected into the column and fractions were collected either manually or using a fraction collector at constant flow

rate (0.2ml/minute). Thirty two 1ml fractions were collected from the column. An ELISA assay was performed on these collected fractions to determine fibronectin positive fractions. ELISA positive fractions were selected and analysed by polyacrylamide gel electrophoresis (PAGE).

2.3.3 Optimisation of Ammonium Sulphate Concentration For Precipitation of Laminin (LM) and Tenascin (TN)

As a first stage of the purification procedure in the initial experiments of this research, ammonium sulphate precipitation was carried out to achieve a partial fractionation of the two proteins (LM, TN) from spent medium of cell culture. The first strategy was to attempt to optimise the ammonium sulphate concentration for precipitation of the proteins. Hence, eight aliquots containing 3ml of conditioned medium were taken into universal tubes and pre-chilled to 4°C. Suitable quantities of ammonium sulphate required to give 20, 30, 40, 50, 60, 70, and 80% saturation were added (Harlow and Lane, 1988) to each aliquot while slowly stirring. Each aliquot was left stirring for 2 hours at 4°C and was then centrifuged at 1060g for 40 minutes. The supernatants of the aliquots were aspirated and the pellets redissolved individually in 2ml PBS, pH 7.4. The extracts were then dialysed against PBS and were collected into universal tubes. The extracts were assayed for total protein using the Lowry method, and the total amount of TN and LM were determined using ELISA. The results of ELISA suggested that a 40% saturation of ammonium sulphate was most efficient for the precipitation of both two proteins.

For the precipitation of spent medium proteins, ammonium sulphate (40% saturation) was slowly added to the pre-chilled conditioned media samples collected from TS and normal fibroblasts, which were slowly stirred to prevent frothing. The mixtures were incubated overnight at 4°C. The

precipitate from each mixture was collected by centrifugation at 17.989g for 90 minutes and resuspended in 6ml of PBS, pH 7.4 containing 2mM PMSF, 5mM EDTA. The ammonium sulphate was removed by dialysis against the same buffer, followed by passage of the redissolved pellets through Sephadex G-25 M desalting columns. The extracts of the spent media were ready to be applied to gelatin-agarose chromatography, for FN purification, and finally anion-exchange (Mono-Q-HR 5/5) column chromatography for LM and TN partial purification.

2.3.4 Partial Purification of LM and TN by an Anion-Exchange (Mono Q-HR 5/5) Chromatography

Partial purifications of laminin and tenascin from spent medium of fibroblasts (TS and normal) were the aims in early experiments of this work. After gelatin- agarose affinity chromatography ammonium sulphate precipitates of medium, concentrated and filtered samples were applied to a Mono Q-HR 5/5 (Pharmacia) column in a FPLC system. The column was equilibrated with 20mM Tris-HCl, pH 7.9, supplemented with 1mM PMSF and 2mM EDTA (starting buffer) using the standard 15 minute "wash program". The column was washed with 10ml of the same starting buffer (at a flow rate of 1ml/min) followed by 20ml of the same buffer containing 1M NaCl (eluting buffer). Full equilibration and a stable base-line was obtained by washing with 5 column volumes of starting and eluting buffer. The anion-exchange column was re-equlibrated using 20-30ml of the starting buffer before application of the samples. All starting and elution buffers were prepared freshly and filtered through 0.22μm micropore filters prior to use. Filtered samples were injected (t = 0 min) onto the column and allowed to equilibrate for 5 minutes in the presence of the starting buffer whilst allowing non-binding entities to be washed off. Following this, a 60ml linear gradient of 0 to 1M NaCl was initiated and continued to completion in 60 minutes (at a

flow rate of 1ml/1min). The column was then washed with an additional 5ml of 100% eluting buffer. Seventy 1ml fractions were collected. Elution of proteins was detected by continuous absorbance measurement at 280nm and the responses recorded on a chart recorder.

2.3.5 Protein Desalting Methods

a) Dialysis : Purified fibronectin from the Prosep-Gelatin column was dialysed against PBS with several changes in 2 cm visking tubing. Dialysed fibronectin was freeze dried and concentrated in 2.5 ml of 20mM CAPS buffer, pH 11, and finally desalted using PD-10 desalting columns. Prior to use, visking tubing was boiled for 1 hour in 10 mM sodium bicarbonate and 1mM EDTA. The tubing was also boiled for 10 minutes in de-ionized water prior to use.

b) Gel Filtration : Pre-packed PD-10 desalting columns containing Sephadex G-25 M (Pharmacia) were used for rapid desalting of protein samples. The column was equilibrated with 50 ml of nanopure water. Exactly 2.5 ml of sample (purified fibronectin from the Prosep-Gelatin or cell extracts) was applied to the PD-10 desalting column. Proteins were eluted with 3.5 ml of nanopure water.

2.3.6 Protein Purity Check,Visualising and Transfer Methods

2.3.6.1 Polyacrylamide Gel Electrophoresis (PAGE)

Sodium dodecyl sulphate polyacrylamide-gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970) with a Mighty Small II SE250 Vertical Slab Electrophoresis unit (Hoefer Scientific Instruments, USA). A discontinuous gel system was used in which two different compositions of gels were involved: an upper stacking gel which

concentrates the protein and a lower small-pore resolving gel separates the denatured proteins depending on molecular weight.

Stock Solutions

The gel recipes are summarised in *Table 2.1.*

Resolving Gel Buffer: 1.5 M Tris-HCl, pH 8.8 (18.17 g of Tris and approximately 48 ml of 1M HCl were mixed and brought to 100 ml final volume with distilled water). The solution was filtered through Whatman No.1 paper and stored at 4°C.

Stacking Gel Buffer: 1M Tris-HCl, pH 6.8 (12.11 g of Tris was dissolved in 40 ml of distilled water, titrated to pH 6.8 with HCl and finally brought up to a volume of 100 ml with distilled water). The solution was filtered through Whatman No. 1 paper and stored at 4°C.

Reservoir (running) Buffer: 0.25 M Tris, 1.92 M glycine, 1% (w/v) SDS, pH 8.3 (30.3 g of Tris, 144g of glycine and 10g of SDS were dissolved in 1 liter of distilled water) stored at 4 °C.

Acrylamide-bisacrylamide solution (30:0.8): Purchased from BDH Chemicals Ltd.

Sample Application Buffer: 0.0625 M Tris-HCl, pH 6.8, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, 0.02% (w/v) bromophenol blue, pH 6.8.

Sample preparation for SDS-PAGE: samples were prepared by mixing with an equal volume of sample application buffer and heating at 100°C for 5 minutes.

Reagents for Polymerisation: 1.5% ammonium sulphate (freshly prepared), and N,N,N',N'-tetramethylethylenediamine (TEMED) was purchased from BDH Chemicals Ltd.

5% resolving gel solutions were prepared using the recipe given in *Table 2.1* and pouring into the mini gel electrophoresis unit, then overlaying with water-saturated butanol.

Table 2.1. Recipe for gel preparation

	Resolving Gel (5%) volume/ml	Stacking Gel volume/ml
30% Acrylamide-bisacrylamide	5	1.7
Deionised water	16.9	6.8
Resolving gel buffer	7.5	----
Stacking gel bufer	----	1.25
10% (w/v) SDS	0.3	0.1
10% (w/v) ammonium persulphate	0.3	0.1
TEMED	0.03	0.01

When the resolving gel had set, the butanol was removed by inversion and the gel rinsed with distilled water. Next, stacking gel solution was poured on top of the resolving gel and a comb was inserted in order to form the sample wells. When the stacking gel had set, the comb was removed, the cast was dismantled and two gels were then transferred to the running apparatus. Following this, reservoir buffer was added to the apparatus and the samples were applied into the wells. Finally electrophoresis was performed at a constant current of 40 mA (20mA/gel) at 4°C for 3-4 hours until the dye-front had reached the bottom of the gel. After electrophoresis, one gel was stained with Coomassie fixing solution or silver staining, and the other Western blotted to nitrocellulose membranes as described in the following pages.

In some cases, continuous gradients gels (30ml, 1.5mm thick) were also prepared using a gradient caster. Typically, a mixture of 4-15%

polyacrylamide resolving gels were prepared using large glass plates. The gel was run at a current of 30mA (Vokam power pack) until the dye front reached the bottom (typically 4-6 hours).

2.3.6.2 Coomassie and Silver Staining of Polyacrylamide Gels

Proteins were visualised by either one of two methods:

a) Coomassie staining of Gels :

Polyacrylamide gels were stained with Coomassie blue fixing solution (5:1:5 (v/v/v) methanol : acetic acid : water containing 0.1% (w/v) Coomassie Brilliant Blue R-250) overnight at room temperature. Coomassie stained gels were destained in a solution A (50% (v/v) methanol and 10% (v/v) acetic acid) for 1 hour and then Solution B (10% v/v methanol, 5% v/v acetic acid) for several hours to visualise proteins.

b) Silver Staining of Gels:

The silver staining method is more sensitive than Coomassie staining for visualising proteins on polyacrylamide gels. Therefore, this method was used in some cases, where a higher detection sensitivity was required.

Gels were washed in fixing solution (80% (v/v) ethanol, 20% (v/v) glacial acetic acid) from 30 minutes to overnight. Fixing solution was then replaced with an incubating solution (7% (w/v) sodium acetate $3H_2O$, 0.2% (w/v) sodium thiosulphate $5H_2O$, 0.13% (w/v) glutaraldehyde, 25% (v/v) ethanol) for 30 min. Gels were washed three times with distilled water (10 minutes each). The gels were incubated with silver solution (0.1% (w/v) silver nitrate, 0.02% (v/v) formaldehyde) for 40 minutes at room temperature with gentle shaking. After this period, $AgNO_3$ solution was discarded and the gel briefly washed in a stream of distilled water for 20 seconds. Proteins were visualised by the addition of a development solution (2.5% (w/v) sodium carbonate, 0.01% (v/v) formaldehyde) for several minutes until protein bands

became prominent. Finally, the reaction was stopped in 1.5% (w/v) EDTA. $2H_2O$ for 15 minutes and stored in distilled water.

2.3.6.3 Transfer of Proteins onto Membrane Filters

After proteins had been separated by SDS-PAGE, it was possible to transfer them to nitrocellulose (NC) or polyvinylidene difluoride (PVDF) membranes (Western blotting) for further analysis of proteins/glycoproteins such as antibody and lectin staining, and amino acid analysis or sequencing. To date, numerous companies have provided equipment for the electrophoretic transfer of proteins from acrylamide gels to solid matrices such as NC and PVDF. There are two basic types of electrophoretic transfer systems used in this research: the "tank" buffer apparatus and semi-dry blotting apparatus.

a) The tank-buffer system:

This system has been designed from the first reports (Towbin *et al.*, 1979) which demonstrated the successful transfer of proteins to membranes and their subsequent detection by antibodies. This type of electrophoresis apparatus is fairly simple, in that an electrophoretic field is generated to transfer proteins from acrylamide to a more stable and permanent matrix such as NC or PVDF. Prior to blotting, both polyacrylamide gel and NC were soaked separately in transfer buffer for 10 minutes, and then prepared in a transfer sandwich consisting of filter papers soaked in buffer, the blotting membrane, and the gel, as shown in *Figure 2.1.* The transfer cassette was submerged in a "tank" of buffer for electrophoretic transfer of proteins. Transfer of proteins was achieved in a Biorad transblot apparatus using Tris-glycine-methanol transfer buffer (25mMTris HCl (pH 8.3) 192mM glycine, 20% (v/v) methanol) at 100mA, at 4°C overnight.

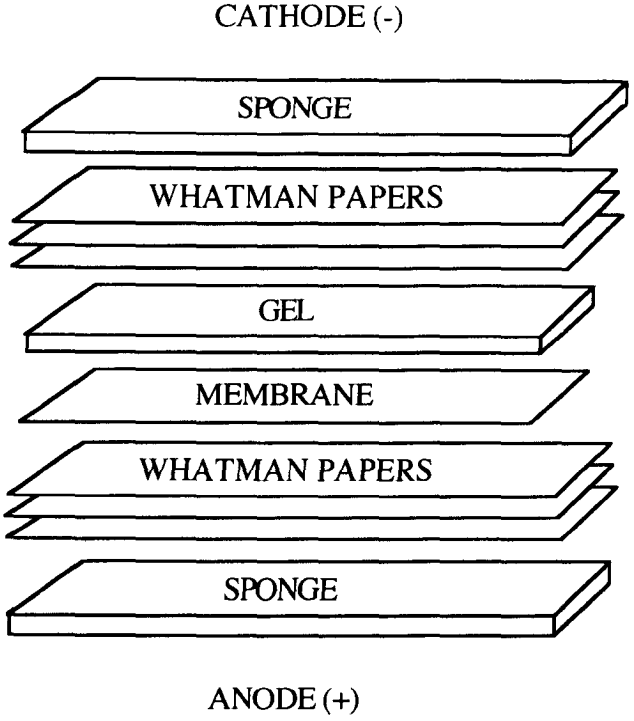


Figure 2.1. Schematic diagram showing the assembly of the blotting sandwich.

b) Protein Blotting Using Semi-Dry Electrophoretic Transfer Equipment

In a semi-dry transfer, a stack of wetted Whatman No 1 filter papers in Tris-glycine buffer surrounding the gel and the blotting membrane was used as a buffer reservoir, instead of a tank buffer system as described above. The main advantages with semi-dry transfer were the ease of handling, the short time required (1 hour) for the transfer, and the low buffer consumption. A transfer sandwich without sponges was prepared as shown in *Figure 2.1* and placed between semi-dry equipment plates containing electrodes which were previously wetted with Tris-glycine buffer (25mM Tris HCl (pH 8.3) 192mM glycine, 20% (v/v) methanol) or 10mM CAPS buffer. An apparatus of Hooffer Scientific Instruments USA, for semi-dry electrophoresis of proteins was used for an efficient transfer at 100mA for 1 hour in a cold room at 4°C.

2.3.7 Quantification Methods

2.2.7.1 Lowry Microprotein Assay

This assay, first described by Lowry *et al.* (1951), was used to determine the amount of protein present in cell and tissue extracts of tuberous sclerosis and normal skin samples.

Required stock solutions

Solution A : 2% (w/v) Sodium carbonate in 0.1M NaOH.

Solution B : 2% (w/v) Sodium potassium tartrate + 1% (w/v) copper sulphate in 0.1M NaOH.

Solution C : 0.1ml solution B + 4.9ml solution A

Folin-Ciocalteu phenol reagent : dilution 1: 1 with distilled water

Standard protein : Bovine serum albumin (BSA) 1mg/ml in distilled water.

Method

The assay detects 0-20μg of protein. Duplicate determinations were performed on each sample fraction and the sample volume made up to 20μl. Standard calibration curves were constructed on the same microtitre plate using 5,10,15, and 20μg of BSA suspended in the same buffer as the samples. 250μl of solution C was added to each well and mixed. The mixture was then left for 10 minutes. 30μl of Folin-Ciocalteu phenol reagent was subsequently applied and incubated for 30 minutes to allow colour development. The absorbances of samples were measured at 620nm using a plate reader (Dynatech MR5000). The BSA standards allowed the determination of a standard curve, which was used to estimate unknown protein concentrations.

2.3.7.2 Enzyme-Linked Immunosorbent Assay (ELISA)

The quantification of glycoproteins was made by enzyme-linked immunosorbent assay using a method described by Rennard *et al.* (1980).

96 well microtitre (Costar) plates were coated with fibronectin and laminin standards in the range 1 to 110ng/110 µl to give a standard curve. After 2 hours at room temperature, the wells were emptied of samples and washed twice with PBS. Blocking buffer (200 µl) made of PBS, pH 7.4 with 0.5% (w/v) BSA and 0.05% (v/v) Tween-20 was then applied to each microtitre well and incubated for a further 2 hours at room temperature or left overnight at 4 °C. Following this, 100 µl of rabbit anti-FN, anti-LM or anti-TN diluted 1:1000 (v/v) in blocking buffer was applied and incubated at room temperature for 2 hours.

The antibody solutions were removed and the wells washed twice with PBS and twice again with 200 µl blocking buffer. 100 µl of the secondary antibody, containing peroxidase-conjugated swine anti-rabbit IgG (1/3000, (v/v) in the blocking buffer) was added and incubated for 1 hour at room temperature.

Finally, 100 µl of enzyme substrate (0.01% (w/v) ABTS, 0.01% (v/v) H_2O_2 in 0.1M citrate phosphate buffer, pH 4.2) was added to each well and the reaction allowed to progress. Peroxidase enzyme catalyses the reaction : ABTS + H_2O_2 $\longrightarrow$ ABTS-O (chromophore) as demonstrated in *Figure 2.2.* After 30 minutes incubation at room temperature, the enzyme reactions were stopped with 50 µl 4 N H_2SO_4, and the amount of cromophore produced was measured at 405nm using a plate reader (Dynatech MR5000).

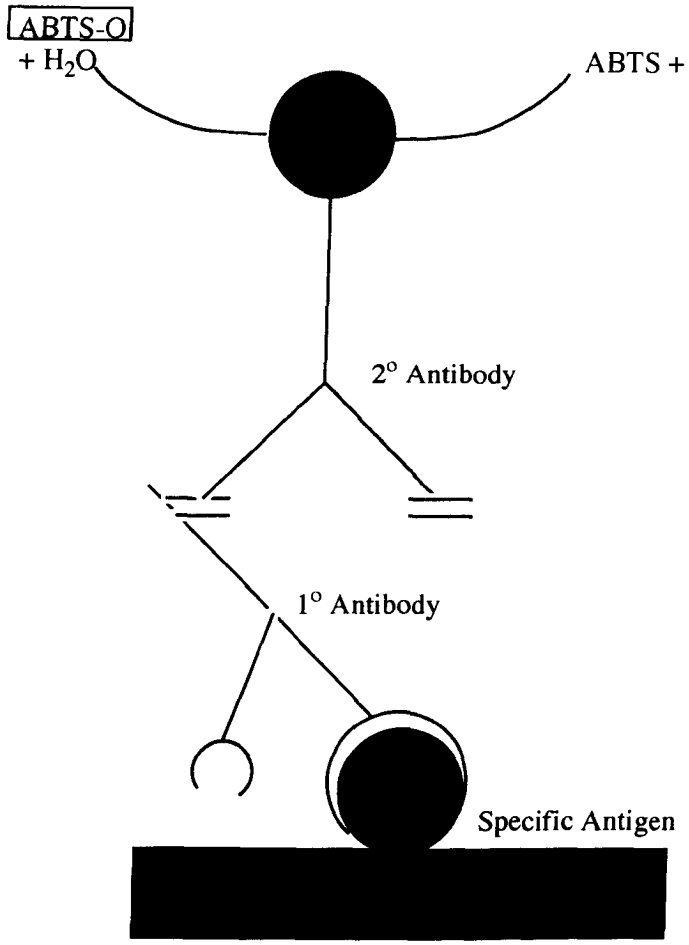


Figure 2.2. Schematic representation of ELISA method.

2.3.7.3 Flow Cytometry

Flow cytometry is a highly sensitive method by which certain properties of cells are measured in a "flow system". Cells in a rapid stream of fluid are orientated in a single column as they pass through a precisely focused laser beam. The cells pass a source of light, usually an argon laser, giving a single wavelength of 488nm and the amount of forward scatter (FSC) and side scatter (SSC) can be measured (see *Fig. 2.3*). FSC (at 2° to 18°) gives an indication of the size of the cells and SSC (at 90°) shows the structure of the

cell, particularly its granularity. Every cell that passes through the laser is measured individually and the data stored. Fluorescent substances are excited by the 488 nm (blue) incident laser light and emit longer wavelength light. Through the use of appropriate dichroic reflectors and narrow band-pass filters, light of specific wavelengths can be directed to specific photomultiplier tubes, thus allowing the use of up to three different fluorescent markers. Fluorescent 1 (FL1) corresponds to a wavelength of 510-530 nm (green), FL2 corresponds to 550-590 nm (red) and FL3 to > 600 nm (red). Thus, cells can be differentiated by size and granularity as well as up to three fluorescent markers allowing detailed analysis of cell phenotypes. Moreover, a flow cytometer can be used quantitatively for measuring the fluorescence intensity of particles in a liquid stream in which cells are injected and measurements made.

Flow cytometry is commonly used for the analysis of suspension cells, in particular blood cells (lymphocytes, monocytes and erythrocytes etc.), to diffferentiate them. However, we have adapted a method for the quantitative analysis of major extracellular matrix glycoproteins expressed by skin fibroblasts attached to the cell culture plate. Therefore, a FACScan (Becton-Dickinson, CA, USA) was used for the quantitative analysis of TS and normal skin fibroblasts samples for the expression of fibronectin (FN), laminin (LM) and tenascin (TN) quantitatively. 2000 events were collected using Consort 30 program and standard fibroblasts settings. A logical fibroblasts gate was set before data analysis. FlowMATE (Dako) was used to analyse fluorescence intensity of the cells.

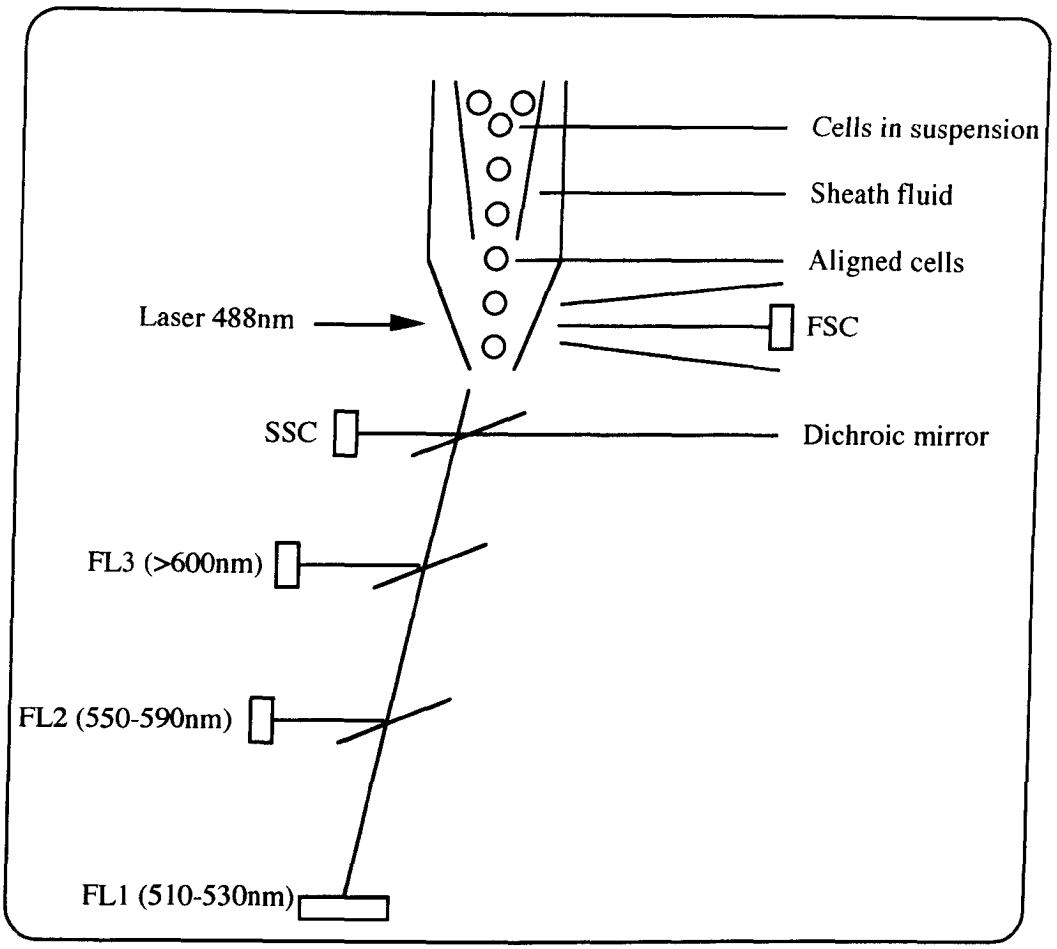


Figure 2.3. A simple schematic diagram of a flow cytometer.

An adapted protocol for the preparation of cultured skin fibroblasts for flow cytometry was as follows;

An Adapted Protocol for the Preparation of Cultured Skin Fibroblasts for Flow Cytometry (Fluorescence-activated cell sorter, FACS) :

1) Spent medium was removed from culture dishes and cells washed twice in PBS.

2) Cells were harvested at confluence using 5mM (or 0.1-0.2%) EDTA (as an alternative to trypsin) in PBS for 20min. at 37° C.

3) Cells were transfered into a universal tube and centrifuged at 150g for 7 minutes in a bench centrifuge (Sartories).

4) Supernatants were removed and cell pellets were gently disrupted by tapping the tube or vortexing.

5) Cells were washed twice with 0.1% bovine serum albumin (BSA) in PBS.

6) Cells were resuspend in 3.8% formaldehyde in PBS (1ml formaldehyde + 9ml PBS) for 10 min. on ice.

7) Cells were centrifuged at 150g for 7 minutes, supernatent was removed and the cell pellet was gently disrupted by tapping the tube or vortexing.

8) Wash cells twice with 0.1% bovine serum albumin in PBS.

9) Cells were resuspend in permeation solution in 70% ethanol (or 1mg/ml glycine + 0.1% Triton X-100) for 10 minutes on ice.

10) Cells were centrifuged at 150g for 7 minutes, supernatent was removed and the cell pellet was gently disrupted by tapping the tube or vortexing.

11) Cells were resuspended in 0.1% BSA in PBS.

12) Cells were washed once by centrifugation in 1% BSA/PBS solution.

13) Pellets were resuspended in BSA/PBS solution and cells counted, then made to $8x10^5$ cells per ml solution in 1% BSA/PBS and transfered into eppendorf microfuge tubes as duplicates. The following incubations were performed.

a) 125µl of $8x10^5$ cells/ml solution (10^5 cells total): incubated with rabbit antibody to human fibronectin (1/50 dilution) for 1 hour on ice.

b) 125µl of $8x10^5$ cells/ml solution (10^5 cells total): incubated with rabbit antibody to tenascin (1/50 dilution) for 1 hour on ice.

c) 125µl of $8x10^5$ cells/ml solution (10^5 cells total): incubated with rabbit antibody to laminin (1/50 dilution) for 1 hour on ice.

d) 125µl of $8x10^5$ cells/ml solution (10^5 cells total): incubated with 1% BSA/PBS solution for 1 hour on ice.

e) 125µl of $8x10^5$ cells/ml solution (10^5 cells total): incubated with 1% BSA/PBS solution for 1 hour on ice.

14) Cells were centrifuge for 4 seconds, supernatant was removed and cells washed twice with 1% BSA/PBS, supernatant was removed, and cell pellet was gently disrupted by tapping tube or vortexing eventually.

15) The following; (a), (b), (c) and (d) were incubated with secondary fluorescein isothiocyanate-FITC conjugated antibody (FITC-swine anti-rabbit) in 1%BSA/PBS at a dilution of 1/30 for 1 hour on ice.

Tube (e) was incubated with 1%BSA/PBS only at a dilution of 1/30 for 1 hour on ice.

a) 125µl of $8x10^5$ cells/ml solution (10^5 cells total): incubated with secondary antibody to fibronectin (1/50 dilution) for 1 hour on ice.

b) 125µl of $8x10^5$ cells/ml solution (10^5 cells total): incubated with secondary antibody to tenascin (1/50 dilution) for 1 hour on ice.

c) 125µl of $8x10^5$ cells/ml solution (10^5 cells total): incubated with secondary antibody to laminin (1/50 dilution) for 1 hour on ice.

d) 125µl of $8x10^5$ cells/ml solution (10^5 cells total): incubated with secondary antibody for 1 hour on ice (to check nonspecific binding).

e) 125µl of $8x10^5$ cells/ml solution (10^5 cells total): incubated only with 1% BSA/PBS solution for 1 hour on ice (to check autofluorescence).

16) Cells were centrifuged for 4 seconds, supernatant removed and cells washed twice with 1% BSA/PBS, removed supernatant and the cell pellet disrupted by tapping tube or vortexing.

17) Cells were resuspended in 0.5 ml 1% BSA/PBS solution.

18) Flow cytometry was performed (FACScan, Becton Dickinson): a gate was set for fibroblasts and 2000 events were collected. a Consort 30 software was used for acquisition of data.

2.3.8 Detection Methods

2.3.8.1 Immunodetection of Glycoproteins on Nitrocellulose Membrane

After Western blotting, the nitrocellulose was processed for antigen detection as follows. Non-specific protein binding sites were blocked using 0.5% (w/v) bovine serum albumin, 0.05% (v/v) Tween-20 in PBS pH 7.4 for 2 hours at room temperature, or overnight at 4°C. Primary antibodies (rabbit anti-human FN, anti-human TN, anti-human LM, and mouse anti-HNK-1) were diluted in blocking buffer to 1:1000 (v/v) and applied to the nitrocellulose blot for 2 hours at room temperature. After this period, the nitrocellulose was washed three times (10 minutes each) with PBS, pH 7.4 supplemented with 0.05 % (v/v) Tween-20. As the secondary antibody, peroxidase conjugated swine antirabbit immunoglobulin (diluted 1/3000, v/v, in blocking buffer) was applied onto nitrocellulose for FN, LM, and TN detection, and peroxidase conjugated anti mouse antibody was applied for HNK-1 carbohydrate detection for 1 hour each. The blot was washed three times (10 minutes each) with PBS containing Tween-20.

Finally, the blot was developed using fresh 3, 3' -diaminobenzidine (DAB) solution (12mg DAB in 18ml of Tris-HCl, pH 7.6, 1ml of 0.3% (w/v) $NiCl_2$). This solution was filtered through Whatman No.1 filter paper and 20µl of H_2O_2 added to the filtered solution. The blot was applied to this solution and the reaction allowed to proceed until the brown bands were clearly visible (this usually took less than 5 minutes). The reaction was stopped by rinsing with PBS, and the membrane was then dried in air.

In some experiments instead of using 3-3' diaminobenzidine, an enhanced chemilunescence kit was used. Luminol, in the presence of hydrogen peroxide was added to the blot and allowed to react for one minute. The excess detection reagent was drained off and the blot was wrapped in cling

film and exposed to Hyperfilm^{TM} MP (Amersham) for a period of 30 seconds to one minute. The film was then removed and developed. This sensitive detection system was used for detection of HNK-1 carbohydrate epitope and laminin B1 chain with monoclonal antibodies.

2.3.8.2 Lectins and Glycan Differentiation On Nitrocellulose Membrane

Lectins can be defined as proteins of non-immune origin that precipitate glycoconjugates and agglutinate cells. Lectins are able to bind to carbohydrate moieties of proteins. The specific binding of lectins to carbohydrate moieties allows the identification of sugar residues of proteins. Hence, lectins conjugated to digoxigenin (DIG) were used to label and detect specific sugars on a Western blot. A Boehringer Mannheim Biochemica Glycan Differentiation Kit (Cat. No. 1210238) was used for this purpose. The lectins applied were conjugated with the steroid hapten digoxigenin which enables immunological detection of the bound lectins following incubation with secondary alkaline phosphatase conjugated anti-digoxigenin antibodies. Brown colour development was performed in the presence of a suitable substrate (NBT and X-phosphate).

Specificities of the lectins included in the kit:

MAA (*Maackia amurensis agglutinin*): recognizes sialic acid linked α(2-3) to galactose.

GNA (*Galanthus nivalis agglutinin*): recognizes terminal mannose, α(1-3), α(1-6) or α(1-2) linked to mannose.

SNA (*Sambucus nigra agglutinin*): recognizes sialic acid linked α(2-6) to galactose.

PNA (*Peanut agglutinin*): recognizes the core disaccharide galactose β(1-3) N-acetylgalactosamine.

DSA (*Datura stramonium agglutinin*): recognizes galactose β(1-4) N-acetylglucosamine.

Required solutions for lectin staining

TBS (Tris buffered saline): 0.05M Tris-HCl, 0.15M NaCl, pH 7.5.

Buffer 1: 1mM $MgCl_2$, 1mM $CaCl_2$, 1mM $MnCl_2$ in TBS, pH 7.5.

Buffer 2: 0.05M $MgCl_2$, 0.1M NaCl in 0.1M Tris-HCl, pH 9.5.

Method

1) The samples were first run on a polyacrylamide gel and then transferred onto a nitrocellulose membrane by the Western blot method.

2) The dried nitrocellulose was incubated for at least 30 minutes in 20ml blocking buffer (0.25g blocking reagent in 50ml TBS).

3) The blocking solution was removed and the nitrocellulose washed twice for 10 minutes each in 50ml TBS and then once with buffer 1.

4) The nitrocellulose was exposed to lectins: 50µl of MAA or 10µl of GNA was added to 10ml of buffer 1 and incubated with the filter for 1 hour in this solution.

5) The lectin solution was removed by washing the blot three times with 50ml TBS.

6) 10µl of conjugate (alkaline phosphatase conjugated anti-digoxigenin) was added to 10ml TBS and nitrocellulose was incubated in this solution for 1 hour.

7) The conjugate solution was removed by washing the nitrocellulose three times with 50ml TBS.

The glycoproteins on the nitrocellulose were stained using 10ml of buffer 2 containing 50µl NBT (100mg 4-nitroblue tetrazolium chloride in 1.3 ml dimethylformamide) and 37.5µl X-phosphate (50 mg 5-bromo-4-chloro-3-indolyl-phosphate in 1ml dimethylformamide. The formation of brown colour was usually completed within a few minutes and the filter was rinsed with distilled water to stop the reaction.

2.3.8.3 Glycan Detection

This method was used to detect glycoproteins on Western blots. A DIG Glycan Detection Kit from Boehringer Mannheim Biochemica was used. The principle of this method is described here; Sodium metaperiodate was added to the filter to oxidise vicinal hydroxyl groups, on the glycoprotein oligosaccharides, into aldehyde groups. Added digoxigenin (DIG) was then covalently attached to these aldehydes via a hydrazide group. A DIG-specific antibody conjugated with alkaline phosphatase was then used to detect the DIG labelled glycoproteins.

Required solutions:

Sodium acetate buffer, 0.1mol/1, pH 5.5:

Tris buffered saline (TBS), pH 7.5;
Tris-HCl, 0.05 mol/l; NaCl, 0.15 mol/l

Tris buffer, pH 9.5;
Tris-HCl, 0.1 mol/l, pH 9.5; $MgCl_2$, 0.05 mol/l; NaCl, 0.1 mol/l.

PBS (phosphate buffered saline), pH 6.5 ;
potassium phosphate K_2PO_4, 50 mmol/l; NaCl, 150 mmol/l.

Method

1) The filter was first washed twice in approximately 50 ml PBS, pH 6.5.

2) The filter was incubated in approximately 10 ml of 10 mM sodium metaperiodate (dissolved in sodium acetate buffer) for 20 minutes at room temperature.

3) The filter was washed 3 times for 10 minutes each with approximately 50 ml PBS.

4) The filter was incubated with 1 µl DIG-succinyl-ε-amidocaproic acid hydrazide dissolved in 5 ml sodium acetate buffer for 1 hour at room temperature.

5) The filter was washed 3 times (10 minutes each) with approximately 50 ml TBS.

6) The filter was incubated in the blocking solution (0.25g blocking reagent in 50 ml TBS, pH 7.5) for at least 30 minutes.

7) The filter was washed 3 times (10 minutes each) with approximately 50 ml TBS.

8) The filter was incubated with 10 µl anti-DIG-alkaline phosphatase dissolved in 10 ml TBS for 1 hour.

9) The filter was washed 3 times (10 minutes each) with approximately 50 ml TBS.

10) The filter was immersed without shaking in staining solution (10 ml TBS pH 9.5, 37.5 µl X-phophate, 50 µl NBT) until colour developed.

11) Finally, the filter was rinsed several times with distilled water to stop the reaction.

2.3.9 Carbohydrate Analysis of Fibronectin: High-pH Anion Exchange Chromatography With Pulsed Amperometric Detection (PAD)

The Dionex Corporation has developed a technique for the separation of carbohydrates. This has been commonly described in the literature as HPAEC-PAD (High performance, or High-pH, Anion Exchange Chromatography-Pulsed Amperometric Detection).

2.3.9.1 Principles of the Method

Carbohydrates are weak acids with p*K*a values of 12-14. In HPAEC, a strong alkaline solution, usually NaOH, is used as eluent. Under these conditions the hydroxyl groups of carbohydrates are transformed into oxyanions, thereby all carbohydrates are negatively charged from oxyanion formation; this is the basis for their retention on strong anion-exchange columns. The HPAEC-PAD system uses sodium hydroxide as the main

eluent to ionise the weakly-acidic hydroxyl groups of carbohydrates which are then separated by a high performance anion exchange column. The elution order of monosaccharides correlates well with their p*K*a values. For example, glucose and mannose have p*K*a values of 12.28 and 12.08 respectively. Mannose elutes later than glucose. Carbohydrates can be detected with high sensitivity (in the low picomole range) by PAD (Hardy and Townsend, 1994).

2.3.9.2 Reagents for HPAEC-PAD

Sodium hydroxide: A 50% solution of NaOH (w/v) was purchased from BDH Chemicals Ltd, Poole. 8ml of 50% NaOH was added to 1 litre of helium degassed nanopure water to give 100 mM solution. It was important not to disturb the carbonate which precipitates to the bottom of this solution during the pipetting of the eluent.

Acetate: Sodium acetate (HPLC grade) was obtained from BDH Chemicals. A 0.5 M solution in nanopure water was filtered using a 0.22 μm Millipore filter and sparged with helium for 1/2 hour, and used for sialic acid analysis.

2.3.9.3 System Settings and Analysis of Carbohydrates by HPAEC-PAD

Monosaccharides of fibronectin were analysed using a Dionex BioLC system consisting of a CarboPacPA-1 anion exchange column of pellicular resin (4x250mm) with pulsed amperometric detection (PAD) equipped with a gold electrode. Hydrolysis with 2M trifluoroacetic acid (TFA) and heat was performed to release neutral and amino sugars from glycoproteins. Samples were dissolved in 100 μl with water, to which freshly prepared 4M TFA (100 μl) was added. These samples were then heated at 100°C for 5 hours, and then dried under nitrogen gas at 30 °C. Dried samples were dissolved in nanopure water at a concentration of 1μg/1μl and injected onto the Dionex-

HPAEC-PAD. TFA, a strong, volatile acid, was handled in a fume hood to avoid skin contact and inhalation.

A standard mixture of fucose (Fuc), galactosamine (GalN), glucosamine (GlcN), galactose (Gal), glucose (Glc) and mannose (Man) at a concentration of 1 nM each was also prepared with 2M TFA and injected onto HPAEC-PAD with unknown monosaccharide samples. The retention times and integrals of the individual peaks of this standard mixture were used for the identification and quantification of monosaccharides present in fibronectin samples. 10-40 μl of monosaccharide samples obtained from fibronectin of TS and normal fibroblasts were loaded via a Spectra-Physics SP8880 autoinjector and eluted with helium sparged eluants at a constant flow rate of 1ml/min at 30°C. Monosaccharides were separated by an isocratic solution of 17 mM NaOH from 0 to 15 min, followed by a 10 min wash step with 200 mM NaOH and the column was then re-equilibrated with 17 mM NaOH. Total run time was 37 minutes. Data were collected and stored using a RaChel (LabLogic, UK) software program with an Ahkter 386SX computer. The manipulation of data and calculation of integrals was performed with the RaChel program.

For the sialic acid (N-acetyl-neuraminic acid, Neu5Ac) analysis, a hydrolysis of Neu5Ac derived from purified fibronectin and cell extracts was performed with 50 mM trifluoroacetic acid (TFA) in a heating block for 1hr at 80°C. A 1 nM Neu5Ac standard was run as duplicate with unknown samples. A sodium acetate SA3-H gradient was required to elute the acidic sugars (Neu5Ac) from the CarboPac PA-1 anion exchange column. The elution of Neu5Ac was by 100 mM NaOH with a 50-500 mM sodium acetate concentration gradient. The gradient was as follows: 0-7.5 minutes 50-150mM sodium acetate, 7.5-15 minutes 150 mM sodium acetate, 15-20 minutes 150-500mM sodium acetate, 20-25 minutes 500 mM sodium acetate

(wash step), 25-35 minutes 50 mM sodium acetate (equilibration step) with 100mM NaOH.

The PAD settings with gold electrode were as follows : E_1=0.05 V, E_2=0.8 V, E_3=-0.15 V, pulse durations; T_1=420 ms (range 2, position 4), T_2=180 ms (position 3), T_3=360 ms (position 6) with a response time of 1 s and at a range of 1000 nA.

For the oligosaccharide analysis, aliquots of fibronectin samples purified from TS and control fibroblasts, plasma fibronectin, bovine fetuin and human transferrin as positive control standards were digested using N-glycanase (see section 4.3.1 for the details of the samples). The samples were dried using a speed Vac, dissolved in nanopure water and analysed directly by HPAEC. Oligosaccharides were seperated under OXALSA gradient of 100 mM NaOH. 0-15 min. isocratic 15 mM sodium acetate (NaAc), 15-70 min. gradient from 15mM-255 mM NaAc, 70-80min. isocratic wash step 500 mM NaAc, 80.1 min. re-equilibrates 15 mM NaOAc. PAD settings: E_1=0.05 V, E_2=0.8 V, E_3=-0.15 V, pulse durations; T_1=420 ms., T_2=180 ms., T_3=360 ms.

2.3.10 Comparative Immunocytochemical Studies of Skin Fibroblasts with Antibodies to Fibronectin,Tenascin and Laminin

To investigate the expression and distribution of fibronectin, laminin and tenascin in skin fibroblasts derived from patients with TS and normal individuals, I carried out immunofluorescence studies on cultured fibroblast cells after 5-6 passages following primary cultures of skin tissues. For this purpose, TS fibroblasts and normal fibroblasts (control) were allowed to attach to glass coverslips in 6 well cell culture dishes in the presence of medium containing 10% foetal calf serum. The following second or third day

of cell culture, immunofluorescence staining of fibroblasts was undertaken as below;

1) The medium was removed, and cell layers were washed three times with phosphate buffered saline (PBS), pH 7.4.

2) Fibroblasts were fixed with 3.8% formaldehyde in PBS (1:10) for cell surface staining of fibronectin, laminin, and tenascin. Cells were then permeabilised with a solution of 0.1% (v/v) Triton-X-100 and 1mg/ml (w/v) glycine in PBS when cytoplasmic staining of these three glycoproteins was required.

3) Fixed fibroblasts were washed with PBS three times, 3 minutes each.

4) Cells were then blocked with 1% (w/v) bovine serum albumin in PBS for 1hr at room temperature or overnight at 4°C.

5) Fibroblasts were incubated with specific primary antibodies (rabbit polyclonal anti-human fibronectin, laminin or tenascin at 1: 50 dilution and monoclonal mouse anti-human laminin at 1: 30 dilution in blocking buffer) for 1 hour at room temperature (RT). These primary polyclonal and monoclonal antibodies were omitted for the negative staining of fibroblasts as control.

6) Cell layers were washed extensively with PBS three times, and incubated for 1 hour at RT with a fluoresceinated secondary swine anti-rabbit antibody for laminin, fibronectin, tenascin, and fluoresceinated secondary rabbit anti-mouse for laminin staining (at 1:30 dilution) in the dark.

Finally cells were washed twice with PBS, and cover-slips were mounted in 1:1 (v/v) PBS/glycerol solution with 8 mg/ml (w/v) 1-4 diazabicyclo-2.2.2-octane (DABCO) to prevent photobleaching. Cover-slips were attached to slides using nail-varnish. Fluorescence was observed in a Nikon microscope (Labophot Y2A). Photographs were taken using Kodak EPD-200 film, with same exposure times

In some cases, the photographs of the cells were taken using a Bio-Rad DVC-250 Confocal laser scanning microscope in the Department of Obstetrics and Gynaecology with permission of Dr. Steven Fleming.

2.3.11 Amino Acid Analysis of Fibronectin

Standard plasma and cellular fibronectin, and purified fibronectin samples from the (collected medium of) fibroblasts of patients with tuberous sclerosis and from normal individuals were run on the 5% PAGE. Proteins of the gel were blotted onto a polyvinylidene difluoride (PVDF) membrane with semi-dry blotting equipment using 10mM CAPS buffer, 3-[cyclohexylamino-1-propanesulfonic acid], pH 11 for 1 hour. In this analysis, CAPS buffer was used to facilitate transfer of proteins (standard transfer buffer contains glycine which would complicate amino acid analysis) on to PVDF membrane (Millipore Ltd). Fibronectin bands were excised from the PVDF membrane after amido black staining as described in section 2.3.12, and then amino acid analysis were carried out using an Applied Biosystems model 420H automated amino acid analysis system. Hydrolysis with 6M hydrochloric acid at 200°C and subsequent pre-column derivatisation were carried out automatically. Amino acids were identified and quantified against known standards. This work was done in collaboration with Mr. K. Bailey in the Departmental Bio-polymer Synthesis and Analysis Unit (BSAU).

2.3.12 Procedure for Amido Black Staining

After semi-dry blotting of proteins, polyvinylidene difluoride (PVDF) membrane was stained with Amido Black just before amino acid analysis and sequencing of the fibronectin.

1) Blotted PVDF membrane was removed from the blotting sandwich and rinsed with distilled water.

2) The membrane was then saturated with 100% methanol for a few seconds to wet it.

3) Membrane was stained with 0.1% (w/v) Amido Black in 40% (v/v) methanol and 1% (v/v) acetic acid to visualise the protein bands.

4) Finally, the membrane was destained by rinsing extensively with distilled water.

2.3.13 Digestion of Fibronectin with Chymotrypsin and Separation of Gelatin-binding Fragments

600 μg Plasma fibronectin was digested by chymotrypsin (Calbiochem) in 0.05 M Tris-HCl buffer pH 7.0 containing 0.15 M NaCl and 0.01 M $CaCl_2$ at enzyme/substrate ratio of 1:50 for 60 min at 23°C. The reaction was terminated by adding 2mM PMSF and soybean trypsin inhibitor. The digests were applied to a Prosep-gelatin column. The unbound fractions were pooled, dialysed and then concentrated. Bound fibronectin was eluted with phosphate-buffered saline PBS (pH 7.5) containing 4M urea, dialysed against water and lyophilised in a vacuumed freeze drier, and concentrated in PBS before its further analysis for protein sequencing as described below.

2.3.14 Amino-terminal Protein Sequencing of Fibronectin

Amino-terminal protein sequencing (Edman degradation) of plasma fibronectin was determined by K. Bailey (BSAU) using an Applied Biosystems model 473A automated protein sequencer. Data were collected and analysed using 610A data analysis software (Applied Biosystems, Foster City, California). Plasma fibronectin was run on the 5% PAGE and blotted onto the PVDF membrane with semi-dry blotting equipment using 10 mM CAPS buffer, pH 11. The fibronectin band was excised from the PVDF membrane after amido black staining, and sequenced directly. Sequencing of the excised fibronectin band showed that fibronectin was blocked due to

pyroglutamic acid from the N-terminal site and did not give any sequence of amino acids.

In the second attempt of protein sequencing, fibronectin was proteolysed using chymotrypsin. The gelatin-binding domain of fibronectin was purified from its proteolytic fragments using Prosep-gelatin column chromatography. The purified domain of fibronectin was run on the 10% PAGE and blotted onto the PVDF membrane with semi-dry blotting equipment using 10mM CAPS buffer, pH 11. The excised gelatin-binding domain was not blocked and was sequenced successfully.

Chapter 3

Chapter 3

Purification of Fibronectin From Conditioned Medium of TS and Control Fibroblasts

3.1. Introduction

Fibronectins are extracellular matrix adhesive glycoproteins that are important in embryonic development, wound healing and tumorigenesis. Fibronectin molecules act as bridges between cell and extracellular materials. There are at least two major classes of fibronectins, namely plasma fibronectin and cellular fibronectin. In the blood, fibronectin is dimeric, but in tissues it forms disulphide crosslinked fibrils (Hynes, 1990, 1986). Plasma and cellular fibronectins are very similar in amino acid and carbohydrate composition and in certain biological activities involving cell attachment to both collagen and plastic. However, the forms of fibronectin clearly differ in certain other biological activities that involve cell-cell interactions, cell morphology and hemagglutination. They have also slightly different subunit polypeptide sizes, and major differences in solubility. Plasma fibronectin migrates on SDS-polyacrylamide gels under reducing conditions as a doublet, implying that at least two different (220-230kDa) subunits exist. However, both cellular and amniotic fibronectins appear larger than plasma fibronectin and do not show a well-resolved doublet pattern (Hynes, 1990).

One of the main objectives of this thesis was to characterise the cellular fibronectin, in particular with respect to glycosylation, from the conditioned

medium synthesised by cultured skin fibroblasts derived from patients with tuberous sclerosis (TS) and normal individuals. In order to achieve this objective it was necessary to purify fibronectin in substantial quantities for carbohydrate analysis. In this chapter, the results of the isolation of cellular fibronectin from conditioned medium of cultured skin fibroblasts from patients with TS and normal individuals will be presented and discussed.

This chapter also describes the partial purification of laminin and tenascin from conditioned medium to demonstrate their synthesis and secretion by fibroblasts into culture media as extracellular matrix glycoproteins.

3.2 Use of Fibroblast Cultures as an Experimental System

The macromolecules that constitute the extracellular matrix are mainly secreted locally by cells in the matrix. In most connective tissues these macromolecules are secreted by fibroblasts. Fibroblasts are principal cells of the dermis (Alberts *et al.*, 1989), their cell culture systems having been used in many studies of disease and also in fibronectin research (Sly and Grubb, 1979, and Hynes, 1990). There are several reasons for the popularity of fibroblasts systems, one of which is that they can be grown in culture from small amounts of tissue explants.

Since TS appears to be a genetic disorder and that one of its major manifestations occurs in the skin of most patients, it would appear that cultured dermal fibroblasts derived from such individuals would provide a convenient and accessible system for the study of this disease which might, in turn, contribute to the elucidation of the possible molecular mechanisms of the disease process.

In this work, fibroblasts were established from primary cultures of skin explants of patients with tuberous sclerosis (TS). In some cases this was from skin lesions and in other cases from unaffected skin. A cell culture

system has been employed to study the adhesive glycoproteins fibronectin, laminin and tenascin synthesised by these established TS fibroblasts. Control fibroblasts, for comparison, have been grown from explants of people who are not known to be suffering from TS nor any other disease or disorder (see *Fig. 3.1*).

Established fibroblasts from TS and normal skin were grown at 37° C until they reached confluence. At confluence, the standard medium was replaced by fibronectin-depleted medium to avoid any plasma fibronectin contamination of the fibroblasts cellular fibronectin. After 2-3 days, the conditioned medium was harvested from the cell culture dishes in the presence of protease inhibitors, 2mM diaminoethanetetra-acetic acid (EDTA) and 2mM phenylmethylsulfonyl fluoride (PMSF). Harvested medium of TS and normal fibroblasts was stored at -80°C until sufficient medium was available for fibronectin purification.

3.3 Purification of Fibronectin by Gelatin affinity-Gel Chromatography

As described in chapter 2 (section 2.2.2.1), cellular fibronectin was isolated by affinity chromatography on a Prosep-gelatin (Bioprocessing) column as a first step purification procedure, by a method established by Engvall and Ruoslahti (1977).

Fibroblasts secrete much of the fibronectin they synthesise into the culture medium in preference to retaining it on the cell surface.

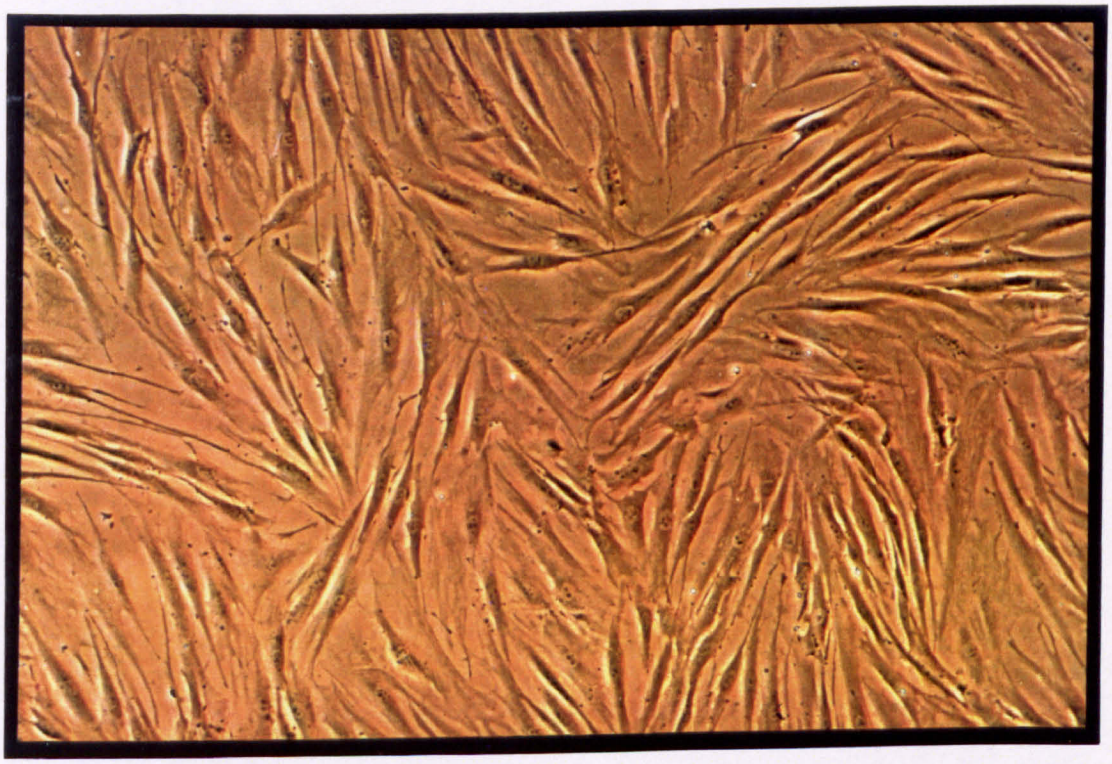


Figure 3.1. The figure shows the typical control fibroblasts established from normal human skin. These established fibroblasts were cultured to act as controls for fibroblasts established from the skin of the TS patients, 26x magnification.

Spent culture medium was therefore a logical source of fibronectin which could be purified by gelatin-affinity and gel filtration as well as anion-exchange chromatography techniques.

Foetal calf serum previously depleted of fibronectin was used in the preparation of the medium to ensure that the product is pure cellular fibronectin. Fibronectin-containing culture medium (500-1500ml) was collected from skin fibroblasts of different TS patients and normal skin fibroblasts, and stored at -80° C until required.

Typically, a 10ml Prosep-gelatin column (Bioprocessing Ltd.) with 2mg/ml fibronectin binding capacity was equilibrated with phosphate-buffered saline (PBS) containing 2mM EDTA and 2mM PMSF, pH 7.5. Fibronectin-containing conditioned medium of fibroblasts was defrosted and filtered with Whatman (No. 50) filter paper, then passed through the Prosep-gelatin column at room temperature (5-12 hours). The Prosep-gelatin column was washed with 5 column volumes of PBS, then washed with 1M NaCl and 1M urea in PBS, pH 7.5 to remove other contaminants. Bound fibronectin was eluted from Prosep-gelatin with 4M urea in 20 mM CAPS, pH 11, containing 0.15M NaCl, 2mM PMFS, 2mM EDTA, and absorbances of collected 2ml fractions were measured at 280 nm spectrophotometrically as shown in *Figure 3.2.*

Eluted fractions were assayed by ELISA, and fibronectin-positive fractions were dialysed, freeze-dried and concentrated in a vacuum dryer. The pellet was redissolved in 0.5 ml 20mM CAPS buffer, pH 11. To analyse the purified protein, samples were denatured and resolved by 4-15% gradient polyacrylamide gel electrophoresis.

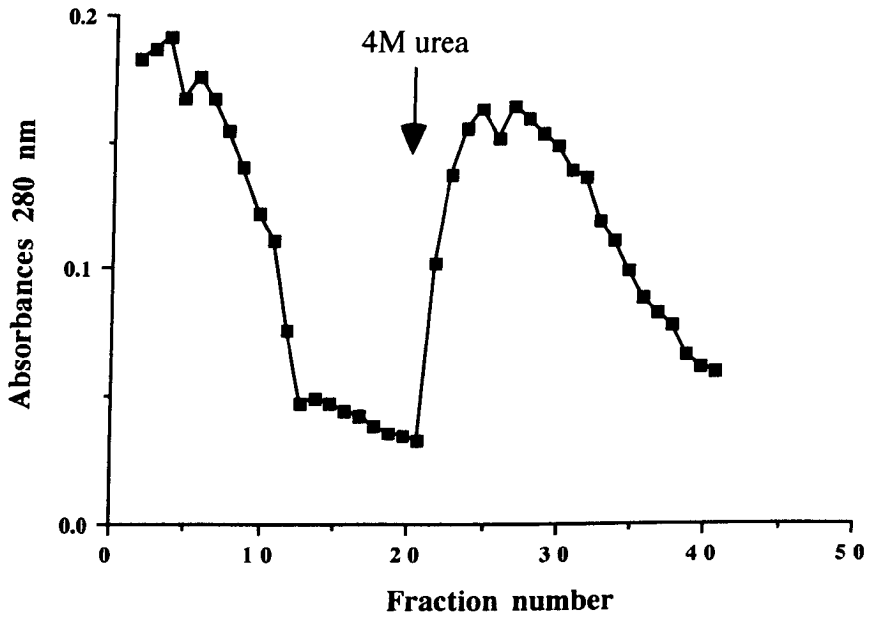


Figure 3.2. Elution of protein from Prosep-gelatin. The figure shows the affinity chromatography of cellular fibronectin from culture medium on a column of Prosep-gelatin. 2ml fractions were collected. The arrow indicates the beginning of the elution of fibronectin with 4 M urea.

Figure 3.3 shows a polyacrylamide gel of the partially purified cellular fibronectin from both TS and control skin fibroblasts from the Prosep-gelatin affinity column. Bands, of the correct molecular weight of fibronectins (approx. 230kDa) were clearly visible on the gel (see *Fig. 3.3*).

The final yield of cellular fibronectin after the Prosep-gelatin column was typically 85% of that from conditioned medium applied to the column, as determined by ELISA (*Table 3.1*).

* Amount of FN in 1000 ml conditioned medium	: 0.408mg
* Amount of FN after prosep-gelatin affinity chromatography	: 0.346mg
* % yield of FN	: 85

Table 3.1. The table shows an example of the recovery of fibronectin assayed by ELISA obtained from conditioned medium of TS skin fibroblasts before and after Prosep-gelatin affinity chromatography.

It is worth noting that tenascin was found to be eluted by 1M urea from the Prosep-gelatin column during the fibronectin purification (see *Figure3.4*). Tenascin was eluted by 1M urea wash from the column following washing with five column volume of PBS before the elution of fibronectin. Eluted tenascin from Prosep-gelatin was collected in 40-50 ml of 1M urea solution and dialysed overnight against PBS, pH 7,5, at 4° C. Dialysed sample was freeze dried and dissolved in 200µl PBS, pH 7.5, and mixed with equal amount of sample buffer containing mercaptoethanol for resolving by 5% SDS PAGE. Interestingly, tenascin purified in this way did not bind to a new Prosep-gelatin column. This suggests that tenascin binds or interacts with fibronectin on the Prosep gelatin column.

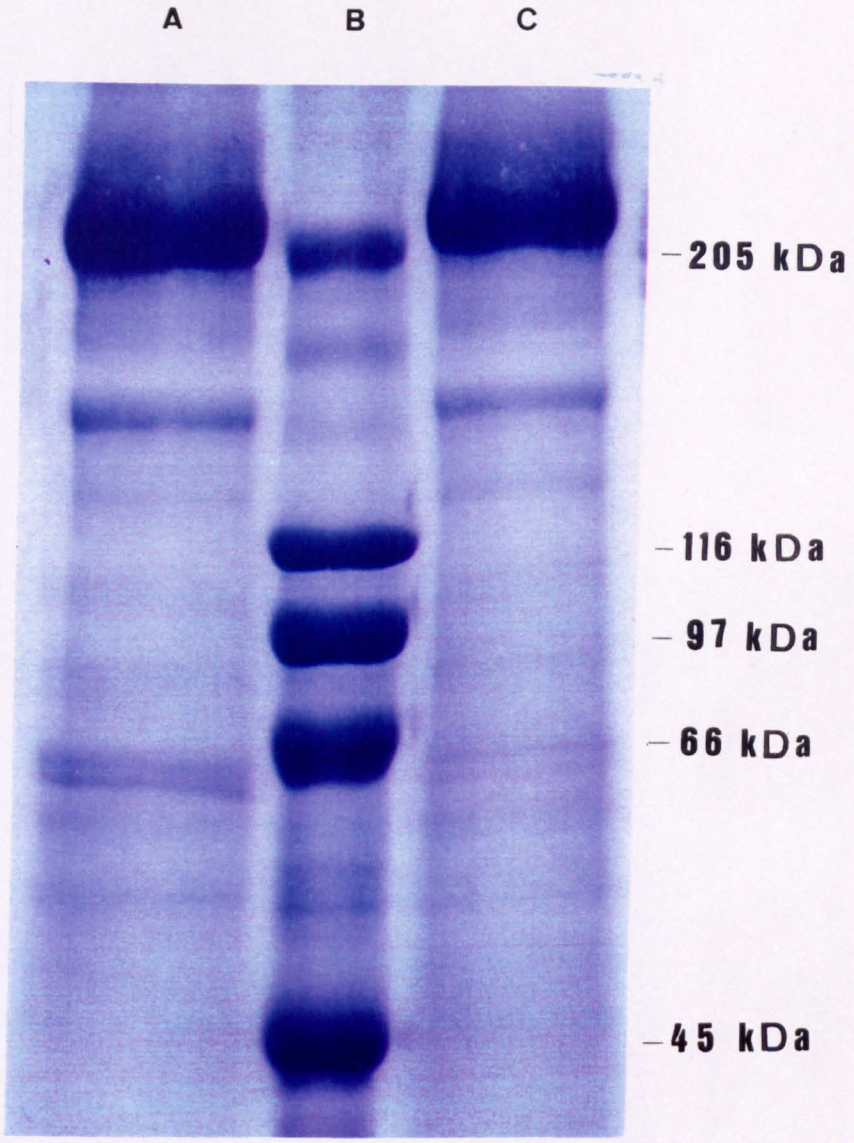


Figure 3.3. SDS 4%-15% gradient polyacrylamide gel electrophoresis pattern of partially purified fibronectin (16μg each) from the cell culture medium of (A) normal and (C) TS skin fibroblasts, using Prosep-gelatin beads. Also shown is (B) high molecular weight marker mixture, with the actual molecular weights indicated to the right. The gel was stained for protein using Coomassie Brilliant Blue.

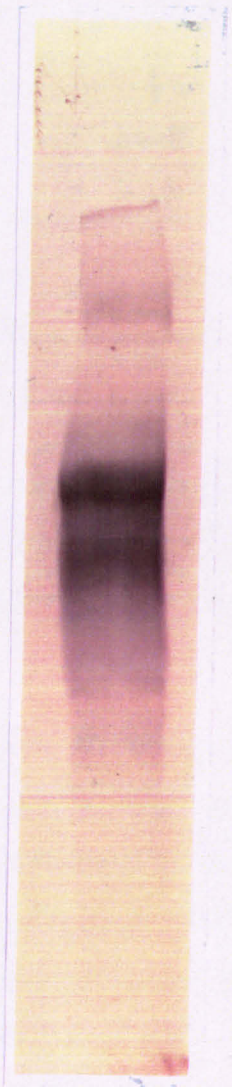


Figure 3.4. 1M urea wash of Prosep-gelatin elutes tenascin from the affinity column. The figure is a western blot of the SDS-PAGE (5%) stained using anti-tenascin as described in chapter 2 (section 2.3.8.1). Molecular weight of tenascin bands was determined by comparison with a standard mixture of proteins (not shown). This tenascin sample was prepared from conditioned medium of fibroblasts grown from the unaffected skin of a TS patient.

3.4 Final Purification of Fibronectin By Gel-filtration using a Fast Performance Liquid Chromatography (FPLC) System

For the next purification step of the fibronectin, FPLC with a Superose 12 gel filtration column was used. Partially-pure fibronectin from the Prosep-gelatin column was resolved on this sizing column.

The Superose 12 column (24ml bed volume) was first equilibrated with 5 bed volumes of 20mM CAPS buffer, pH 11. After obtaining a flat baseline on the chart recorder, 200µl of fibronectin solution was injected into the column and fractions were collected either manually or using a fraction collector, at constant flow rate (0.2ml/minute) (see *Fig. 3.5*). Proteins were detected by measuring the absorbance at 280nm.

Thirty-two 1ml fractions were collected from the column. An ELISA assay was performed on these collected fractions to determine fibronectin positive fractions (see *Fig. 3.6*). ELISA positive fractions were selected and analysed by SDS-PAGE.

Coomassie blue (See *Fig. 3.7*), silver (See *Fig. 3.8*), and antibody (See *Fig. 3.9*) staining of the reduced purified fibronectin from TS and normal fibroblasts analysed by 5% SDS-PAGE showed a single broad band with approximate molecular weight of 230 000. The mobility of fibronectin isolated from fibroblasts grown from skin lesions of TS patients was identical to that from fibroblasts of unaffected skin.

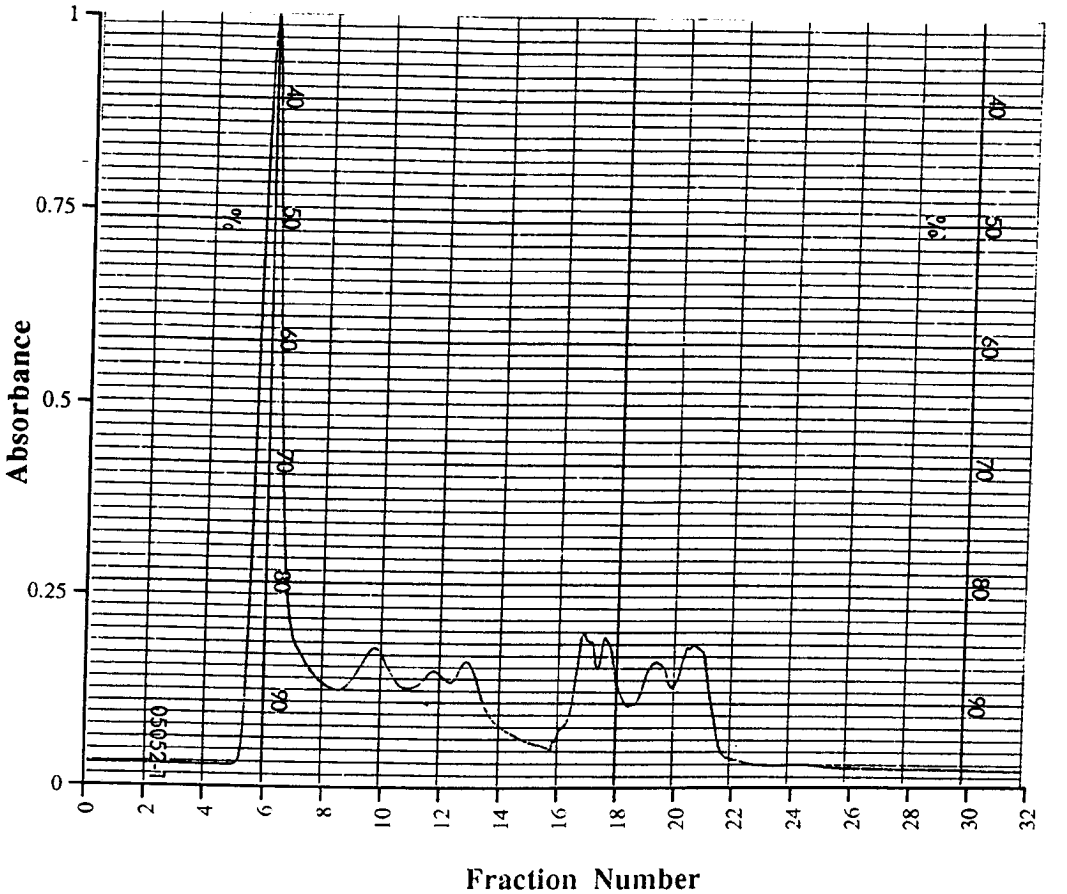


Figure 3.5 Elution profile of fibronectin positive fractions (21-40) from Prosep-gelatin resolved on the Superose 12 gel filtration column. Eluted proteins were detected by measuring the absorbance at 280nm.

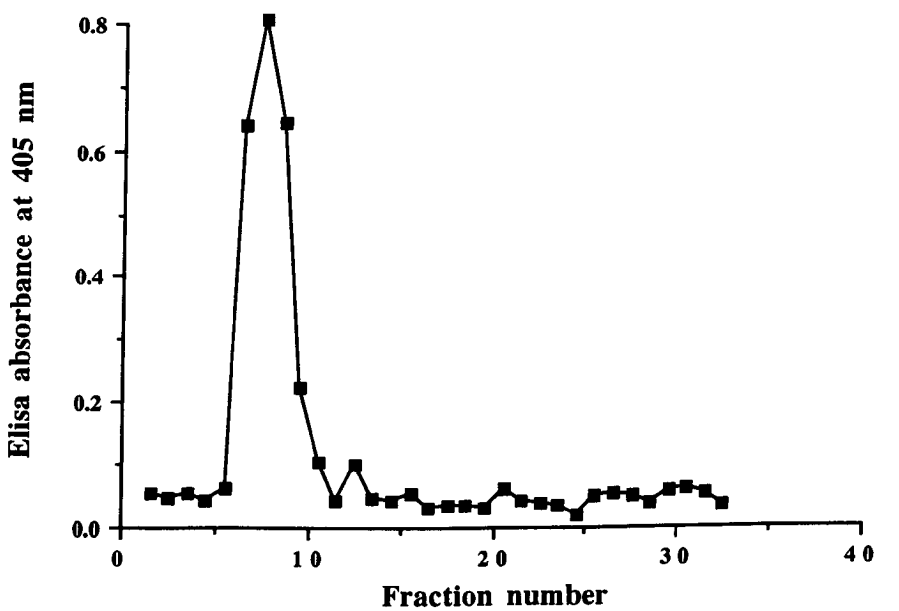


Figure 3.6. ELISA absorbances at 405nm of collected fractions from the Superose 12 gel filtration column (figure 3.5) assayed using anti-fibronectin. Note the elution of fibronectin in the void volume of the column.

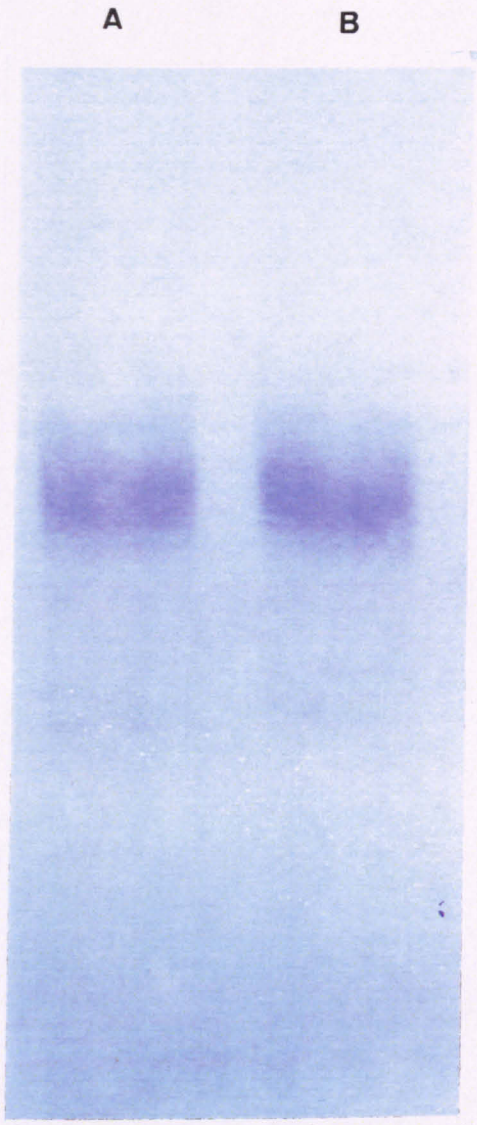


Figure 3.7. SDS-PAGE (5% gel) analysis of purified cellular fibronectin (8µg each) from (A) normal skin fibroblasts, (B) TS unaffected skin fibroblasts after the Superose 12 stage of purification. Purified fibronectin was detected by staining with Coomassie Brilliant Blue as described in chapter 2 (section 2.3.6.2-a).

Figure 3.8. SDS-PAGE (5% gel) analysis of purified cellular fibronectin (4µg each) from the cell culture medium of (A) normal skin fibroblasts (B) TS unaffected skin fibroblasts after the Superose 12 stage of purification. Purified fibronectin was detected by the silver staining method as described in chapter 2 (section 2.3.6.2-b).

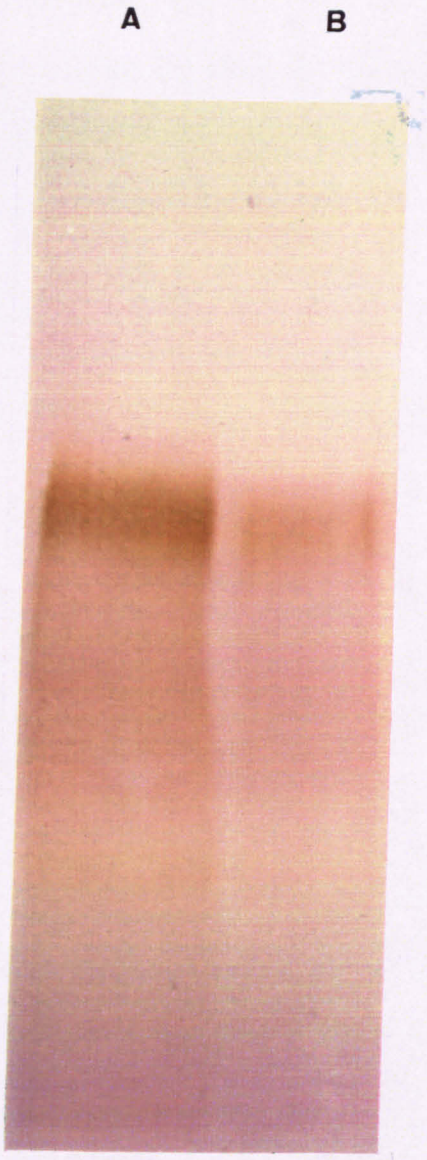


Figure 3.9. Western blotting (5% SDS-PAGE) analysis of purified cellular fibronectin from the cell culture medium of (A) TS unaffected skin fibroblasts (B) normal skin fibroblasts after the Superose 12 stage of the purification. Purified fibronectin (lane A=12µg, lane B=5µg fibronectin) was detected by staining with polyclonal anti-fibronectin as described in chapter 2 (section 2.3.8.1).

Typically, the final yield of fibronectin from the Superose-12 column was 70% of that applied, as shown in *Table 3.2.* However, the overall recovery of fibronectin following Prosep-gelatin and Superose-12 columns was ~60% of that in the conditioned medium used.

* Amount of FN after Prosep-gelatin affinity chromatography	: 0.346mg
* Amount of FN after gel filtration (Superose-12) column chromatography	: 0.242mg
* % yield of FN	: 70

Table 3.2. The table shows an example of the final recovery of fibronectin obtained from conditioned medium of TS skin fibroblasts after the Superose-12 gel filtration column.

The purified fibronectin from conditioned medium of both TS and control cell lines was then ready for further carbohydrate and amino acid analysis.

3.5 Partial Purification of LM and TN from Conditioned Cell Culture Medium by Anion-Exchange (Mono Q-HR 5/5) Chromatography

a) Ammonium Sulphate Precipitation of the Conditioned medium

The major extracellular matrix (ECM) glycoproteins secreted by fibroblasts were investigated by a series of purification procedures using the spent medium of cultured fibroblasts. Ammonium sulphate precipitation was carried out to achieve a partial fractionation of the two ECM glycoproteins, laminin (LM) and tenascin (TN) from conditioned medium as a first stage of the purification procedure. The first strategy was to attempt to optimise the ammonium sulphate concentration for precipitation of LM and TN. Successive 20, 30, 40, 50, 60, 70 and 80% saturation of ammonium sulphate was used to precipitate medium obtained from TS fibroblasts (TSFM) (see section 2.3.3). After centrifugation, the pellets were resuspended in 0.5ml PBS and dialysed.

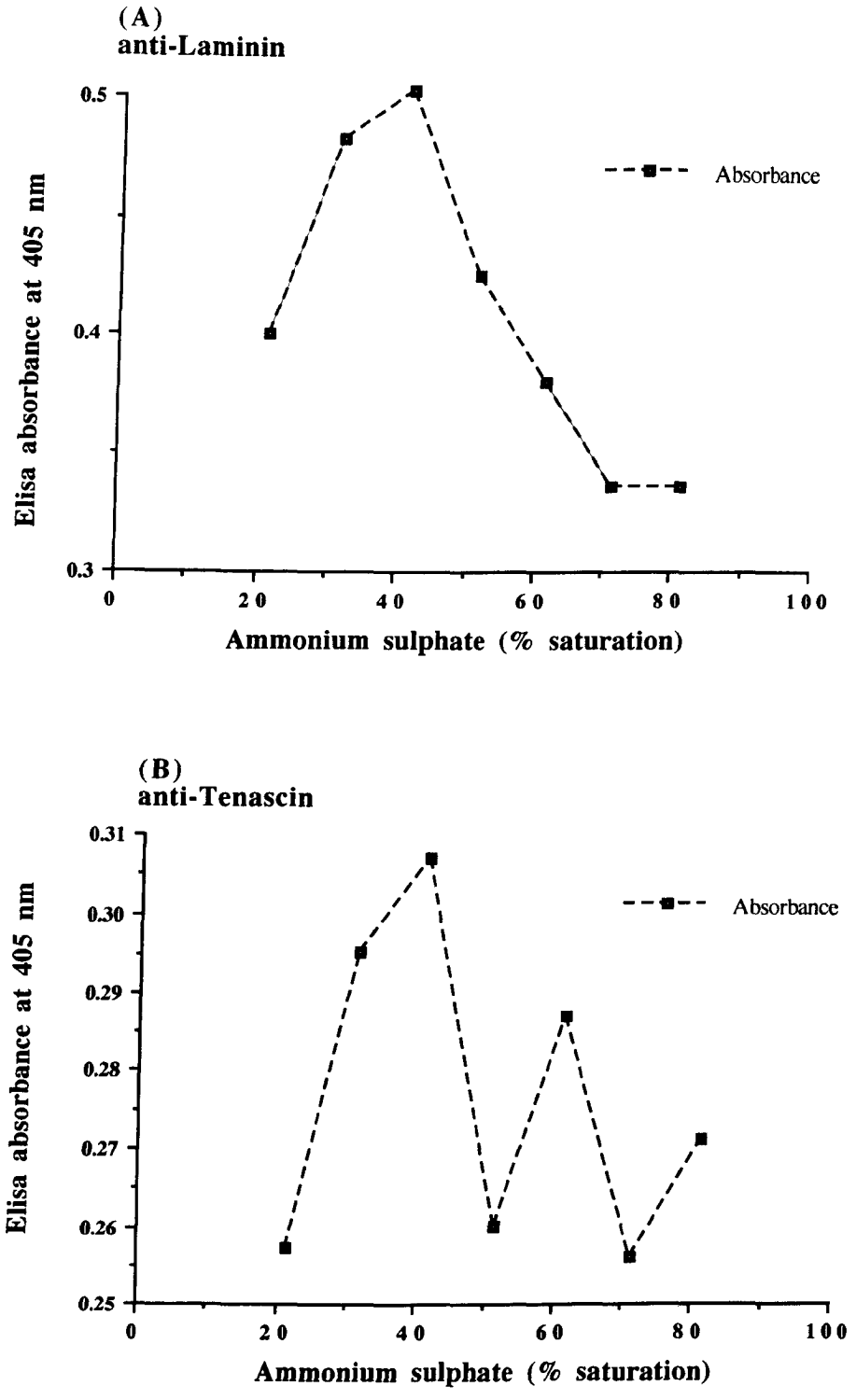


Figure 3.10. Ammonium sulphate precipitation of conditioned medium of TS fibroblasts and absorbances of precipitates for (A) LM and (B) TN measured by ELISA.

Dialysed samples were assayed for LM and TN by the ELISA method as described earlier. *Figure 3.10* shows that 40% saturation gave the highest absorbance at 405nm by the ELISA method for LM and TN, although appreciable quantities of both LM and TN were precipitated at all concentrations of ammonium sulphate.

After optimising the precipitation procedure, the required amount of ammonium sulphate to reach 40% saturation was added slowly by mixing to each sample of conditioned medium, followed by incubation at 4° C overnight. After centrifugation, the pellets were redissolved in 6ml of PBS with 10mM sodium citrate, 2mM PMSF, pH 7.4. The samples were desalted using Sephadex G-25M columns (Pharmacia), giving final volumes of 15ml for each sample. The total protein of the precipitates from the conditioned medium from two different TS patients abbreviated as TSFM (520 ml), TSFC (496 ml), and normal fibroblasts (NSFS, 389 ml) samples was determined by the Lowry method to be 20.4, 54.6 and 25.5mg respectively.

To use the samples efficiently, first of all, each collected medium was subjected to the gelatin-agarose (Sigma) affinity chromatography for the partial purification of fibronectin as described before (see section 2.3.2.1). The fractions eluted by 4M urea were pooled together and dialysed. Samples were then freeze-dried and resuspended in 2ml of PBS containing 2mM PMSF, pH 7.5 and kept at -20° C until required.

Laminin and tenascin were then isolated by anion exchange chromatography from the remaining sample of the gelatin-agarose column .

b) Anion-Exchange (Mono Q-HR 5/5) Chromatography

Remaining medium and the first 19 (2ml) fractions of the 1M NaCl and 1M urea wash from the gelatin-agarose affinity column were pooled and desalted by dialysis against distilled water at 4° C. Following freeze-drying,

samples were resuspended in 20 ml Tris-HCl, pH 7.9 containing 1mM PMSF (chromatography buffer) and filtered using 0.22μm filters. Following this, samples were loaded onto a Mono Q HR 5/5 high performance anion-exchange (FPLC) column pre-equilibrated with 20mM Tris-HCl, pH 7.9 containing protease inhibitors. The column was washed with 5ml buffer at a flow rate of 1ml/min and then eluted using a linear gradient of 0 to 1M NaCl (1 hour). Collected 1ml fractions were assessed by ELISA for tenascin and laminin (see *Figures. 3.11* and *3.12* respectively). The salt gradient eluted 2 main peaks of immunoreactivity for anti-tenascin in fractions 35-42 and 48-52, at just greater than 0.45M NaCl. The main anti-laminin immunoreactivity occured as a broad peak in fractions 55-65, eluted at just over 0.80 M NaCl.

In early experiments of these purification studies, the degree of any possible cross-reactivity of fibronectin with anti-tenascin and anti-laminin was determined using ELISA and dot-blot methods. No evidence of cross-immunoreactivity was seen (results not shown). Moreover, there was also no cross-reactivity of laminin with anti tenascin and anti-fibronectin.

The combined LM fractions from the mono-Q column were negative when tested with the ELISA method for FN and TN. The combined TN fractions from this column were also negative when tested with the ELISA method for FN but showed the presence of a traces of LM. Both fractions of LM and TN also contained some other proteins and as could be described as only partially purified.

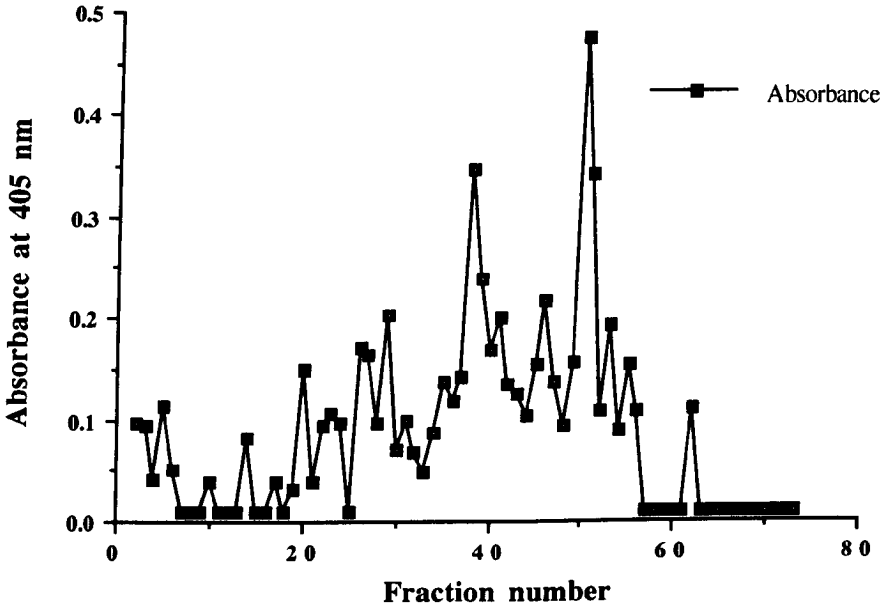


Figure 3.11. Elution profile of fractions containing tenascin determined by ELISA following Mono-Q anion exchange chromatography of conditioned medium from normal fibroblasts. The main anti-tenascin immunoreactivity occured fractions 35-42 and 48-52, corresponding to elution conditions of 0.45 M NaCl or higher.

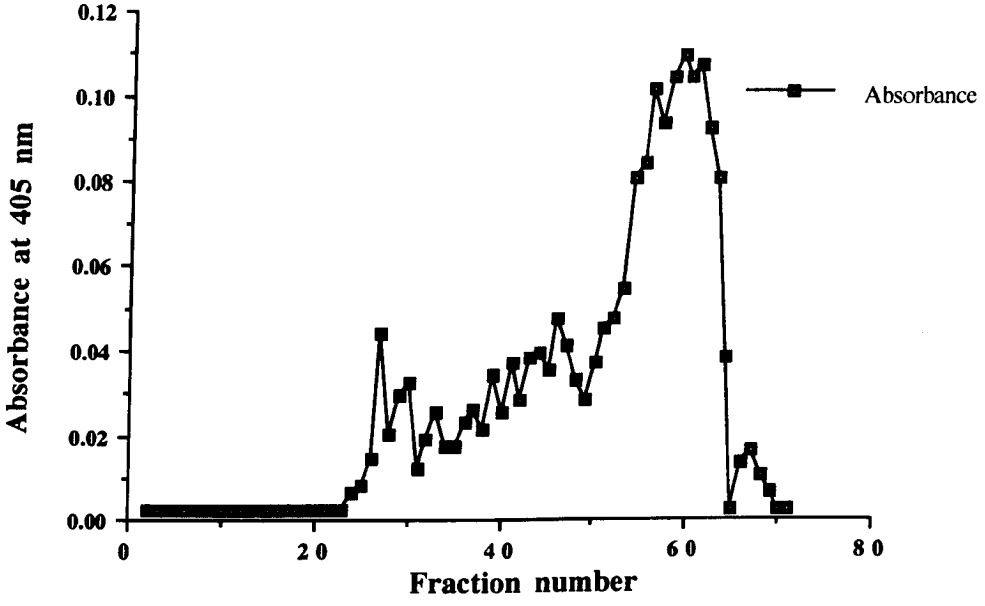


Figure 3.12. Elution profile of fractions containing laminin determined by ELISA following Mono-Q anion exchange chromatography of conditioned medium from TS fibroblasts. The main anti-laminin immunoreactivity occured as a broad peak in fractions 55-65, corresponding to elution conditions of 0.80 M NaCl or higher.

The eluted 1 ml fractions with the highest absorbances at 405 nm were selected for further examination. Typically, the strongly positive fractions for LM (57-62 fractions) were pooled together and mixed with equal amount of sample buffer containing mercaptoethanol. The TN fractions (49-51) were also combined in a similar way. These fractions were then subjected to analysis by SDS-PAGE followed by western blotting. *Figure 3.13 (D)* and *3.14* show the western-blots probed with anti-laminin and anti-tenascin respectively.

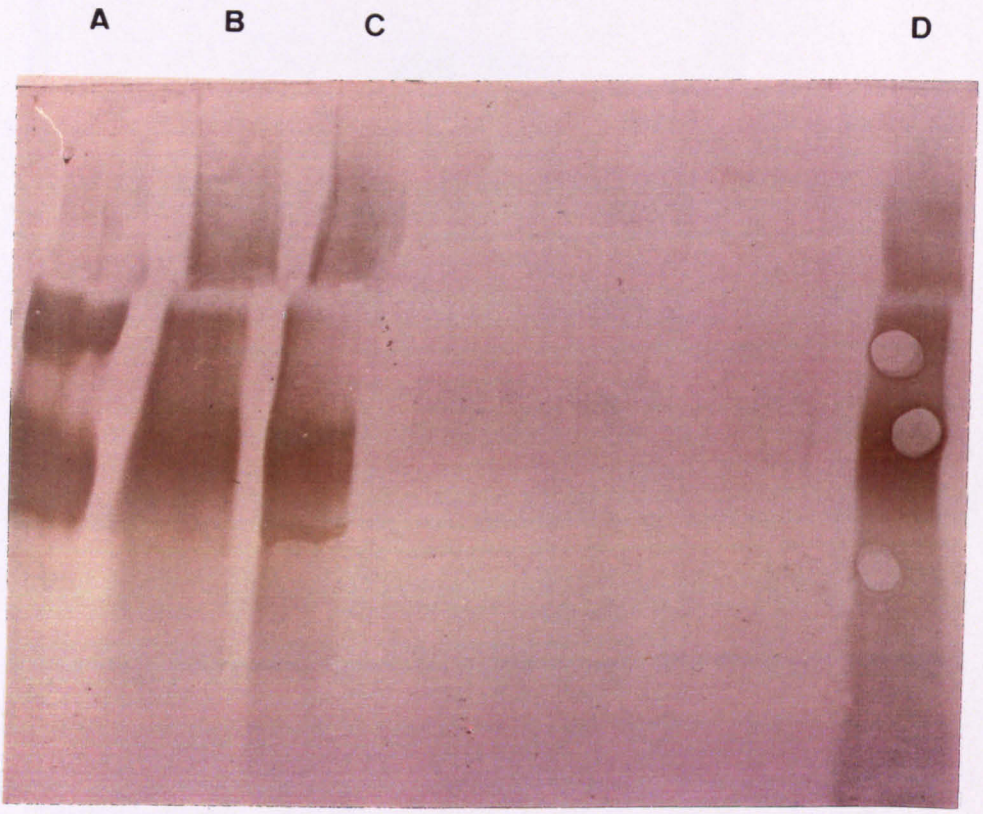


Figure 3.13. Western blotting analysis of (D) a sample of combined fractions 57-62 from the mono-Q column, (B) concentrated conditioned medium, (C) extracts of TS fibroblasts, and (A) standard laminin. The samples were separated on 5% SDS-polyacrylamide gel electrophoresis, the gel blotted on nitrocellulose and laminin bands were detected with anti-laminin. as described in chapter 2 (section 2.3.8.1). For the extraction; fibroblasts were washed with PBS and treated with sample application buffer described in section 2.3.6.1.

Figure 3.14. Western blotting analysis of duplicate samples of combined fractions 48-52 from the mono-Q column. The samples were separated on 5% SDS-polyacrylamide gel electrophoresis, the gel blotted on nitrocellulose and tenascin bands were detected with anti-tenascin as described in chapter 2 (section 2.3.8.1).

Western blot analysis of standard laminin (from Telios) showed the two bands expected at 400 and 200 kDa (*Fig. 3.13-A*). The anti-laminin antibody also showed specific labelling of these two bands in fibroblasts, and in culture medium, as seen in *Figure 3.13* (C and B) respectively.

The western blot analysis of the tenascin fractions eluted from mono Q column showed two major tenascin bands (280kDa and 190kDa, respectively) in fibroblasts from affected skin (ungual fibroma) of a TS patient, as seen in *Figure 3.14.*

Here, the western blot analysis of tenascin and laminin (ELISA positive fractions from the Mono Q column) again demonstrated the presence of these two extracellular matrix glycoproteins, in addition to fibronectin, in conditioned medium of cultured TS and control fibroblasts.

3.6 Discussion

In summary, ammonium sulphate precipitation, Prosep-gelatin affinity chromatography, gel filtration (superose-12) and mono-Q anion exchange chromatography were used for the isolation of the adhesive glycoproteins fibronectin, laminin and tenascin.

Foetal calf serum previously depleted of fibronectin was used in preparation of the cell culture medium to ensure that the product is pure cellular fibronectin. The purification of cellular fibronectin was achieved from conditioned medium of established TS and control fibroblasts using Prosep-gelatin affinity chromatography and gel filtration chromatography techniques. The use of gelatin columns for purification of fibronectin was initially developed by Engvall and Ruoslahti (1977). However, purified fibronectin by this method was only partially pure (see *Fig. 3.3*). Therefore, fibronectin was purified further by gel filtration chromatography to give pure protein for carbohydrate analysis.

The affinity of fibronectin for gelatin allowed the isolation of relatively pure fibronectin in the first step of purification. The washing steps of the Prosep-gelatin affinity column with 1M NaCl and 1M urea in PBS were useful to remove some other minor contaminants such as tenascin. 4M urea was sufficient to elute fibronectin from the Prosep-gelatin affinity column. Interestingly, tenascin was found to contaminate and interact with fibronectin bound to Prosep-gelatin and was detected during the 1M urea wash of Prosep-gelatin affinity column. Although the binding of tenascin to fibronectin has been described before (Yamada, K.M, 1991) this phenomenon has also been recognised in the purification of these compounds. It is possible that this interaction between tenascin and fibronectin might be important in the assembly of the extracellular matrix, and in the functions of these two glycoproteins.

Fibronectin was further purified by gel filtration chromatography which separates molecules according to size. Fibronectin was eluted in the void volume (5-7 ml fractions) of a Superose 12 size column because of high (440 kDa) native molecular weight. The purified fibronectin from conditioned medium of both TS and control cell lines showed a single broad band with approximate molecular weight of 230 000 (denatured) on SDS PAGE as seen in *Figures 3.7*, *3.8*, and *3.9*. The purified fibronectin from TS and control fibroblasts was then ready for further carbohydrate and amino acid analysis.

Generally speaking, the purification of cellular fibronectin from conditioned medium requires intricate and extensive cell culture work because of the lower expression of this protein than of plasma fibronectin. Storage of fibronectin can sometimes be a problem because of its insolubility at neutral pH after purification, particularly during the lyophilisation procedure, and sensitivity of fibronectin to proteolysis. The dissolution of fibronectin was

achieved by using cyclohexylaminopropanesulfonic (CAPS) buffer, pH 11, and protection from proteolysis using PMSF and EDTA. The solution was stored at -80° C in plastic vials for further analysis.

In this thesis, the expression of laminin and tenascin in addition to fibronectin by TS and control skin fibroblasts was investigated by ELISA, immunofluorescence, and flow cytometry techniques, these results are discussed later. This chapter has demonstrated the synthesis and secretion of tenascin and laminin into cell culture medium in addition to fibronectin by TS and control skin fibroblasts. Seperation of these proteins was achieved by ammonium sulphate precipitation, gelatin-agarose affinity chromatography and Mono-Q anion exchange chromatography techniques. Expression of laminin by fibroblasts was particularly important for this research as there are contrary indications in previous published scientific papers about its expression by fibroblasts (Timpl *et al.*, 1982, Woodley *et al.*, 1988, and Cooper *et al.*, 1993).

Chapter 4

Chapter 4

Carbohydrate and Amino Acid Analysis of Purified Fibronectin From Conditioned Medium of TS and Control Fibroblasts

4.1 Introduction

The fibronectins are large, multifunctional glycoproteins found in the plasma and extracellular matrix. Fibronectin variants isolated from plasma, cultured cells, or tissues have been reported to differ from each other with respect to their carbohydrate content. Fibronectin contains 4-9% carbohydrate, depending on the tissue or cell origin (Petersen *et al.*, 1989). The fibronectin from adult human skin fibroblasts has a low carbohydrate content, whereas fibronectin derived from tumor cells has a higher content (Ruoslahti, 1988). Fibroblast-derived fibronectin contains fucose linked to the innermost N-acetylglucosamine, and its sialic acid is linked α2-3 to galactose, whereas plasma fibronectin lacks fucose and its sialic acid is α2-6 linked (Fukuda *et al.*, 1982). Human amniotic fluid fibronectin has a higher carbohydrate content (6-10%) than plasma or fibroblasts fibronectins from various species including human (Ruoslahti *et al.*, 1981). Fibronectin glycosylation mainly consists of complex type oligosaccharides linked to asparaginyl residues.

This chapter focuses on the carbohydrate analysis and characterisation of peptides of purified cellular fibronectin from conditioned medium of cultured fibroblasts derived from patients with tuberous sclerosis (TS) and normal individuals.

4.2 Monosaccharide Analysis of Purified Fibronectin Samples

4.2.1 Preparation of Samples for Monosaccharide Analysis

As described and demonstrated in chapter 3, fibronectin was purified from conditioned medium of established fibroblasts from patients with TS and normal human skin explants. The purity of each fibronectin sample was established by SDS-PAGE as demonstrated in *Figures 3.6, 3.7,* and *3.8* of chapter 3. Glycosylation of the purified fibronectin was confirmed using a Dig-Glycan detection kit (Boehringer) on blotted membranes (see *Fig. 4.1*). The pure fibronectin samples were de-salted using Sephadex G-25m columns (PD-10-Pharmacia) and concentrated in a freeze dryer prior to carbohydrate analysis. Lyophilised samples were dissolved in nanopure water (100-200μl) and the amounts of fibronectin present were measured by ELISA. Aliquots (25-100μg) of the purified fibronectin were subjected to acid hydrolysis in 2M trifluoroacetic acid (TFA) at 100° C for 5 hours to release the neutral sugars, and 50mM TFA at 80° C for 1 hour to release sialic acid (N-acetylneuraminic acid, Neu5Ac). Monosaccharides were separated by high pH anion-exchange chromatography (HPAEC) using a Carbopac PA100 column eluted with a 50-150 mM sodium acetate gradient in 100 mM NaOH for sialic acid (Neu5Ac), and 17 mM isocratic NaOH for neutral sugars as described previously (section 2.3.9.3). Separated monosaccharides were detected by a pulsed amperometric detector (PAD).

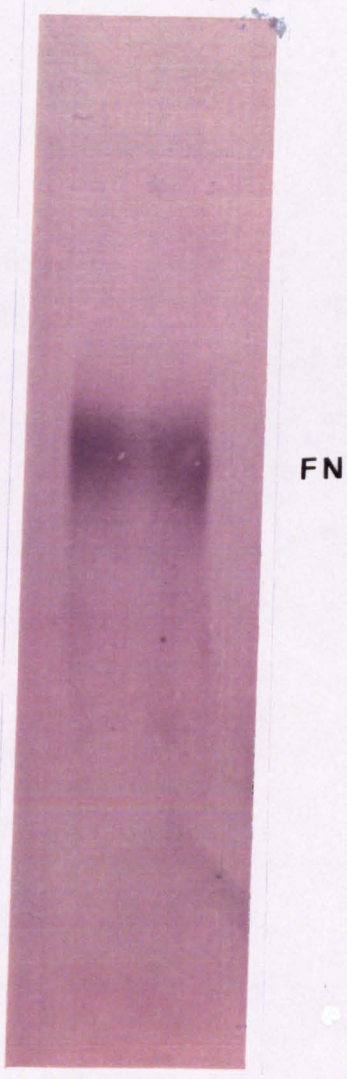


Figure 4.1. The figure shows glycan detection of purified fibronectin from conditioned medium of TSFL-fibroblasts established from neck fibroma of a TS patient. 12µg of fibronectin sample mixed with sample buffer containing SDS and mercaptoethanol was separated by 5% SDS-PAGE, the gel was blotted onto nitrocellulose, and the fibronectin was detected with a DIG-Glycan Detection Kit (Boehringer).

4.2.2 Analysis of Monosaccharides of Fibronectin by HPAEC-PAD

Neutral monosaccharides of fibronectin were analysed according to the method described (see section 2.3.9.3). A standard mixture of monosaccharides containing fucose, galactosamine, glucosamine, galactose, glucose and mannose was used with each at a concentration of 1nanomole. The retention times and integrals of the individual peaks of the standard monosaccharide mixture were used for identification and quantitation of monosaccharides in the unknowns. Glucose is a common contaminant of the samples and is therefore not included in the analyses. Typical elution profiles and retention times of standard plasma fibronectin monosaccharides (2M trifluoroacetic acid (TFA) hydrolysate), and a standard mixture of monosaccharides are given in *Figures 4.2-a* and *4.2-b* respectively. Monosaccharide analysis of 2M TFA hydrolysate of purified cellular fibronectin from conditioned medium of TS fibroblasts, and standard mixture of monosaccharides with their typical retention times are shown in *Figures 4.3-a* and *4.3-b.*

The monosaccharides eluted from each sample were identified by comparing their retention times with the mixture of standards, containing 1nmole each of fucose (Fuc), galactosamine (GalN), glucosamine (GlcN), galactose (Gal), glucose (Glc), and mannose (Man). The standards used for such comparisons were treated in the same way as the unknowns during the sample preparation.

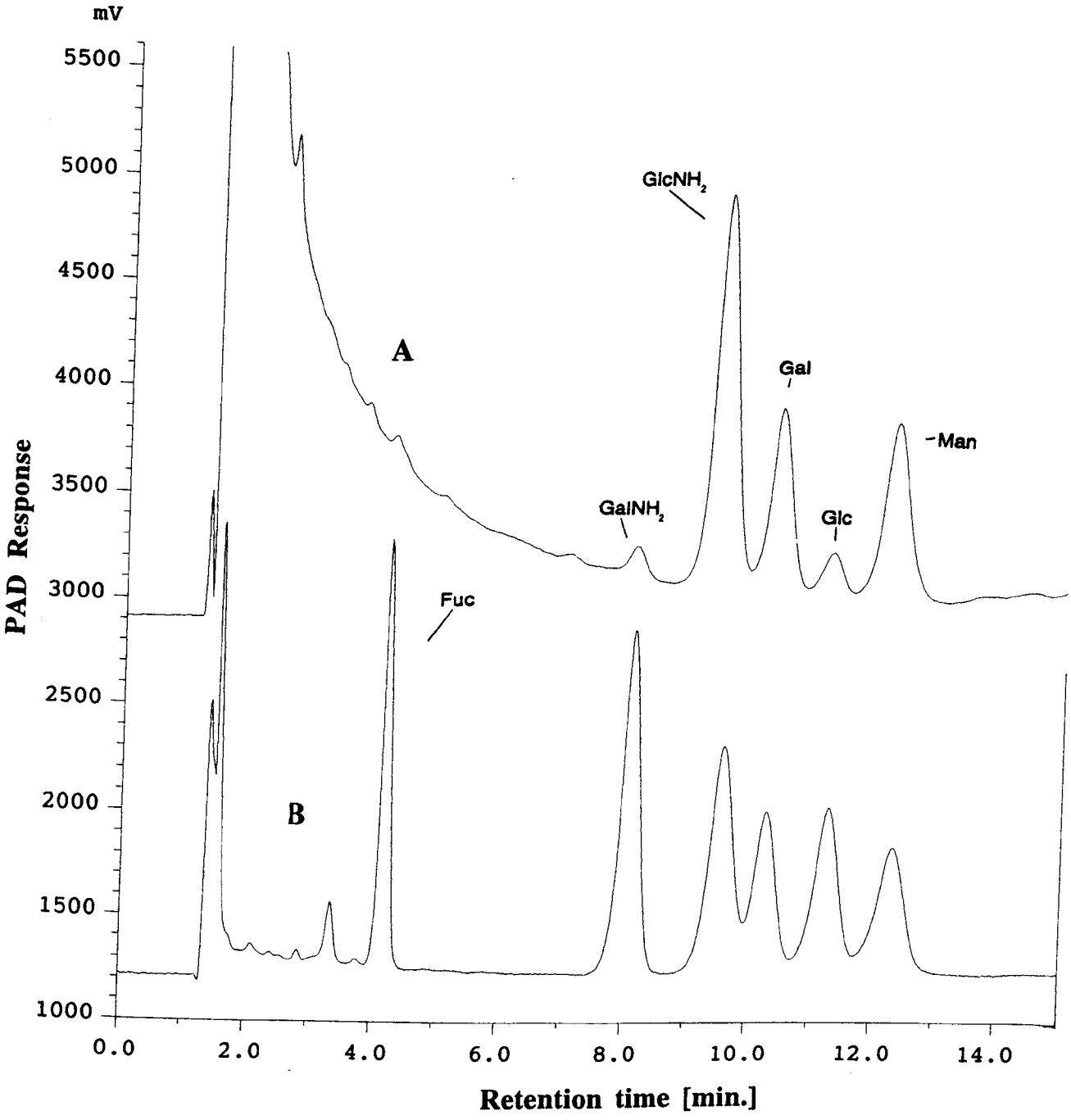


Figure 4.2. Elution profiles from HPAEC-PAD monosaccharide analysis. **(A)** Monosaccharide analysis of TFA hydrolysate of 30μg human plasma fibronectin. **(B)** A standard solution containing 1nanomole each of fucose, galactosamine, glucosamine, galactose, glucose, and mannose. Peaks with similar retention times to standard monosaccharide are annotated.

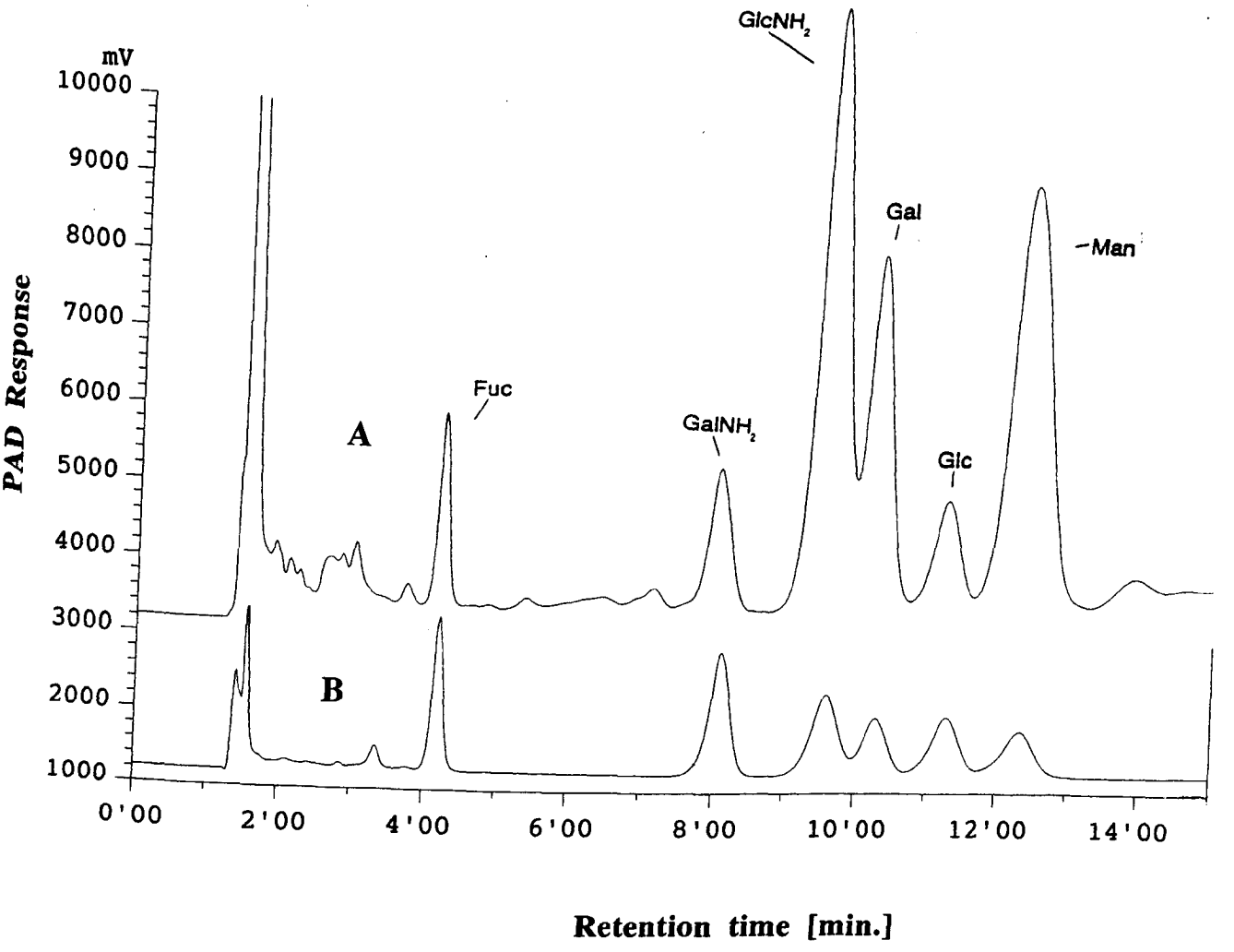


Figure 4.3. Elution profiles from HPAEC-PAD monosaccharide analysis of cellular fibronectin. (**A**) Purified fibronectin from conditioned medium of cultured skin fibroblasts established from neck fibroma of a TS patient. (**B**) A standard solution containing 1nmol each of fucose, galactosamine, glucosamine, galactose, glucose, and mannose. Peaks with similar retention times to standard monosaccharide are annotated.

4.2.3 Sialic Acid (N-Acetylneuraminic Acid) Analysis of purified fibronectin

The sialic acids, which are often the terminal monosaccharides of animal oligosaccharides, are a family of 9-carbon carboxylated sugars (Varki, 1992). There are many different types of sialic acids occurring in nature. In fibronectin, the most common form is called N-acetylneuraminic acid (Neu5Ac).

Purified fibronectin samples from conditioned medium of both established fibroblasts from patients with TS and normal human skin explants were prepared for sialic acid analysis as described in section 4.2.1. Sialic acids, principally Neu5Ac, were analysed using HPAEC-PAD with a sodium acetate gradient elution system. In this system standard N-glycolylneuraminic acid has a retention time of 15 minutes compared with that of 8 minutes for Neu5Ac.

Typical elution profiles and retention times for standard plasma fibronectin, and standard Neu5Ac are given in *Figure 4.4-A* and *4.4-B* respectively. Neu5Ac analysis of 50mM trifluoroacetic acid (TFA) hydrolysate of purified cellular fibronectin from conditioned medium of TS fibroblasts, and standard Neu5Ac with their typical retention times are also shown in *Figures 4.5-A* and *4.5-B*. N-Acetylneuraminic acid in the cellular fibronectin samples was identified by its retention time compared to that of the standard Neu5Ac.

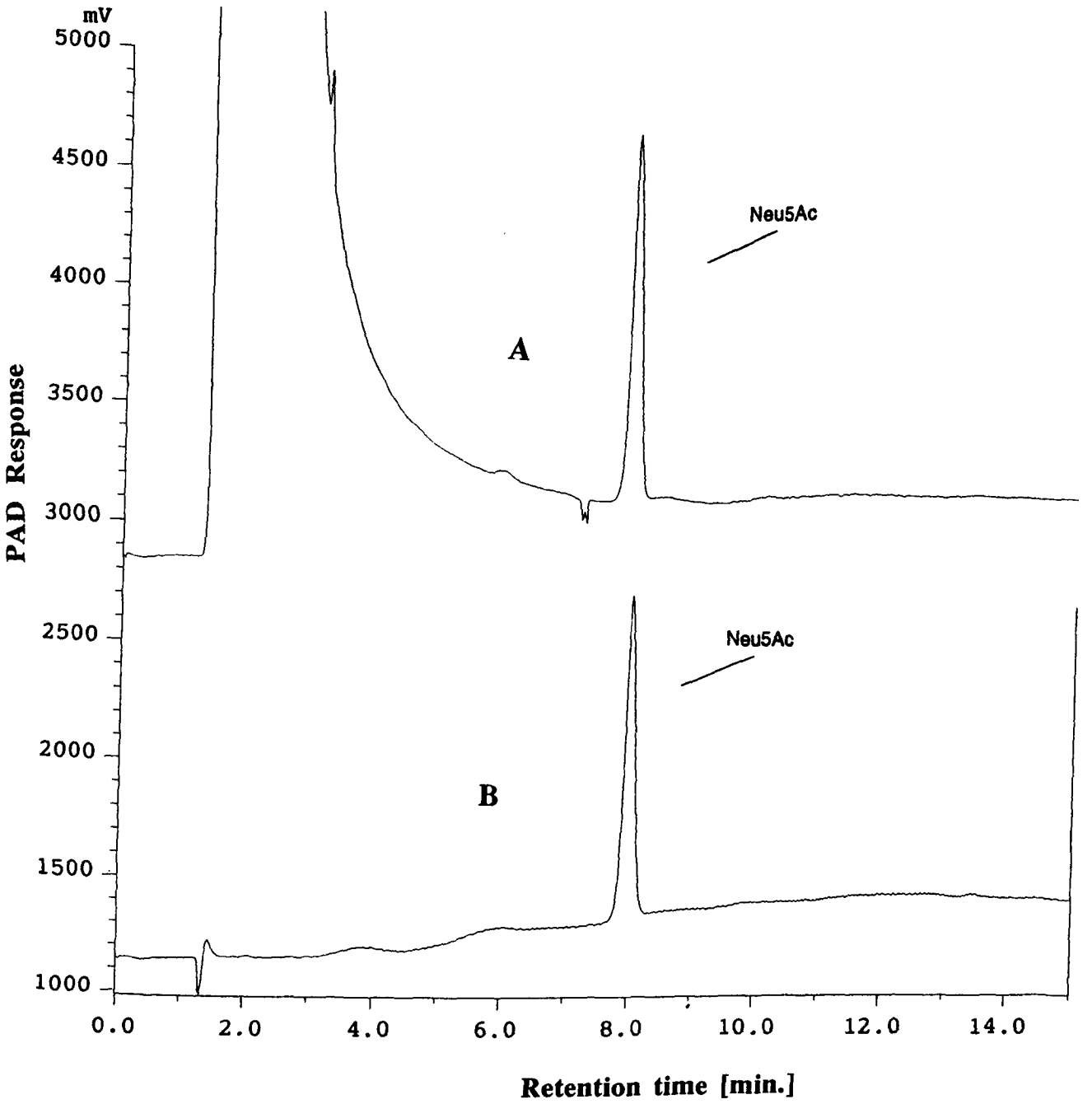


Figure 4.4. Elution profiles from HPAEC-PAD N-Acetylneuraminic acid (Neu5Ac) analysis. A) Monosaccharide analysis of a 50mM TFA hydrolysate of 25μg human plasma fibronectin and B) a standard solution containing 250ng Neu5Ac run on the SA3-H gradient described in chapter 2 (section 2.3.9.3). Retention times of the Neu5Ac peaks were A) 7.58, B) 8.00 minutes. The peak with similar retention time to standard Neu5Ac is annotated.

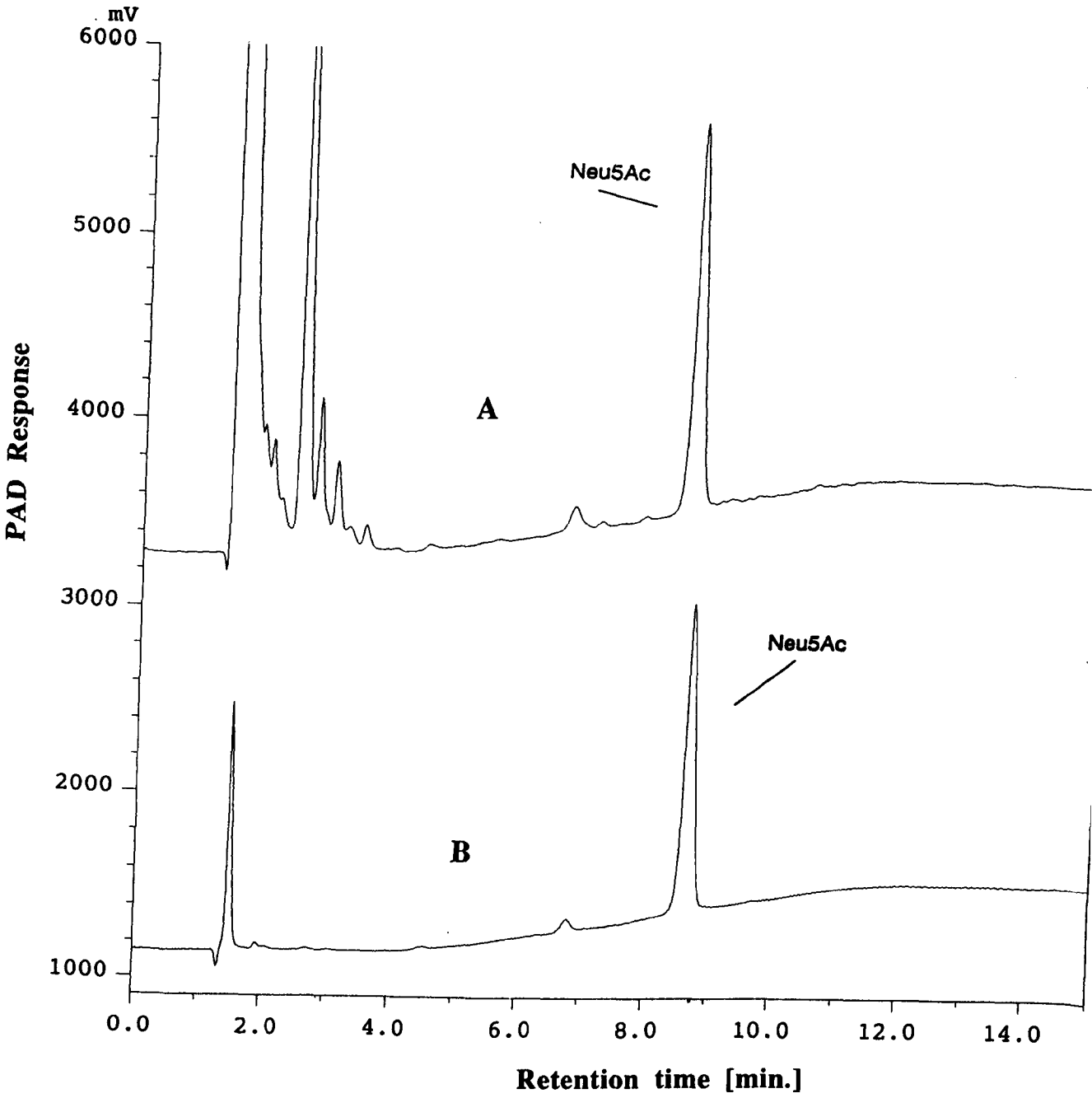


Figure 4.5. Elution profiles from HPAEC-PAD N-Acetylneuraminic acid (Neu5Ac) analysis. **A)** Purified fibronectin from conditioned medium of cultured skin fibroblasts established from a neck fibroma of a TS patient. and **B)** a standard solution containing 1nanomole of Neu5Ac run on the SA3-H gradient described in chapter 2 (section 2.3.9.3). Retention times of the Neu5Ac peaks were A) 8.37, B) 8.41 minutes. The peak with a similar retention time to standard Neu5Ac is annotated.

In summary, *Figures 4.2, 4.3, 4.4* and *4.5* show that the carbohydrate portion of the fibronectin molecule is made up of galactose, mannose, glucosamine, galactosamine, sialic acid, and fucose. As noted earlier, fibroblast-derived fibronectin contains fucose linked to the innermost N-acetylglucosamine, and its sialic acid is linked α2-3 to galactose, whereas plasma fibronectin lacks fucose as seen in *Figure 4.2*, and its sialic acid is α2-6 linked.

Monosaccharide (neutral and amino sugars) composition was determined by HPAEC-PAD after 2M trifluoroacetic acid (TFA) hydrolysis of purified cellular fibronectin from conditioned medium of TS and control skin fibroblasts. Two different purifications of fibronectin from conditioned medium of normal (Jpa), and three established fibroblast lines (TSFL, TSHS, and TSAH) from the skin of three different TS patients were analysed and the results are presented in *Table 4.1.* The three established TS fibroblast lines were derived from neck fibroma (TSFL), ungual fibroma (TSHS), and unaffected skin (TSAH) of different patients (see section 2.3.1.1 for details of these fibroblasts). The carbohydrate composition of plasma fibronectin was also analysed in duplicate and presented in *Table 4.1.*

In another analysis, sialic acid (Neu5Ac) was released from purified fibronectin samples in 50mM TFA for 1hour at 80° C and separated by HPAEC-PAD using a Carbopac PA100 column. The results of the sialic acid analysis from two independent purifications of fibronectin from conditioned medium of three different TS (TSFL, TSHS, and TSAH) and control fibroblasts (JPa) are presented in *Table 4.1.*

Monosaccharides	Human plasma fibronectin % Composition *	Purified fibronectin of JPa- fibroblasts derived from normal skin % Composition *		Purified fibronectin of TSHS-fibroblasts derived from ungual fibroma of a TS patient % Composition *		Purified fibronectin of TSFL-fibroblasts derived from neck fibroma of a TS patient % Composition *		Purified fibronectin of TSAH-fibroblasts derived from unaffacted skin of a TS patient % Composition *	
		A		B	(B/A)	C	(C/A)	D	(D/A)
Fucose	absent	0.19		0.26	(1.40)	0.33	(1.70)	0.14	(0.70)
Galactosamine	0.04	0.12		0.20	(1.70)	0.29	(2.40)	0.09	(0.75)
Glucosamine	1.12	0.82		1.78	(2.20)	2.40	(2.90)	0.72	(0.90)
Galactose	0.72	0.55		1.42	(2.60)	1.22	(2.20)	0.62	(1.10)
Mannose	0.94	0.64		1.31	(2.00)	1.86	(2.90)	0.71	(1.10)
Sialic acid	1.06	0.49		1.61	(3.30)	1.52	(3.10)	0.63	(1.30)
Total % sugar	**3.88**	**2.81**		**6.58**	**(2.30)**	**7.62**	**(2.70)**	**2.91**	**(1.00)**

Table 4.1. Carbohydrate compositions of human plasma, and cellular fibronectins which were purified from conditioned medium of fibroblasts (Jpa) grown from normal skin and fibroblasts grown from ungual fibroma (TSHS), from neck fibroma (TSFL), and from unaffacted skin (TSAH) of different patients with tuberous sclerosis. * Values for composition are given as the mean of duplicate determinations from independent preparations and are expressed as sugar weight per 100 mg of glycoprotein weight. Variations within duplicates were always within ± 5%. Ratios of the TS-fibroblast-derived fibronectin % composition to normal-fibroblast-derived fibronectin % composition are given in brackets. Glucose was excluded due to its presence in control samples.

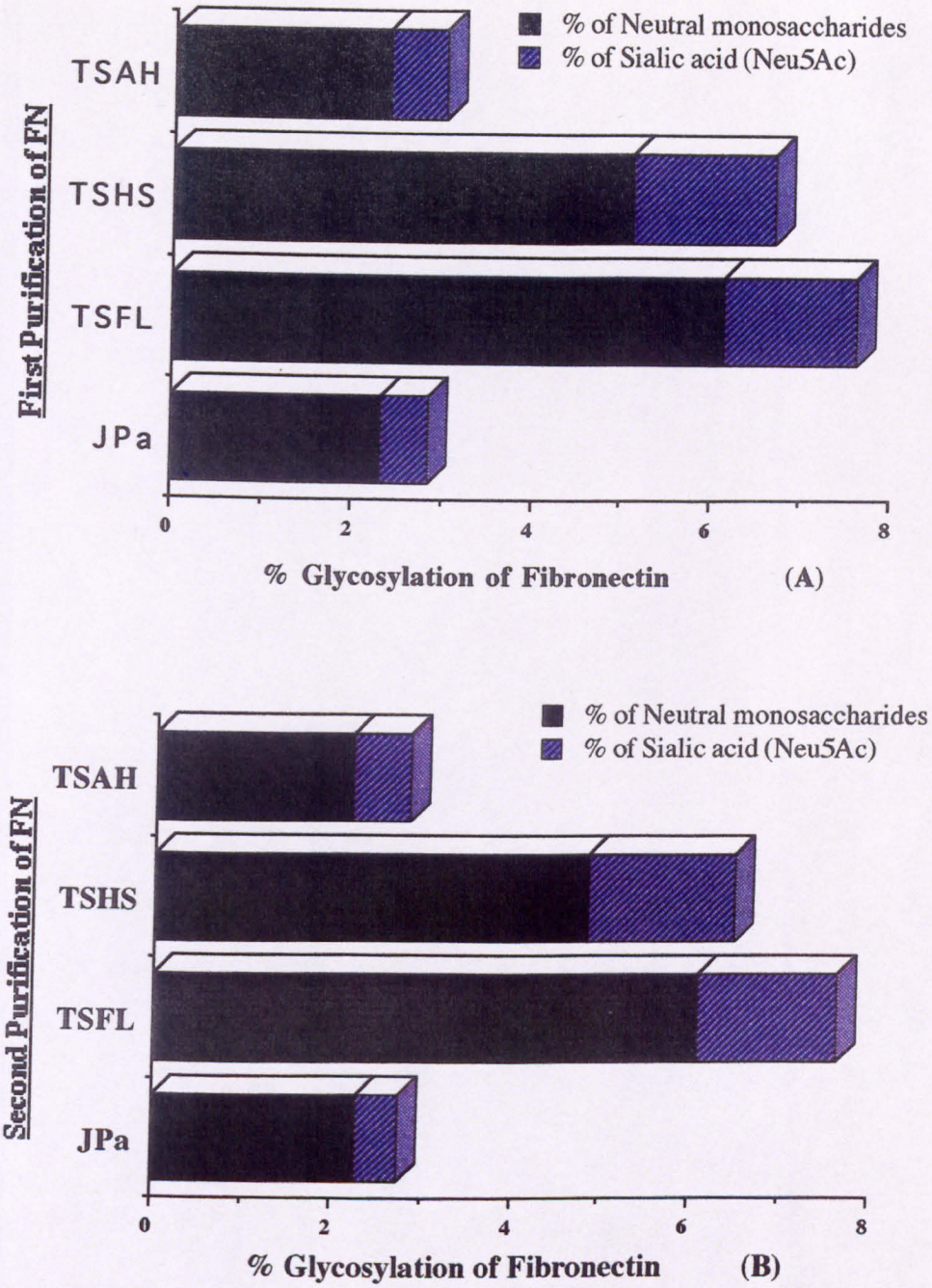


Figure 4.6. The figure shows comparison of the percentage glycosylation of purified fibronectins from conditioned medium of fibroblasts grown from normal skin (JPa) and fibroblasts grown from ungual fibroma (TSHS), from neck fibroma (TSFL) and from unaffacted skin (TSAH) of TS patients. Separately obtained sialic acid data were added to neutral and amino sugars to give total glycosylation. Variations of the % glycosylation between two independent purifications (A and B) of each sample were less than 5%.

The results presented in *Table 4.1* and in *Figure 4.6* establish that there are substantial differences between monosaccharide compositions of fibronectins purified from conditioned medium of fibroblasts grown from normal skin and fibroblasts from skin lesions, but not unaffected skin, of TS patients. In general, total glycosylation of purified fibronectins from skin lesions (ungual and neck fibromas) of TS patients was increased more than two-fold in comparison to control fibroblasts from normal skin. Furthermore, the increased concentration of sugars was general, being observed in sialic acid, galactosamine, glucosamine, galactose mannose and fucose of fibronectin purified from fibroblasts of neck and ungual fibromas of patients with TS. The ratios of these sugars were two to three fold (depending on the sugar) increased in TS lesion-derived fibronectin samples. However, glycosylation of fibronectin purified from the fibroblasts of unaffected skin of TS patient did not change in comparison to skin lesions of TS patients and was at similar levels to control fibroblasts from normal skin.

Interestingly, little N-acetylgalactosamine was detected in plasma fibronectin, with that being three-fold less than cellular fibronectin. However, it was increased one or one and half fold in fibronectins from fibroblasts of ungual and neck fibromas of TS patients respectively in comparison to control fibronectin. N-acetylgalactosamine was also detected in plasma and amniotic fluid fibronectins by other researchers (Pande *et al.*, 1981, Ruoslahti *et al.*, 1981). Skorstengaard *et al.*, (1986-b) detected O-linked side chains in fibronectin. In addition, O-linked oligosaccharide units have also been identified on amniotic fluid fibronectin (Krusius *et al.*, 1985). The presence of N-acetylgalactosamine could be the result of O-glycosylation in fibronectin.

It was previously reported that fucose is present in hamster cellular fibronectin but not in plasma fibronectin. The presence and absence of

fucose was one of the pieces of evidence for chemical diversity between cellular and plasma fibronectin (Fukuda *et al.*, 1982). Here, the results presented in table 4.1 indicate that fucose (0.19%) is present in cellular fibronectin produced by human skin fibroblasts (JPa) in contrast to plasma fibronectin. In addition, the presence of fucose (0.18%) was also observed in another control fibroblasts line (DAKO) from normal skin (data not shown).

Overall, monosaccharide analysis of cellular fibronectin purified from two different control human skin fibroblasts (JPa and DAKO) by HPAEC-PAD revealed that the main components of the glycan portion were mannose, galactose, glucosamine, galactosamine, fucose and sialic acid with less than 3% total glycosylation. Plasma fibronectin was found to be 3.88% glycosylated in this investigation. Vuento *et al.*, (1977) have previously reported that human plasma and cellular fibronectin contains ~4.5% carbohydrate. Different methodology, incomplete TFA hydrolysis or even monosaccharide decomposition under acidic conditions are possible explanations for the lower value found in this investigation.

Plasma fibronectin contains more sialic acid than cellular fibronectin produced by fibroblasts (JPa) as seen in *Table 4.1.* The relatively low sialic acid content of the latter suggests the existence of incomplete branches on the side chains of human cellular fibronectin. Such incomplete branches with terminal galactose residues are observed in the cellular fibronectin produced by human fibroblasts (Ledger *et al.*, 1983) and in hamster cellular fibronectin (Fukuda and Hakomori, 1979).

4.3 N-Glycanase Released Oligosaccharide Analysis of Fibronectin by HPAEC

High-pH anion-exchange chromatography (HPAEC) has been used succesfully to analyse covalently linked carbohydrates from natural and

recombinant glycoproteins. HPAEC is a useful 'oligosaccharide mapping' method since because it gives sufficient chromatographic resolution for all classes of oligosaccharides found on glycoproteins, there is a high probability that each peak represents a single oligosaccharide structure (Hardy and Townsend, 1994).

In addition to the monosaccharide analysis of fibronectin, oligosaccharide mapping of the cellular FN purified from TS and control fibroblasts using HPAEC was highly desirable.

4.3.1 Preparation of N-linked oligosaccharides using N-Glycanase

N-Glycanase digestion of FN to release N-linked oligosaccharides was carried out according to the recent method of Hardy and Townsend (1994). Briefly, purified FN samples (70μg/70μl each) from conditioned medium of fibroblasts grown from normal skin (Rica) and fibroblasts grown from ungual fibroma (TSFC) and from unaffacted skin (TSAH) of TS patients were concentrated using a 'Speed Vac' and dissolved in 48μl of nanopure water and 2μl of 5% (w/v) SDS. 150μg/150μl of plasma FN, 480μg/480μl of bovine fetuin and human transferrin (positive controls) were also concentrated and dissolved in 48μl of nanopure water and 2μl of 5% (w/v) SDS. All samples were denatured at 100 °C for 3 minutes and allowed to cool to room temperature. Then, 2.5μl of 10% (v/v) Nonidet P40 (non-ionic detergent), 24μl of 5x concentrated incubation buffer (phosphate; EDTA pH pH 7.5-8) purchased from Oxford GlycoSystems, 38.5μl nanopure water and 5μl (7 unit/ml) N-Glycanase (Oxford GlycoSystems) were added. The total volume (120μl) of all samples was incubated at 37 °C for 20 hours.

Approximately 30% of the released oligosaccharides are in an unstable glycosylamine form resulting in 2 peaks during HPAEC. Hence, 10 μl of 174mM acetic acid was added and left for 2 hours at RT; this lowers the pH

and converts the glycosylamine to its single reducing form (Hardy and Townsend, 1994). Finally, all samples (130µl) were dried using a 'Speed Vac' and dissolved in 55µl of nanopure water.

4.3.2 Analysis of N-Linked Oligosaccharides of Fibronectin by HPAEC-PAD

Although FN from two cultures of normal cells (JPa and Rica) from three cultures of TS affected tissues TSFC, TSFL and TSHS and from one culture from TS unaffected tissue TSAH was purified there generally was insufficient for this analysis. In fact there was an sufficient for a preliminary experiment with TSFC only. Aliquots (~45µg/35µl) prepared from the fibronectins of TS and control fibroblasts, and a 95.5µg/35µl aliquot of plasma fibronectin were analysed directly by HPAEC. In addition, 305µg/35µl aliquots of fetuin and transferrin was also analysed by HPAEC. Both bovine fetuin with 18% glycosylation and human transferrin with 6% glycosylation are N-linked glycoproteins which were used as positive controls in the analyses. The oligosaccharide map from N-glycanase treated bovine fetuin was identical to the hydrazinolysis released standard from Oxford GlycoSystems (see *Fig. 4.7*). N-linked oligosaccharide finger print patterns of the plasma fibronectin, transferrin and disialylated biantenary standard were demonstrated by HPAEC as shown in *Figure 4.8*. HPAEC chromatograms of N-glycanase released oligosaccharides from TS and plasma fibronectins are presented in *Figures 4.9-A* and *4.9-B* respectively. In general, the analyses of purified fibronectin samples from conditioned medium of fibroblasts grown from normal skin (Rica) and fibroblasts grown from unaffected skin (TSAH), and from ungual fibroma (TSFC) of TS patients was unsuccessful; in summary, only TSFC purified fibronectin chromatograms of these samples were obtained by HPAEC-PAD, and only using a high sensitivity scale of the program in the computer.

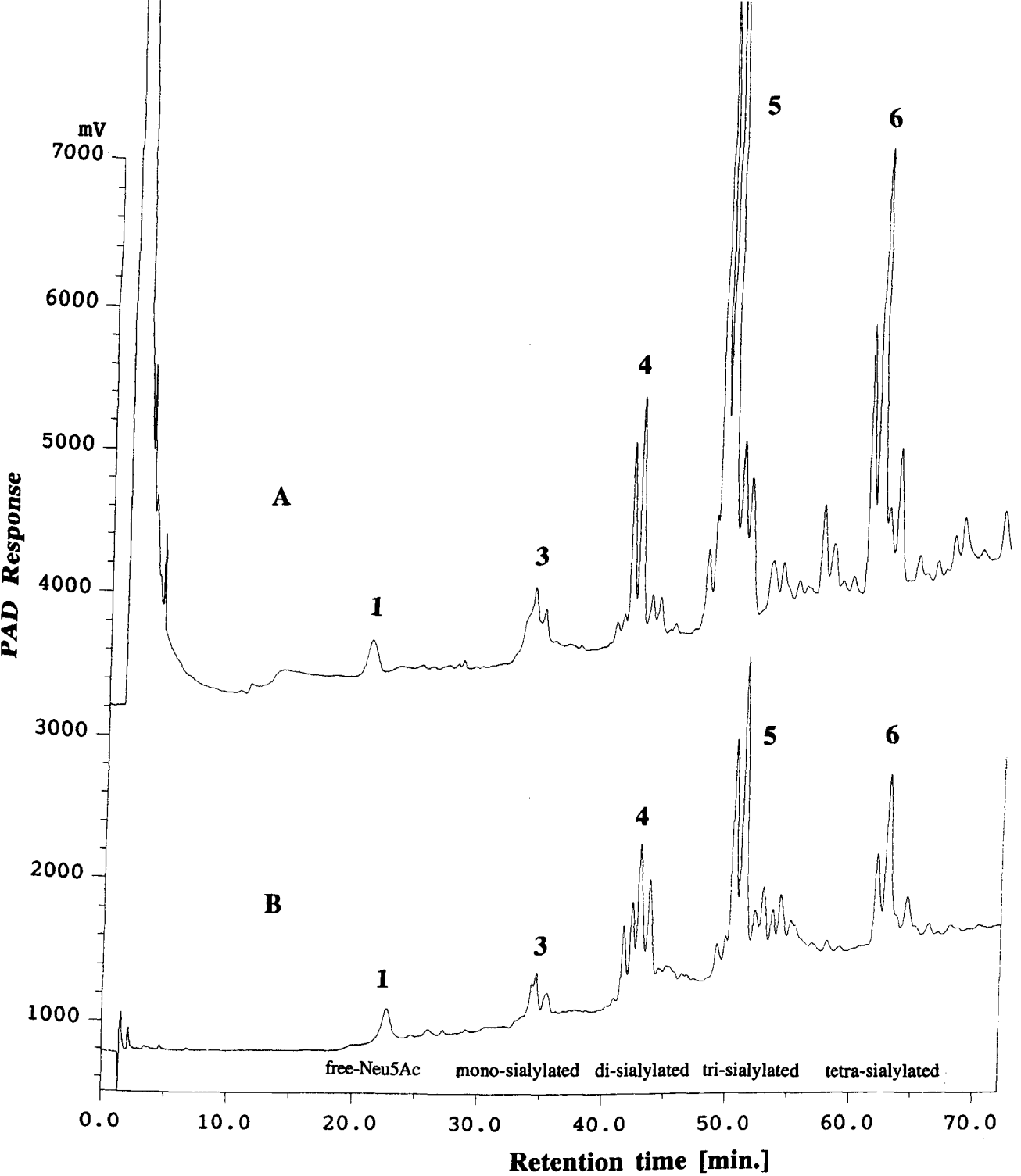


Figure 4.7. Sialyloligosaccharides from bovine fetuin analysed by HPAEC. Commercial preparation released by hydrazinolysis (17.5µg sugar) from Oxford GlycoSystems (A), and PNGase treated fetuin-350µg (B). The assignment of the groups of the peaks in A is based on information supplied by Oxford GlycoSystem accompanying the sample obtained from them. This assignment is indicated at the bottom of the diagram. For simplicity each group has been given a number to aid identification of peaks in traces from other samples. Samples were run on the OXALSA gradient described in chapter 2 (section 2.3.9.3).

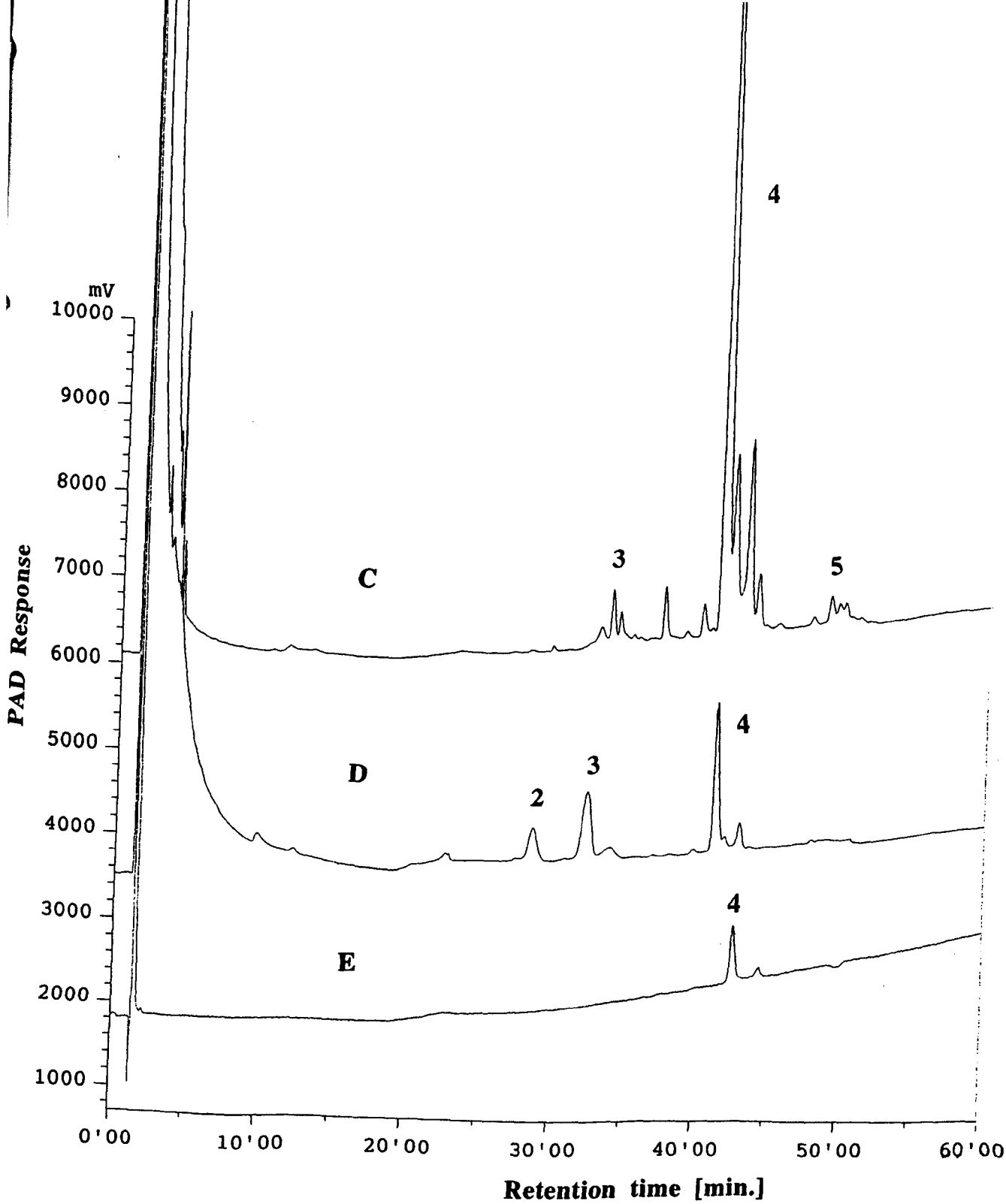


Figure 4.8. A typical HPAEC-PAD chromatogram of PNGase-released oligosaccharides obtained from (C) human transferrin, (D) plasma fibronectin, and (E) disialylated biantennary standard from Oxford GlycoSystem. The numbers alongside peaks are explained in the legend to figure 4.7. Peak 2 indicates the presumed position of asialoglycan. Samples were run on the OXALSA gradient described in chapter 2 (section 2.3.9.3).

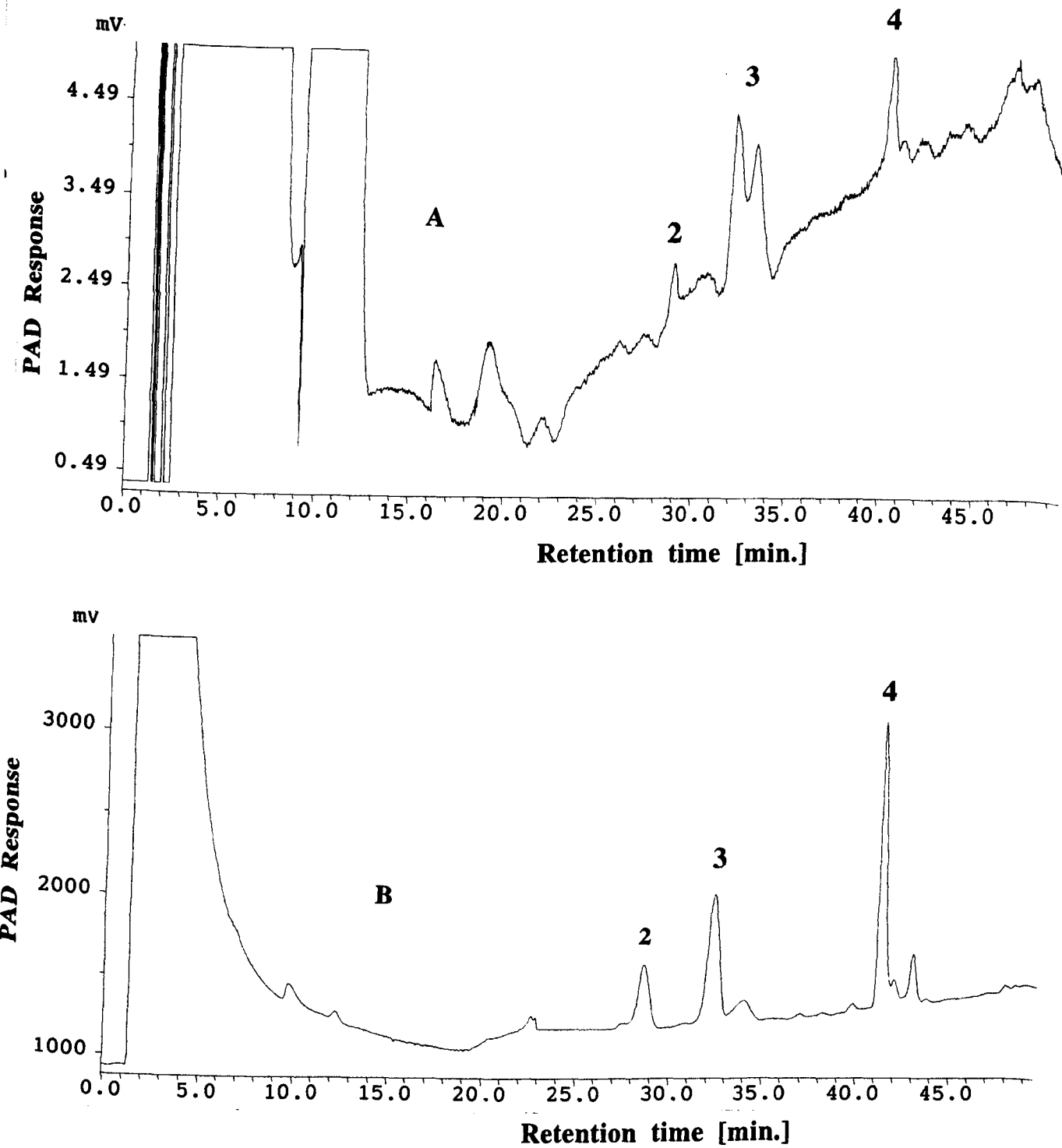


Figure 4.9. The figures show the typical HPAEC-PAD chromatograms of N-glycanase released oligosaccharides from purified fibronectin of TSFC-fibroblasts derived from ungual fibroma of a TS patient (A), and plasma fibronectin (B). The numbers alongside peaks are explained in the legends of figures 4.7 and 4.8. Samples were run on the OXALSA gradient described in chapter 2 (section 2.3.9.3).

Figure 4.10. Comparison of the carbohydrate structures of plasma and purified cellular fibronectin with two digoxigenin-labelled lectins: from *Sambucus nigra agglutinin* (SNA) and *Maackia amurensis agglutinin* (MAA). 7μg of the plasma fibronectin in lanes 1, and 5μg of purified cellular fibronectin from conditioned medium of normal fibroblasts (JPa) in lanes 2 were used. Fibronectin samples were mixed with sample buffer containing SDS and mercaptoethanol and separated by 5% SDS-PAGE, the gel blotted on to nitrocellulose and the fibronectins were detected with the Glycan Differentiation Kit (Boehringer). SNA recognizes sialic acid linked α(2-6) to galactose. MAA recognizes sialic acid linked α(2-3) to galactose.

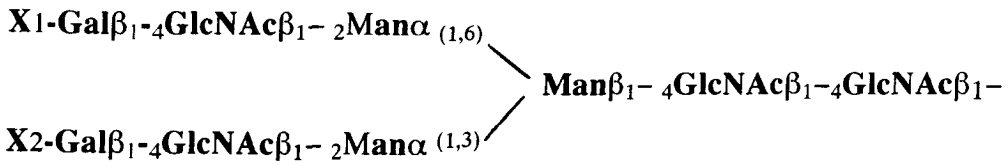


Figure 4.11. Structural assignment of oligosaccharide peaks appearing in figures 4.8 and 4.9. Major peak 4 represents di-sialylated oligosaccharides in which X1 and X2 would be sialic acid residues. Major peak 3 represents mono-sialylated oligosaccharides in which X1 or X2 would be sialic acid residue. Major peak 2 represents asialylated oligosaccharides in which no sialic acid residues occur in the positions of X1 and X2.

PNGase treatment of plasma and TSFC purified fibronectin and other glycoproteins resulted in the successful release of N-glycosides. These were visualised as peaks on the HPAEC chromatograms as seen in *Figures 4.7, 4.8* and *4.9*. The oligosaccharide map from PNGase treated bovine fetuin was identical to hydrazinolysis released standard (see *Figure 4.7*). Oligosaccharides from human plasma fibronectin eluted as 3 sharp peaks. The main one (see peak D4 with retention time ~42min) coincided with the biantenary, disialylated complex-type oligosaccharides from transferrin (C4) and fetuin (A4;B4) as well as the standard from Oxford GlycoSystems (E4) which represents these structures as demonstrated in *Figure 4.11*. Therefore, X1 and X2 in *Figure 4.11* would be sialic acid residues in plasma fibronectin and purified cellular fibronectin from TSFC fibroblasts. Peak D3 eluted in the same position as C3, B3, and A3 which in the latter case was identified as monosialylated glycan with the monosialylated derivative of peak 4. Peak D2 is assumed to be the asialooligosaccharide derivatives, as peaks A1 and B1 represents free sialic acid. Oligosaccharides of plasma fibronectin and TSFC purified fibronectin appeared basically as mono and disialylated biantenary structures (see *Fig. 4.9*).

Figure 4.10 showed that sialic acid linked to galactose with a linkage α(2-6) in plasma, and α(2-3) in cellular fibronectins. However, monosialylated

oligosaccharides in TSFC purified cellular fibronectin appeared more prominent than in plasma fibronectin. As described before, the presence of fucose in purified cellular fibronectins of TS and normal fibroblasts is also another difference from plasma fibronectin carbohydrate structure.

4.4 Cellular Fibronectin Purified from TS Skin Fibroblasts Does Not Express HNK-1 Carbohydrate Epitope

The L2 and HNK-1 (anti-Leu-7) monoclonal antibodies recognise an unusual carbohydrate structure which is expressed on glycolipids, glycoproteins and proteoglycans in the peripheral and central nervous system. It has been reported that a sulphated, glucuronic acid-containing carbohydrate structure is a marker for neural cell adhesion molecules involved in cell-cell and cell-substrate interactions during neural development (Kruse *et al.*, 1984, 1985; Gowda *et al.*, 1989: Chou *et al.*, 1986; Schachner, 1989). Expression of the HNK-1 carbohydrate epitope in tumors of neuroectodermal origin has been reported by Harper *et al.*, (1990). These workers showed that a human neuroectoderm-derived cell line secretes fibronectin that shares the HNK-1 carbohydrate epitope with neural cell adhesion molecules. Preliminary experiments in our laboratory (Ellis private communication) suggested fibronectin from TS patients may also carry this epitope. For these reasons the expression of this epitope on fibronectin produced by cells grown from skin and skin lesions of TS patients was also investigated. Fibronectin was purified from the conditioned medium of TS and normal cells by affinity chromatography on Prosep-gelatin, and gel filtration chromatography techniques. Fibronectin samples mixed with sample buffer containing mercaptoethanol and SDS were resolved by 5% SDS-PAGE and western blotted. The blot was probed with mouse monoclonal HNK-1 (anti-Leu 7) antibody for antigen detection as described in 2.2.8.1. An extract of normal human brain tissue was also subjected to 5% SDS-PAGE and western-

blotting. Although anti-Leu 7 was positive on the brain extracts, we did not observe any reactivity on the blot even when the most sensitive methods such as enhanced chemiluminescence (ECL) were used for the detection of this antigen. The expression of this epitope was also checked on several TS and control fibroblasts by immunofluorescence staining with 1/25 dilution of mouse monoclonal anti-Leu 7 antibody. There was no staining of these cell lines (data not shown). These negative results suggest that the HNK-1 carbohydrate epitope is not expressed by TS and normal skin fibroblasts and their product fibronectin.

4.5 Amino Acid Analysis of Purified Fibronectin

4.5.1 Analysis of Amino Acids by Amino Acid Analyser

As described before (section 2.3.11), standard plasma and cellular fibronectin, and purified fibronectin samples from the collected medium of fibroblasts from patient with TS and normal individuals were resolved by 5% SDS-PAGE. Proteins on the gel were blotted onto polyvinylidene difluoride (PVDF) membrane (millipore) with a semi-dry blotting apparatus using 10mM CAPS buffer, 3-[cyclohexylamino-1-propanesulfonic acid] pH 11 for 1 hour. In this analysis, CAPS buffer was used to facilitate transfer of proteins (standard transfer buffer contains glycine which would complicate amino acid analysis) on to PVDF. Fibronectin bands were excised from the PVDF membrane after amido black staining as described in section 2.3.12, and then amino acid analysis of fibronectin was carried out using an Applied Biosystems model 420H automated amino acid analysis system. Hydrolysis with 6M hydrochloric acid at 200 °C and subsequent pre-column derivatisation were carried out automatically. Amino acids were idendified and quantitated against known standards. The results obtained from the amino acid analysis are shown in *Table 4.2.*

Amino acid	TSJH-FN p***	TSFL-FN p**	TSAH-FN p**	JPa-FN p**	St.Cell.-FN c***	Cell FN Vuento *et. al.* (1977)	Pl. FN Vuento *et. al.* (1977)	St.Pl.-FN c***
E--Glu	11.77	14.47	16.25	15.58	12.51	11.63	11.78	13.57
G--Gly	12.41	9.12	12.01	11.81	10.20	8.85	8.27	10.02
D--Asp	10.5	9.65	10.95	10.45	10.49	9.52	10.08	9.18
S--Ser	7.89	8.19	8.99	9.79	9.14	8.33	8.55	6.80
T--Thr	8.23	10.75	8.52	8.44	10.00	8.42	10.42	8.55
V--Val	6.49	8.34	6.53	6.45	7.44	6.16	6.01	10.19
R--Arg	6.09	5.24	6.42	5.95	6.11	4.51	4.48	5.48
P--Pro	8.92	8.32	5.39	5.63	6.35	8.46	7.01	6.62
A--Ala	5.49	5.17	5.25	5.37	4.96	4.93	5.10	5.55
L--Leu	5.4	6.12	4.88	5.43	5.68	5.52	6.17	6.97
K--Lys	3.46	2.25	3.66	3.40	3.79	5.13	4.35	3.39
Y--Tyr	3.94	3.80	3.36	3.17	3.94	4.21	4.34	3.71
I--Ile	3.2	4.16	3.07	3.33	3.64	3.56	3.12	4.61
F--Phe	2.32	2.00	1.59	1.79	2.15	2.54	2.56	2.13
M--Met	2.00	0.90	1.34	1.67	1.53	0.86	1.09	1.45
H--His	1.89	1.55	1.82	1.77	2.07	1.96	1.80	1.78

Table 4.2. Amino acid compositions (mol %) of human cellular fibronectins purified (p) from conditioned medium of fibroblasts (JPa) grown from normal skin, and fibroblasts grown from the skin (TSJH: forehead plaque (lesion) , TSFL: neck fibroma, and TSAH: unaffected skin) of different TS patients. Commercial (c) plasma and cellular fibronectins purchased from Gibco were analysed as controls. Values for composition are given as the mean of determinations from three (***) or two (**) independent samples, and expressed as mole percentage. Variations within these independently prepared samples were usually within ± 10%.

In general, the amino acid composition of the purified fibronectin from normal fibroblasts (JPa) was very similar to that of purified fibronectins from TS fibroblasts as well as standard plasma (pFN) and cellular fibronectin (cFN) (*Table 4.2*). However, small consistent differences (between TS affected tissue and normal or unaffected tissue) were observed in serine and proline but significance of these changes is not clear. In addition, the amino acid composition of fibronectin showed close agreement between our results and Vuento *et al.*, (1977) as expected.

4.6 Amino Acid Sequence of Fibronectin

In the first attempt, unsuccesful sequencing of excised fibronectin bands from PVDF membrane showed that fibronectin was N-terminally blocked. In the literature, it has been indicated that the amino terminus of the plasma protein from different sources is blocked by pyroglutamic acid (Furie and Rifkin, 1980), thus preventing direct sequence determination. Therefore, it was necessary to cleave the fibronectin into fragments to make available an internal sequence. The original plan was to develop the proteolytic digestion procedure on human plasma fibronectin since more of this was available. As can be seen from the following this worked well. Unfortunately there was never sufficient purified fibronectin from cultured cells to complete this aspect of work.

Plasma fibronectin was digested with chymotrypsin as described in section 2.3.13 (see *Fig. 4.12-a* and *-b*), and the digest were applied to Prosep-gelatin to purify the gelatin-binding domain of fibronectin (*Fig. 4.13*). Amino-terminal protein sequencing (Edman degradation) of the purified gelatin-binding domain of fibronectin was determined using an Applied Biosystems model 473A automated protein sequencer. Data was collected and analysed using 610A data analysis software (Applied Biosystems, Foster City, California).

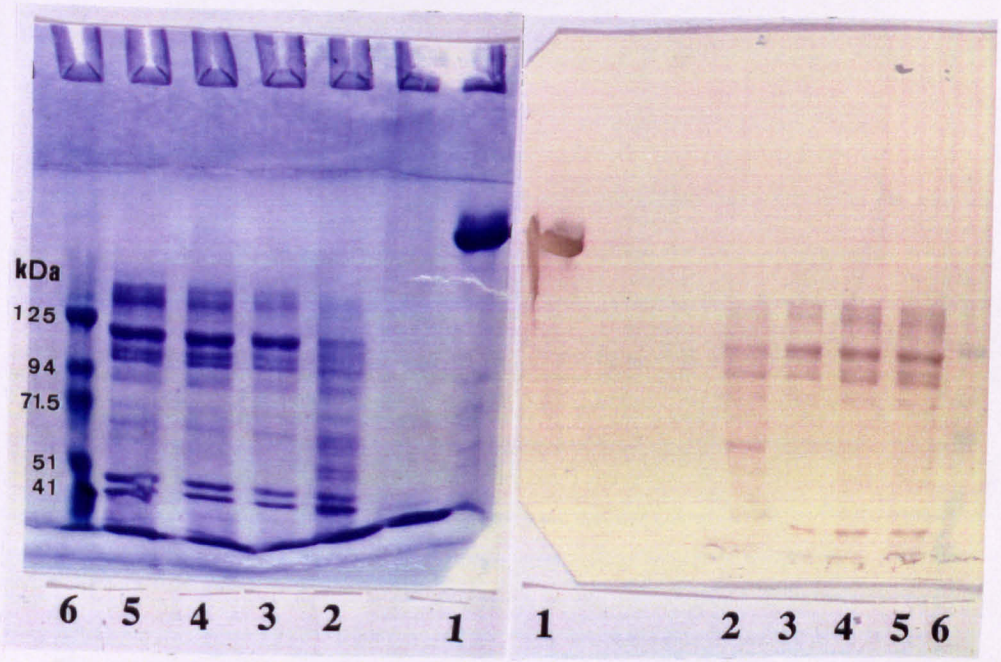


Figure 4.12. SDS-PAGE analysis of plasma fibronectin chymotryptic fragments. *Lane 1* : reduced plasma fibronectin before chymotrypsin digestion, *Lanes 2-5* : fibronectin proteolytic fragments (In lane 2, fibronectin was digested with chymotrypsin in 0.05 M Tris, 0.15 M NaCl, and 0.01M $CaCl_2$, in lanes 3-5, fibronectin was digested with chymotrypsin in 0.05 M Tris, 0.15 M NaCl, and 0.02M $MgCl_2$). *Lane 6* : pre-stained molecular weight standards (125 kDa, 94 kDa, 71.5 kDa, 51 kDa and 41kDa) A) Coomassie staining of 7.5% SDS-PAGE. B) Antibody staining (Anti-FN) as for A.

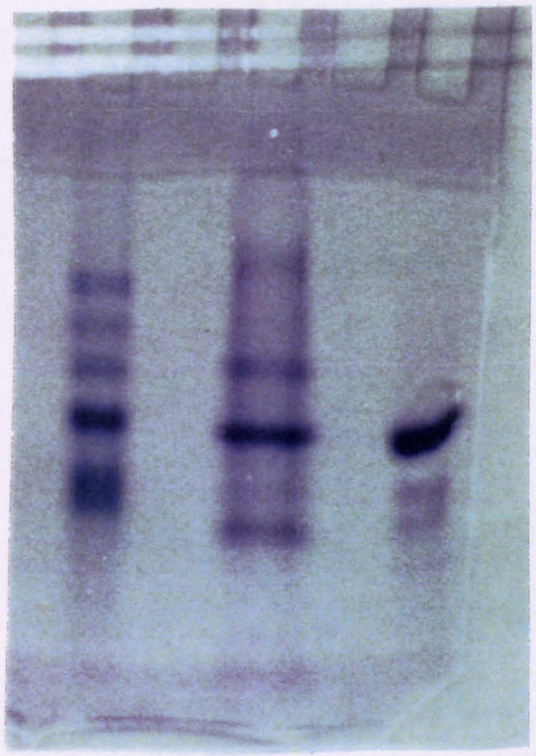


Figure 4.13. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of purified plasma fibronectin chymotryptic gelatin-binding fragments. Coomassie staining of the 10% SDS-PAGE: *Lane 1* : 45 kDa standard gelatin-binding domain (Gibco), *lane 2* : purified chymotryptic gelatin-binding fragments using Prosep-gelatin column, *lane 3* : pre-stained molecular weight standards (125 kDa, 94 kDa, 71.5 kDa, 51 kDa and 41kDa). A similar gel to above was blotted, and the major band in the middle of the lane 2 was excised from PVDF membrane after amido black staining, then sequenced for five amino acid residue using an Applied Biosystems model 473A automated protein sequencer.

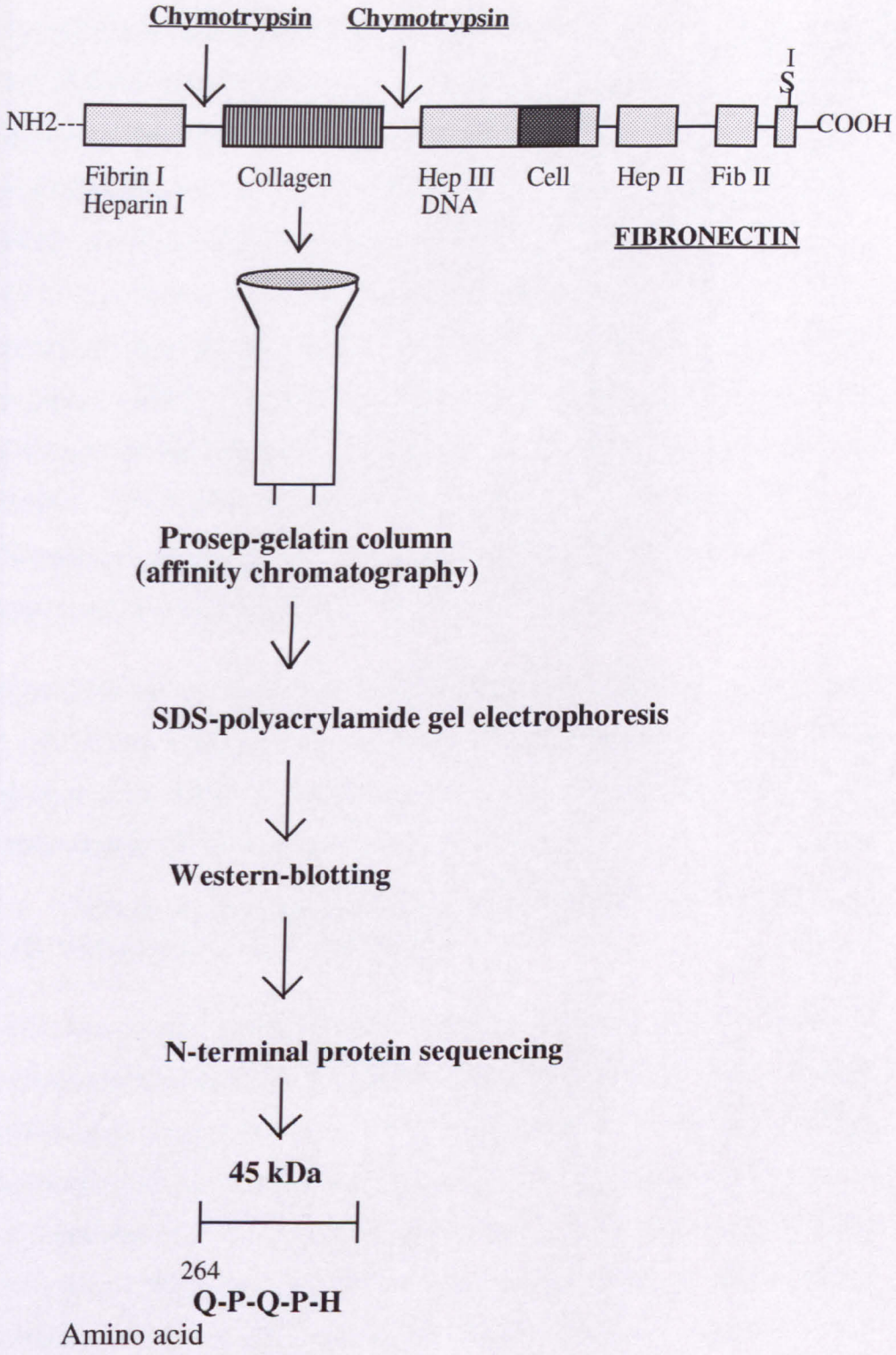


Figure 4.14. N-terminal amino acid sequencing of human plasma fibronectin gelatin-binding chymotryptic fragment.

As indicated before, the gelatin-binding domain of plasma fibronectin was not blocked and was sequenced successfully following the process shown in *Table 4.14*. Plasma fibronectin was digested with chymotrypsin as shown in *Figure 4.12-a* and *-b*, and the digest was applied to Prosep-gelatin to purify gelatin-binding domain of fibronectin as seen the result in *Figure 4.13*. Amino-terminal protein sequencing (Edman degradation) of purified gelatin-binding domain of fibronectin was determined. The results of sequence analysis showed the N-terminal sequence glutamine-proline-glutamine-proline-histidine (Q-P-Q-P-H) in the 45 kDa collagen-binding domain. These five residues were compared to the sequence of plasma fibronectin reported by Petersen *et al.*, (1989), and found to be equivalent to amino acid residues 264-268.

It was also demonstrated that the purified gelatin-binding domain (*Figure 4.13*) carries many but not all of the carbohydrate side chains of the fibronectin as judged by using digoxigenin-labelled lectins in the Glycan Differentiation Kit-Boehringer (results not shown).

4.7 Effects of Plasma and Purified Cellular Fibronectins on Cell Morphology and Spreading

Cells can attach, spread, and migrate on a variety of extracellular glycoproteins including fibronectin. These interactions occur through specific cell surface receptors. Cell adhesion receptors belong to a large superfamily of related cell surface complexes called the integrins (for reviews see Ruoslahti and Pierschbacher, 1987; Hynes, 1987, Buck and Horwitz, 1987; Hay,1991; Bosman, 1993). Many of the adhesive glycoproteins contain a common tripeptide sequence (RGD) which is recognised by the cell surface integrins and helps bind cells to the extracellular matrix (ECM). The ECM plays an important role in the proliferation, differentiation and attachment of many cells including fibroblasts.

To investigate the effects of cellular fibronectins purified from conditioned medium of TS and control fibroblasts, and plasma fibronectin (Gibco) on fibroblasts, cell culture plates were coated with different concentrations of purified cellular and plasma fibronectins. A procedure according to Freshney (1992) to coat tissue culture plastic with fibronectin was used;

i) Different concentrations of plasma and purified cellular fibronectins ($1.5\mu g/cm^2$, $3\mu g/cm^2$ and $6\mu g/cm^2$ in distilled water) were transferred into tissue culture multi-well plates with covers (Flow Laboratories) and were left to air-dry at 40 °C for 10 days.

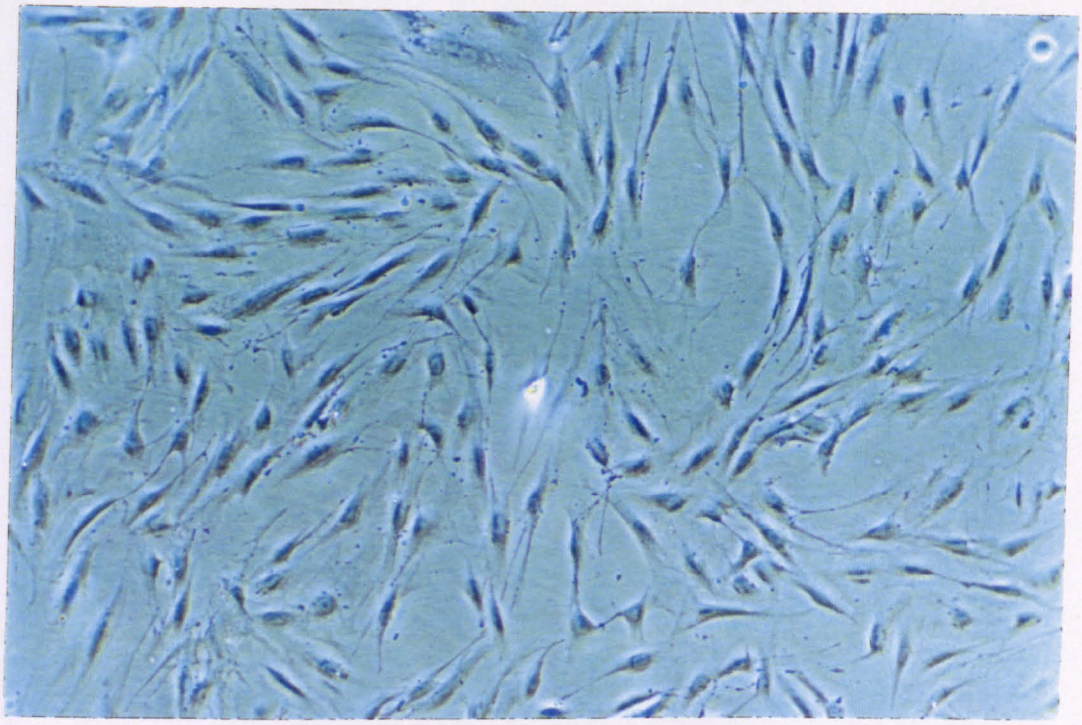
ii) When the fibronectin solution was dried completely in each well, all wells were rinsed extensively with sterile distilled water and again air dried.

iii) Equal number ($0.15x10^4$) of normal fibroblasts (DAKO) in 3ml fresh medium (DMEM) containing fibronectin depleted 10% foetal calf serum and 2mM L-glutamine were transferred to each group of wells coated with the plasma and cellular fibronectins from TS and normal fibroblasts.

iv) Cells were incubated at 37 °C in a humidified incubator with 5% CO_2 in air for 48 hours.

After 48 hours, fibroblasts were examined under the light microscope. Photographs of the fibroblasts were taken using a Nikon camera connected to the light microscope. Examples of these photographs are shown in *Figures 4.15* and *4.16*.

A



B

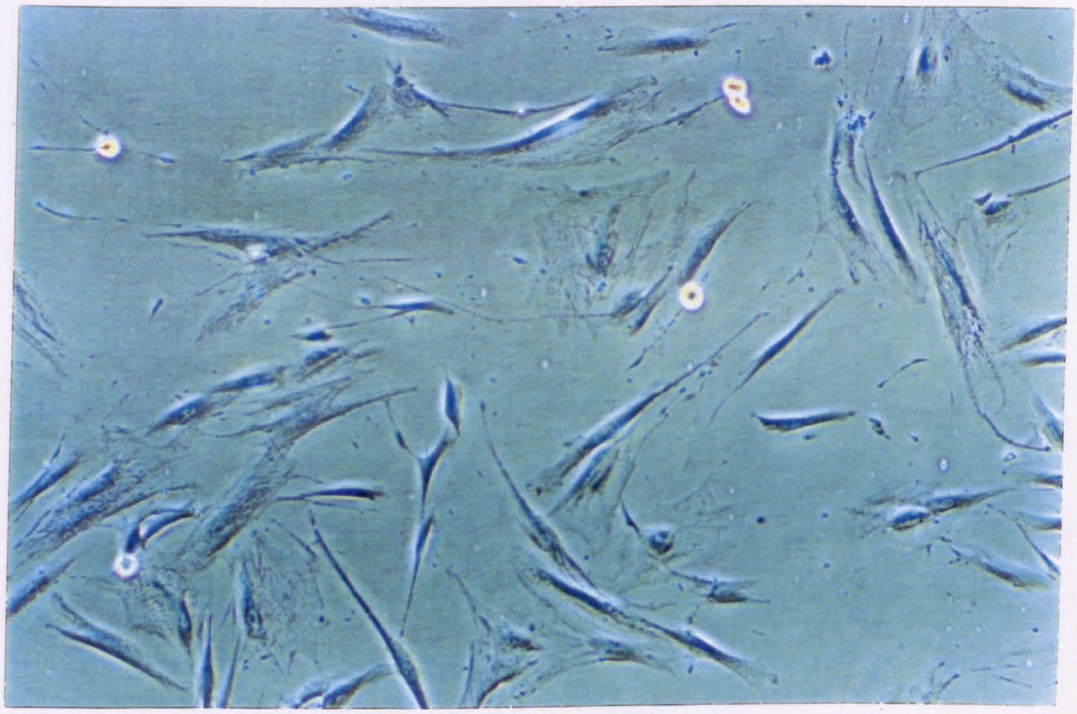
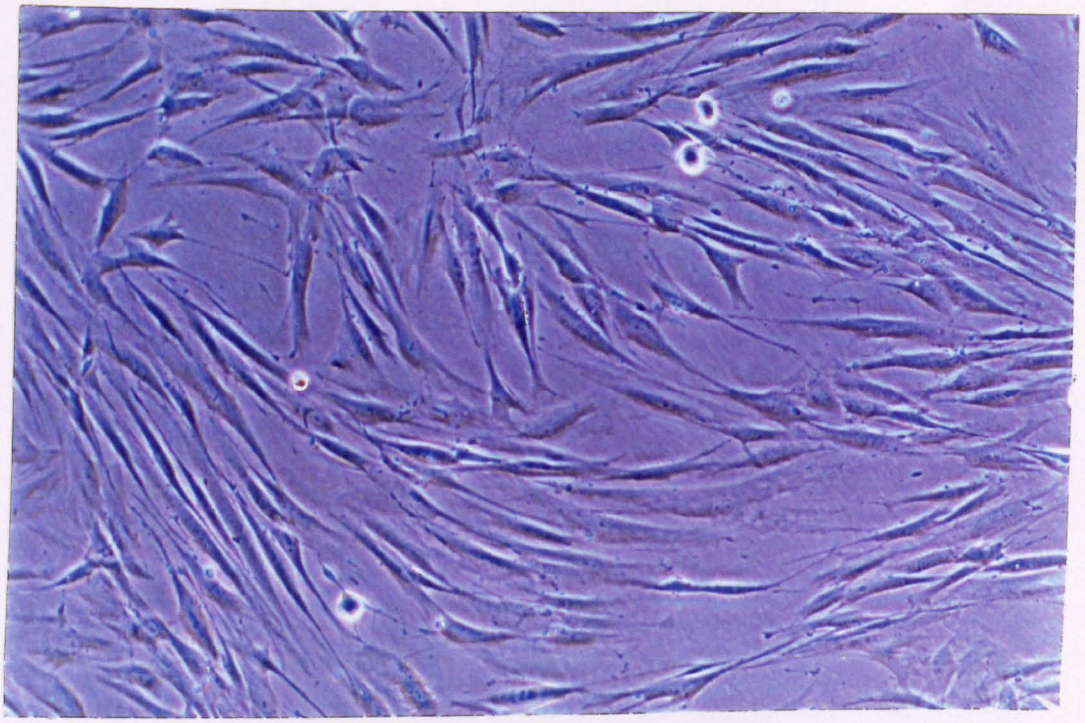


Figure 4.15. Morphological effects on normal skin fibroblasts of the purified fibronectins from (A) conditioned medium of normal skin fibroblasts, and (B) conditioned medium of TSFC-fibroblasts derived from skin lesion of a TS patient. Photographs were taken at 26x magnification on 3µg/cm^2 fibronectin-coated plates.

A



B

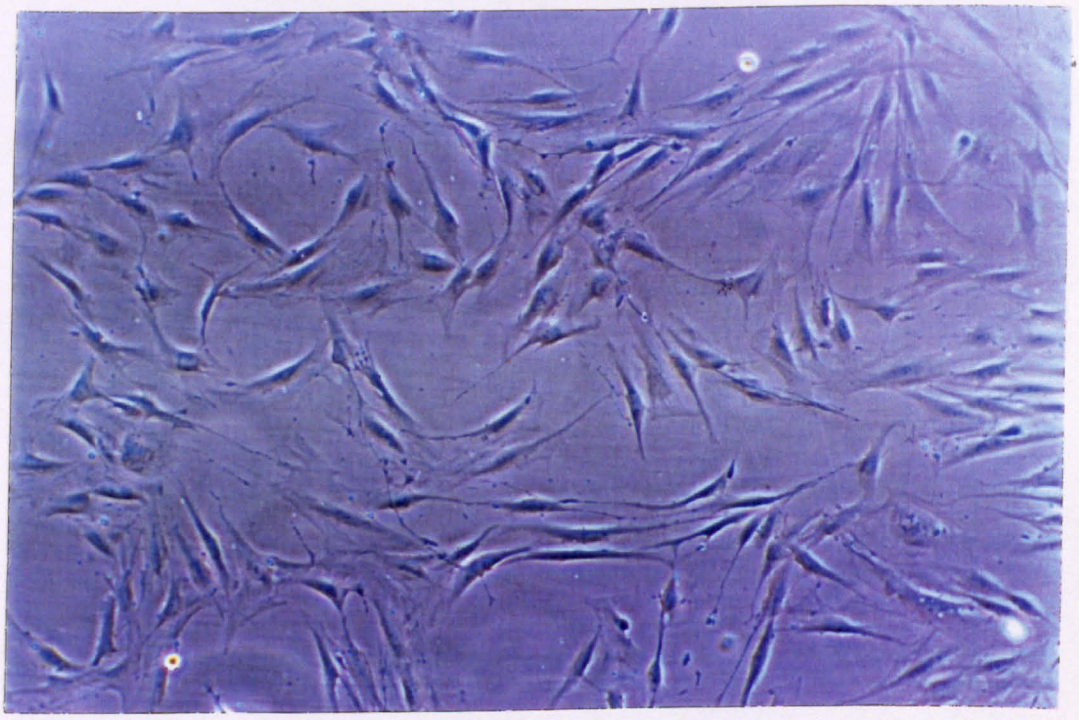


Figure 4.16. Morphological effects on normal skin fibroblasts of the purified cellular fibronectin from conditioned medium of normal skin fibroblasts (A), and plasma fibronectin purchased from Gibco (B). Photographs were taken at 26x magnification on 6µg/cm^2 fibronectin-coated plates.

In the presence of plasma and cellular fibronectins, increased attachment and flattening of cells are both likely to be consequences of the increased adhesivity of the cells observed in early stages of the cell culture. In general, cell adhesion and spreading was promoted in all fibronectin coated cell culture plates after 48 hours.

Interestingly, altered morphology and less confluence were observed in normal fibroblasts cultured on the purified fibronectin ($3\mu g/cm^2$ and $6\mu g/cm^2$) from conditioned medium of fibroblasts derived from skin lesion of a TS patient. The appearance of the normal fibroblasts was often changed and cells became larger and expanded on the TS derived cellular fibronectin (*Fig. 4.15-B*). Altered morphology and reduced proliferation of fibroblasts grown on the TS derived cellular fibronectin may be the result of the increased glycosylation of fibronectin derived from fibroblasts of TS skin lesions. The morphology of the cells was normal, well established, and the cell culture plates were nearly confluent on the cultured normal cellular fibronectin purified from conditioned medium of normal skin fibroblasts (*Figures 4.15-a* and *4.16-a*). In plasma fibronectin coated cell culture plates at all concentrations, the morphology of the cells was normal, however, plates were more confluent than TS-derived fibronectin coated plates, but were less confluent than normal cellular fibronectin coated plates (*Figure 4.16-B*). Moreover, plasma and cellular fibronectins at all concentrations ($1.5\mu g/cm^2$, $3\mu g/cm^2$ and $6\mu g/cm^2$) were equally active in promoting cell attachment. However, cellular fibronectin from normal fibroblasts appeared more active in promoting the spreading of fibroblasts than plasma fibronectin, and this is consistent with the literature (Yamada and Olden, 1978). Overall, these results suggest that all fibronectins (plasma and cellular) were equally active in promoting cell attachment. However, these fibronectins, especially the TS derived cellular fibronectin, were not equally active in promotion of the normal cell morphology and confluency.

4.8 Discussion

In summary, the results of the carbohydrate analysis and characterisation of peptides of purified fibronectin from conditioned medium of TS and normal fibroblasts are presented in this chapter.

Analysis of purified fibronectin samples by high pH anion-exchange chromatography (HPAEC-PAD) revealed that the carbohydrate portion of the fibronectin molecule was made up of galactose, mannose, glucosamine, galactosamine, sialic acid and fucose. Oligosaccharides of TSFC-purified fibronectin from fibroblasts of TS skin lesion and plasma fibronectin appeared basically as mono and disialyted biantenary structures respectively (*Fig. 4.9*). The major type of side chain in fibronectin glycosylation is of the biantennary complex asparagine-linked type (*Fig. 4.11*). As expected, fucose was not detected in human plasma fibronectin. Fucose was also not detected in hamster plasma fibronectin by other researchers (Fukuda *et al.*, 1982). In human plasma and fibroblasts derived cellular fibronectin, the most common form of sialic acid was found to be N-acetyl neuraminic acid. Nevertheless, the sialic is N-glycolyl neuraminic acid in hamster plasma fibronectin according to Fukuda and colleagues (1982).

Moreover, there was altered monosaccharide composition of fibronectin purified from conditioned medium of fibroblasts grown from skin lesions of different TS patients in comparison to carbohydrates of fibronectin derived from normal fibroblasts. An increased concentration of sugars was observed in sialic acid, galactosamine, glucosamine, galactose and mannose of purified fibronectin from neck and ungual fibromas of patients with TS. The total increase of carbohydrates of TS fibroblasts fibronectin was more than two fold in comparison to normal fibroblasts-derived fibronectin.

Interestingly, more galactosamine was detected in cellular fibronectin of TS and normal fibroblasts than in human plasma fibronectin. This suggests the occurence of the O-glycosylation in fibronectin, as it was also detected by Skorstengaard *et al.*, (1986-b). Carter and Hakomori (1979) and Fukuda and Hakomori (1979) have also reported the presence of some galactosamine associated with hamster cellular fibronectin.

In this thesis, the results of the purified cellular fibronectin glycosylation represent a more detailed study than preliminary observations in this laboratory. Earlier, Ellis and Hemming (1990) reported the increased glycosylation of the fibronectin on the metabolically labelled (glucosamine and mannose incorporation) cultured fibroblast derived glycoproteins using SDS-PAGE. In the more extensive study presented in this thesis, only fibroblasts from skin lesions but not unaffected skin of TS patients showed an increased glycosylation of purified cellular fibronectin in comparison to normal skin fibroblasts.

Several oligosaccharide-dependent functional alterations of fibronectin have previously been described. It was shown that glycosylation can modulate the interaction of fibronectin with collagen and with the cell membrane. Jones *et al.* (1986) compared glycosylated and non-glycosylated human skin fibroblast fibronectins and found that the deglycosylated form had an increased affinity for gelatin and enhanced adhesion-promoting properties. An important function of the carbohydrate moieties of the fibronectin is also protection of the protein from proteolytic attack. Bernard and colleagues demonstrated that the heavily glycosylated collagen-binding domain of chick embryo fibroblast fibronectin was selectively resistant to a broad variety of proteases (Bernard *et al.*, 1982).

Harper *et al.*, (1990) previously reported that human tumors of neuroectodermal origin secrete fibronectin that contains the cell-adhesion-

related HNK-1 epitope. In this study, the glycoproteins including fibronectin derived from TS skin fibroblasts and normal fibroblasts were found not to express the HNK-1 carbohydrate epitope. This result is not surprising, because the epitope was reported to be a marker for neural cell adhesion molecules involved in cell-cell and and cell substrate interactions during neural development.

Since fibronectin glycosylation is involved in promotion of fibroblasts adhesion and spreading, and playing significant roles in the extracellular matrix (ECM), the abnormal glycosylation of fibronectin found in skin lesions of TS patients may effect the cell-cell adhesion and cell-ECM interaction during the early and later embryonic development.

In general, the amino acid composition of the purified fibronectin from normal fibroblasts (JPa) was very similar to that of purified fibronectins from TS fibroblasts as well as standard plasma (pFN) and cellular fibronectin (cFN) puchased from Gibco and Telios, USA, respectively (*Table 4.2*). Amino acid composition of these fibronectins was also consistent with the scientific literature. This suggests, as expected, no major differences in the primary structure of the fibronectin in TS.

Amino acid sequencing of fibronectin purified from TS and normal fibroblasts was not satisfactorily completed because of the N-terminal blocking and low expression of these fibroblast derived cellular fibronectin. However, N-terminal amino acid sequencing (Edman degradation) was demonstrated on the purified gelatin-binding domain of chymotrypsin digested human plasma fibronectin. The results of sequence analysis showed the N-terminal sequence glutamine-proline-glutamine-proline-histidine (Q-P-Q-P-H) in the 45 kDa collagen-binding domain. It was also found that the purified gelatin-binding domain carries many but not all of the

carbohydrate side chains of the fibronectin as judged by using digoxigenin-labelled lectins in the Glycan Differentiation Kit (Boehringer).

To investigate the effects of cellular fibronectins purified from conditioned medium of TS and control fibroblasts, and plasma fibronectin (Gibco) on fibroblasts, cell culture plates were coated with different concentrations of purified cellular and plasma fibronectins. Adhesion of fibroblasts was promoted in all concentrations of plasma and TS derived cellular fibronectin coated cell culture plates.

Interestingly, altered morphology and less confluence were observed in the culture plates coated with cellular fibronectin derived from TS skin lesions. This altered morphology may be the consequence of the effects of altered (increased) glycosylation of the fibronectin as a ECM macromolecule. The appearance of the fibroblasts was very similar (and normal) on the cultured plates coated with plasma, and normal cellular fibronectin derived from normal skin. However, cellular fibronectin coated fibroblast cultures were more confluent than plasma fibronectin coated culture plates. This indicated that cellular fibronectin is more active than plasma fibronectin in the promotion of the cell spreading, proliferation and morphology.

In general, these results suggest that such changes in ECM composition and organisation may have profound effects on cell spreading, adhesion, proliferation, cell shape and behaviour during the early embryonic development. However, the possible consequences of these effects in relation to TS will be discussed in detail in chapter 6.

Chapter 5

Chapter 5

Expression of Adhesive Glycoproteins Fibronectin, Tenascin and Laminin by Cultures of Skin Fibroblasts From Patients With Tuberous Sclerosis

5.1 Introduction

Cells in connective tissues are embedded in an intricate extracellular matrix that not only binds cells and tissues together but also influences the development, polarity, and behavior of the cells it contacts (Alberts *et al.*, 1994). Fibronectin, laminin, and tenascin are examples of large adhesive glycoproteins in the matrix. By means of their multiple binding domains, such proteins help cells adhere to and become organised by the ECM. Many of these adhesive glycoproteins contain a common tripeptide sequence (RGD), which is recognised by cell surface receptors, called integrins. Through these links, cells influence the nature of the matrix and the matrix affects the physiology of the cells. Moreover, proportions and arrangements of these matrix components play vital roles in the construction and functions of the matrix. Therefore, expression of the major matrix glycoproteins fibronectin, laminin, and tenascin by fibroblasts may be important in hardening of affected tissue, particularly in most common skin lesions of tuberous sclerosis (TS) patients. The interrelations between cells and the surrounding extracellular matrix (ECM) is an important parameter in the degree of hardening. The macromolecules that constitute the

extracellular matrix are mainly secreted locally by cells in the matrix. In most connective tissues these macromolecules are secreted by fibroblasts which are the principal cells of the dermis.

The general procedure of investigating the expression and distribution of extracellular matrix glycoproteins fibronectin, laminin and tenascin was as follows:

I) Immunocytochemical studies on established skin fibroblasts from TS patients and normal individuals were carried out to determine the expression and localisation of the glycoproteins of both cell lines.

II) Quantitative analysis of the three ECM glycoproteins was determined on TS and normal skin fibroblasts using flow cytometry and enzyme-linked immunosorbant assay (ELISA).

5.2 Expression and Distribution of Fibronectin by Skin Fibroblasts from Patients with TS and Normal Individuals

5.2.1 Immunofluorescence Microscopic Localisation of Fibronectin

As described in more detail in section 2.3.10, TS and normal fibroblasts were cultured on glass coverslips in 24 well cell culture dishes. On the second or third day of cell culture, immunofluorescence (indirect) staining of fibroblasts was undertaken. Cells were fixed with 3.8% formaldehyde in PBS (v/v), 1:10 dilution, for cell surface staining. Fibronectin visualised in such cells was primarily extracellular. To detect intracellular fibronectin, the cells were fixed with 3.8% formaldehyde and made permeable with Triton X-100 (0.1% in PBS). Cells were rinsed three times with PBS, pH 7.4, after each of these steps. Then, cells were blocked with 1% bovine serum albumin in PBS (w/v) overnight at 4 °C.

Next day, fibroblasts were incubated with the polyclonal antibody (rabbit anti-human fibronectin at 1: 50 dilution in blocking buffer) for 1 hour at room temperature. This primary antibody was omitted for the negative

control staining of fibroblasts. Cell layers were washed with PBS three times, and incubated for 1 hour with a fluoresceinated secondary swine anti-rabbit IgG antibody (at 1:30 dilution) at room temperature in the dark. Finally cells were washed twice with PBS, and cover slips were mounted in 1:1 (v/v) PBS/glycerol solution with 8 mg/ml (w/v) 1-4 diazabicyclo-2.2.2-octane (DABCO) to prevent photobleaching. Cover slips were attached to slides using nail-varnish. Cells were examined on a Nikon microscope (Labophot Y2A). Photographs using Kodak EPD-200 film were taken at 20X or 40X magnification, all with the same exposure times.

In this study, skin samples were obtained from patients undergoing surgery. The following fibroblast cell lines, established from primary cultures of skin samples, were used for our immunocytochemical studies :

TSFL : Skin tissue was obtained from a neck fibroma of a 13 year old male TS patient.

TSAH : Unaffacted skin was obtained from the lumbo-sacral area of a 65 year old male TS patient.

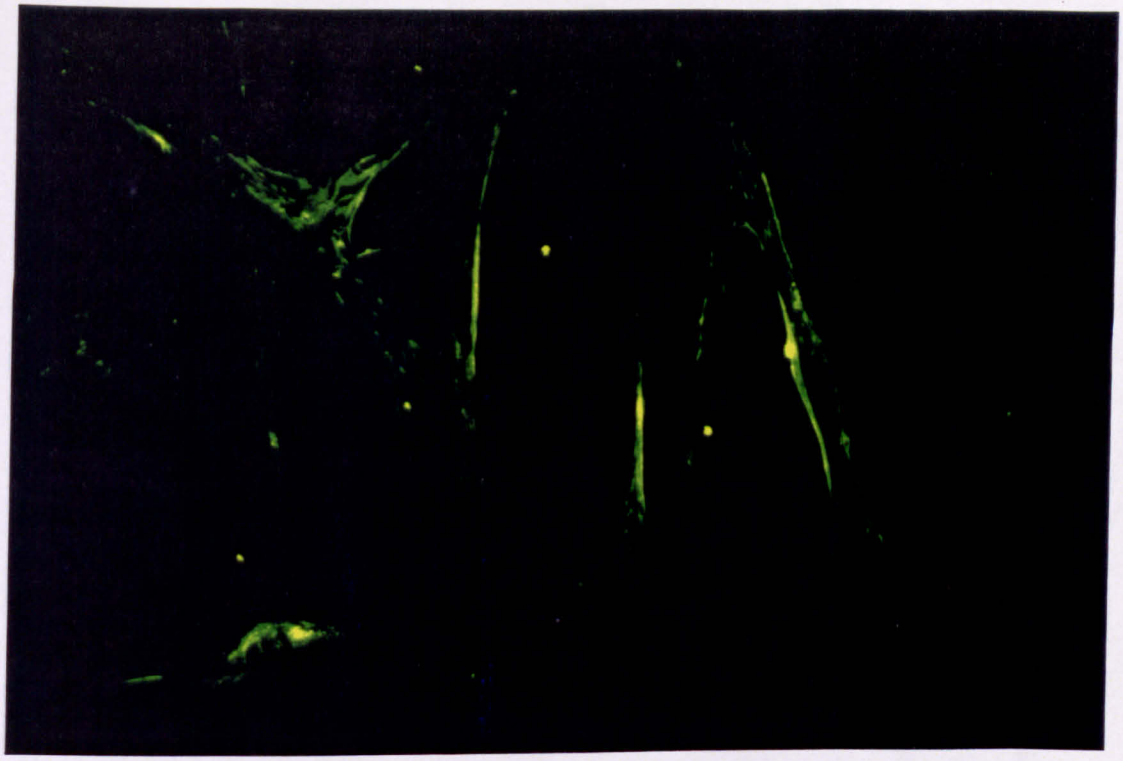
TSFM : Skin tissue was derived from an ungual fibroma of the second phalanx of a 10 year old male.

TSJH : Skin tissue was obtained from a forehead plaque (lesion) of a 14 year old female TS patient.

DAKO and JPA normal skin fibroblasts derived from skin explants of 12 year old female and 8 year old male respectively were used as control.

By means of immunofluorescence, we have analysed the distribution of cellular fibronectin in the above TS and control fibroblast cell lines. *Figures 5.1*, *5.2*, and *5.3* show the results of indirect immunofluorescence studies of these cell cultures.

A



B

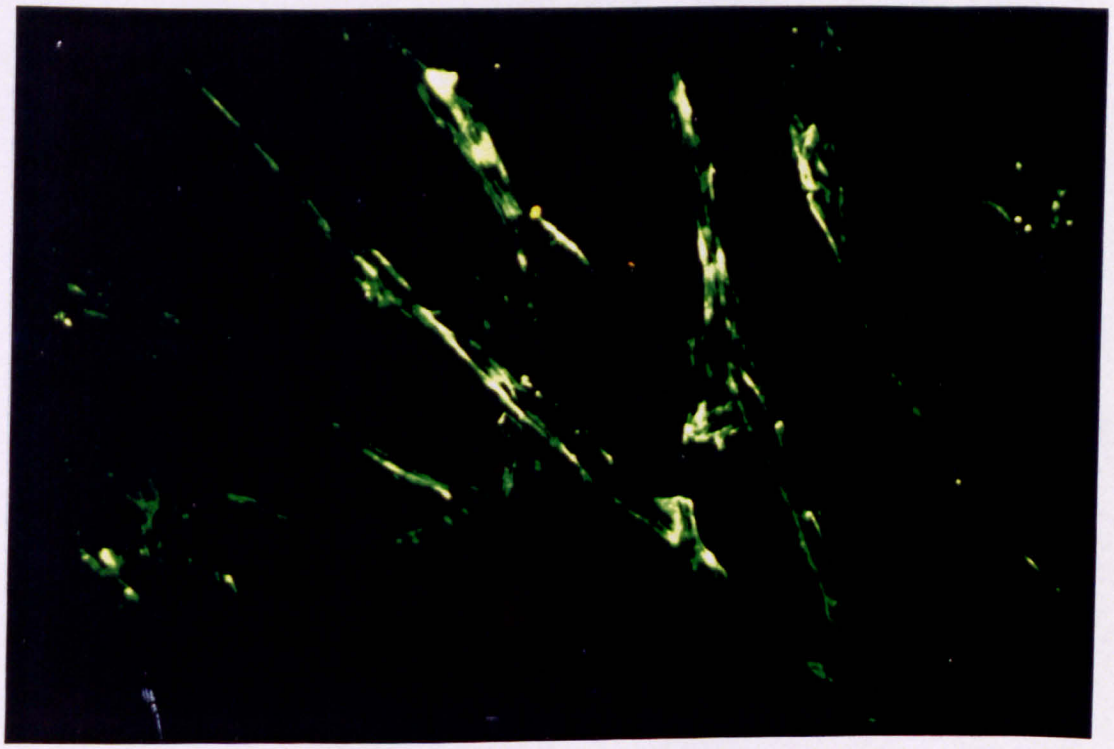
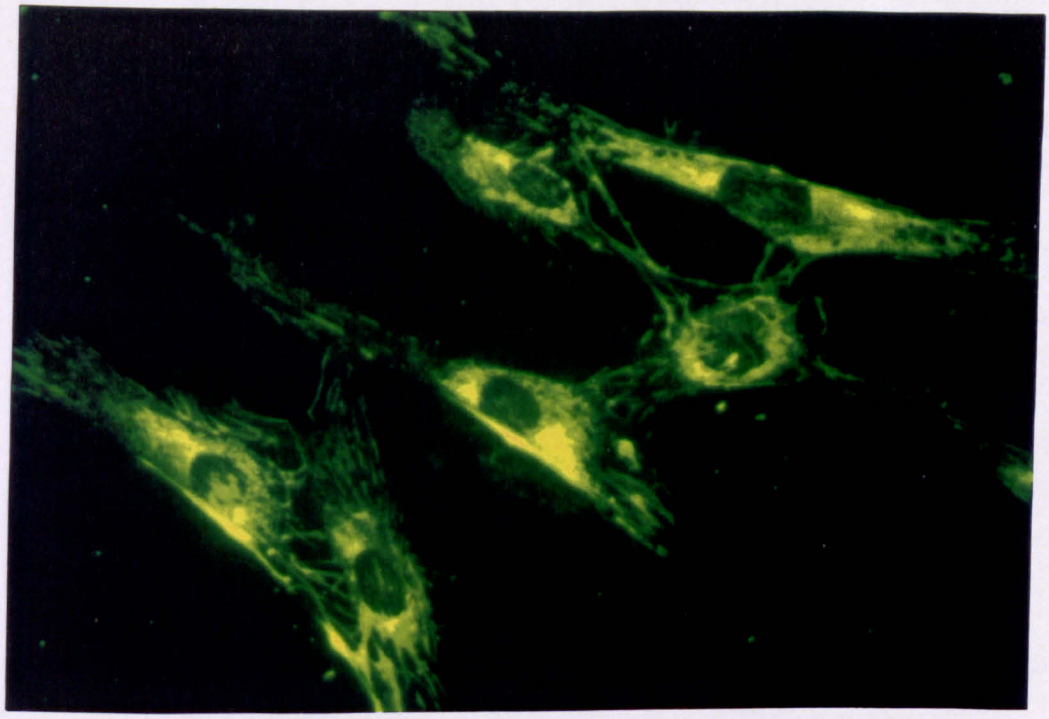


Figure 5.1. Indirect immunofluorescence localisation of cell-surface fibronectin. Normal JPa-skin fibroblasts (a), and ungual fibroma-derived TSFM-fibroblasts (b) were fixed with formaldehyde for cell-surface staining. Photographs were taken at 40X magnification.

A



B

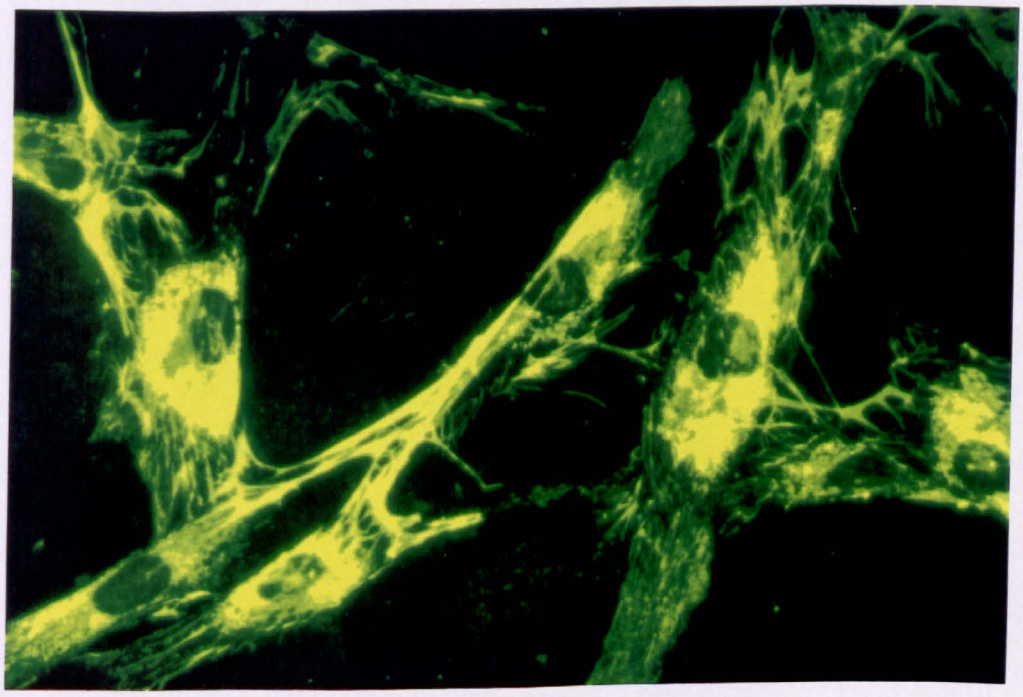


Figure 5.2. Indirect immunofluorescence localisation of intracellular fibronectin. Normal JPa-skin fibroblasts (a), and ungual fibroma-derived TSFM-fibroblasts (b) were fixed with formaldehyde and made permeable with Triton X-100. Photographs were taken at 40X magnification.

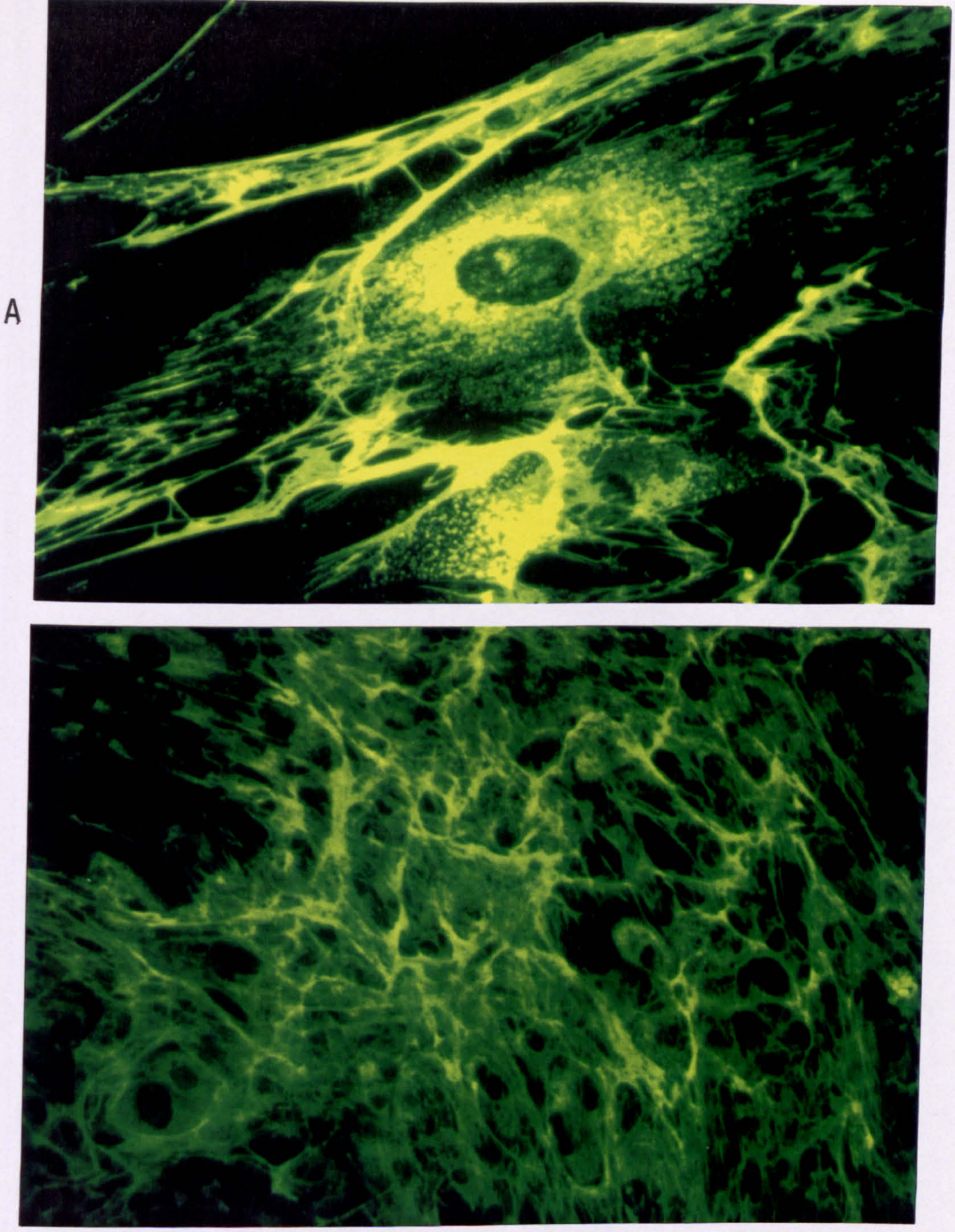


Figure 5.3. The figure shows the indirect immunofluorescence localisation of intracellular fibronectin on a large ungual fibroma-derived TSFM-fibroblast (a), and the fibrillar matrix of fibronectin on cultured normal JPa-fibroblasts (b). This sort of dense matrix (fibril network) is typical of confluent cultures of fibroblasts. Fibroblasts were fixed in formaldehyde and made permeable with Triton X-100 to visualize both fibrillar surface-associated and intracellular fibronectins. Photographs were taken at 40X (a), and 20X magnification (b). Note the increased fibril formation around the large cell derived from the skin lesion (ungual fibroma) of a TS patient (a).

Indirect immunofluorescence studies revealed that the distribution and expression of fibronectin compared to controls was only altered in TSFM ungual fibroma-derived skin fibroblasts of the 10 year old male TS patient. However, the distribution and expression of fibronectin was not changed in other fibroblasts derived from a forehead plaque lesion and neck fibroma of a 14 year old female and 10 year old male TS patient respectively in comparison to control fibroblasts. Unaffected skin fibroblasts (TSAH) derived from the skin of the lumbosacral area of a TS patient also showed similar distribution and fluorescence intensity for fibronectin in comparison to normal (control) skin fibroblasts (data not shown).

The immunofluorescence staining results in *Figures 5.1, 5.2,* and *5.3* show the altered distribution of fibronectin in TSFM-fibroblasts derived from ungual fibroma of a TS patient in comparison to normal JPa-fibroblasts. Cell surface staining of ungual fibroma derived TSFM-fibroblasts with anti-fibronectin was markedly different than normal (control) skin fibroblasts. Moreover, the cell surface distribution of fibronectin was uneven and appeared more dense on the TSFM-fibroblasts derived from ungual fibroma of a TS patient, while it is evenly distrubuted on control JPa-fibroblasts (*Fig. 5.1*).

The intensity of the fluorescence of intracellular fibronectin was increased in the TSFM-fibroblasts derived from ungual fibroma of a TS patient in comparison to normal JPa-fibroblasts. Cells from an ungual fibroma of a TS patient appeared larger than normal fibroblasts in size. In addition, the fluorescence intensity was diffuse and predominantly perinuclear in both TS and normal skin fibroblasts (*Fig. 5.2*). However, the intracellular fluorescence was patchy and also predominantly perinuclear in large cells (called N-cells by others) of TS fibroblasts (*Fig. 5.3*).

In agreement with the literature (Yamada and Olden 1978; Hynes 1990), a fibrillar matrix of fibronectin was also observed in our normal human skin fibroblasts (*Fig. 5.3-b*). These striking fibrillar arrays were better established around the large TS cells derived from ungual fibroma than normal skin fibroblasts (compare *Figures. 5.3-a* and *5.2-a*). The fibrillary matrix of fibronectin was also observed in formaldehyde-fixed confluent TS and normal fibroblasts (data not shown). This suggests that the fibrillary matrix of fibronectin is mainly cell surface associated.

As a negative control, primary antibody (anti-fibronectin) staining was omitted, and fibroblasts were incubated with a fluoresceinated secondary swine anti-rabbit antibody (at 1:30 dilution). There was no staining of secondary antibody in these negative control fibroblasts as expected (*Fig. 5.4*).

Figure 5.4. Negative control staining of fibroblasts with only fluoresceinated secondary swine anti-rabbit antibody (1:30 dilution).

5.2.2 Quantitative Analysis of Fibronectin Expressed by TS and Normal Skin Fibroblasts

5.2.2.1 Flow Cytometric Analysis of Fibronectin

A flow cytometer can be used quantitatively to compare the fluorescence intensity of particles (Givan, 1992). In addition to immunofluorescence studies, quantitative analysis of fibronectin expression was performed using a Becton Dickinson FACScan in the Department of Immunology. 2000 events were collected using the consort 30 program and standard fibroblasts settings. A logical fibroblasts gate was set before data analysis. FlowMATE (Dako) and Consort 30 (Becton Dickinson) programs as PC and FACScan software respectively were used to analyse the mean fluorescence intensity of dual labelled cells for fibronectin.

In this thesis, the fluorescent intensity of TS and normal skin fibroblasts was measured and compared. In the example shown in *Figure 5.5* (A) forward (FSC) versus side scatter (SSC) light measurements (cytogram), and (B) the fluorescence of fibroblasts, is displayed on the gated histogram. Using specific gating procedures in the computer program, a box was drawn around the fibroblast populations. This gate or window is used by the computer to select cells for accumulation of events into the fluorescence histogram.

TSFL (neck fibroma), TSFM (ungual fibroma) and JPa (normal skin) fibroblasts were subjected to the flow cytometric analysis of fibronectin. An adapted flow cytometry protocol was described in detail in section 2.3.7.3.

Figure 5.6 shows a typical photograph of the trypsinised rounded TSFM fibroblasts which were stained with primary (anti-fibronectin) and FITC-conjugated secondary antibodies, and suspended in 0.5 ml of 1% BSA/PBS solution just before flow cytometric analysis.

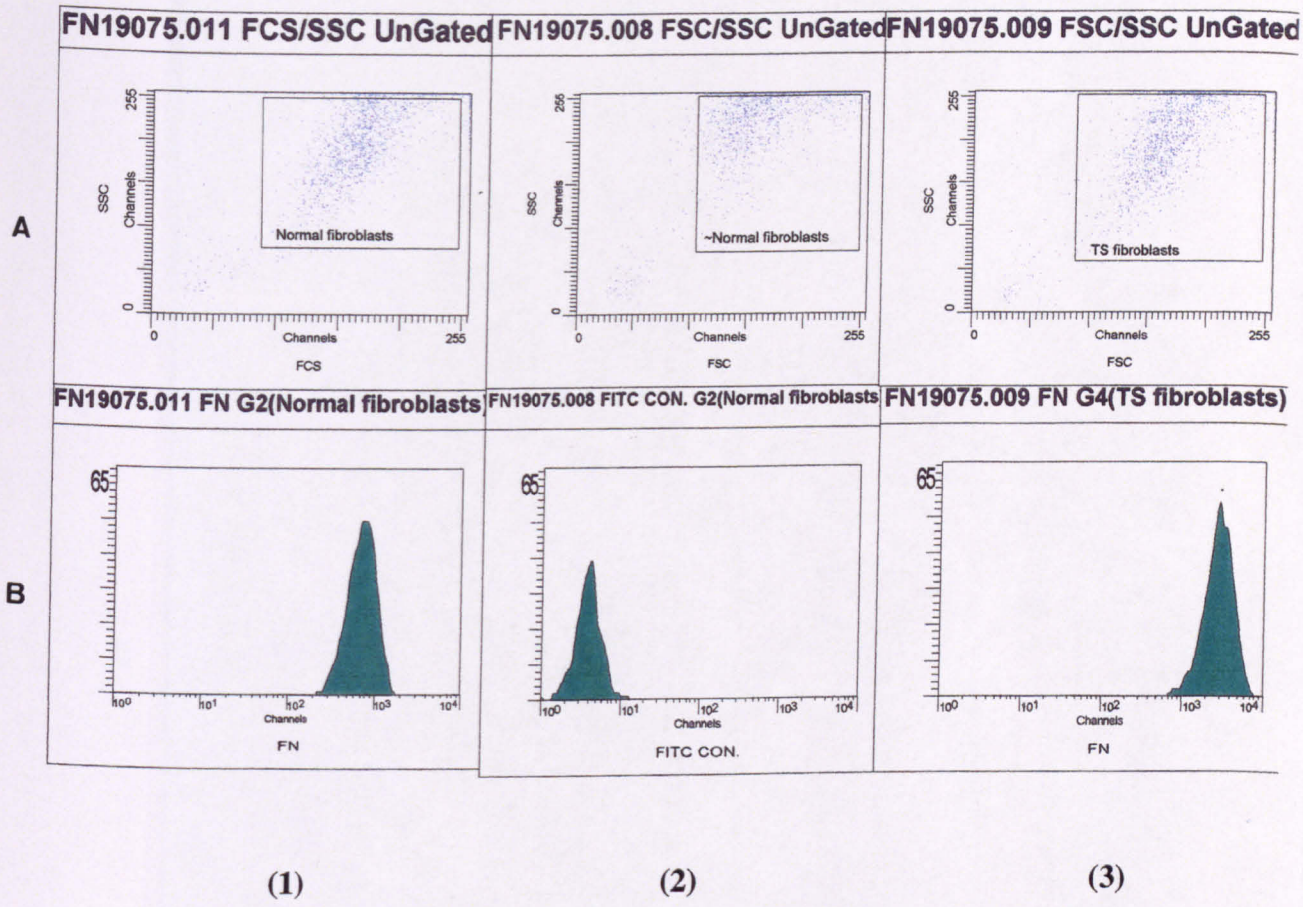
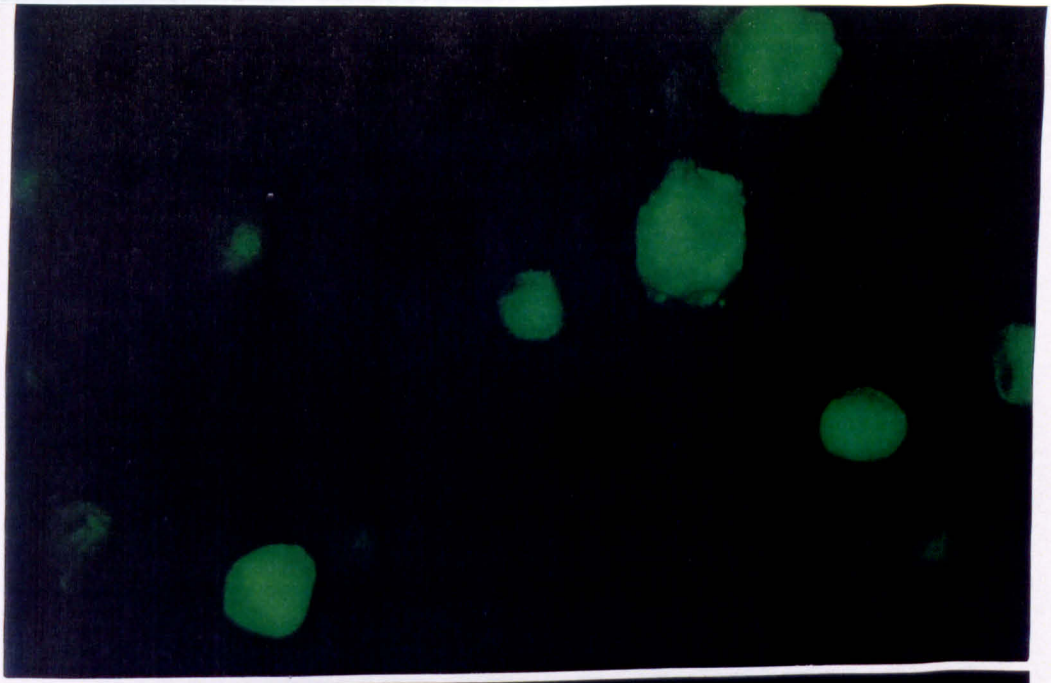


Figure 5.5. The figure shows the typical cytograms and histograms of flow cytometric analysis of fibronectin (FN). Panel A shows the forward (FSC) versus side scatter (SSC) cytograms (dotplots of fibronectin expression) of TS and normal skin fibroblasts. Panel B shows the fluorescence histograms of TS (TSFM) and normal skin fibroblasts (JPa). A box is drawn around the fibroblast cluster and used as a gate so that the fluorescence intensity of only the cells falling within this gate are displayed in the immunofluorescence profile shown in Panel B. For comparison, see panel B-1 and B-3 which represents fluorescence intensity of fibronectin of JPa and TSFM fibroblasts respectively. Panel B-2 represents only FITC-conjugated secondary (anti-rabbit) antibody staining of fibroblasts (omitting primary anti-FN) as negative control staining.

A



B

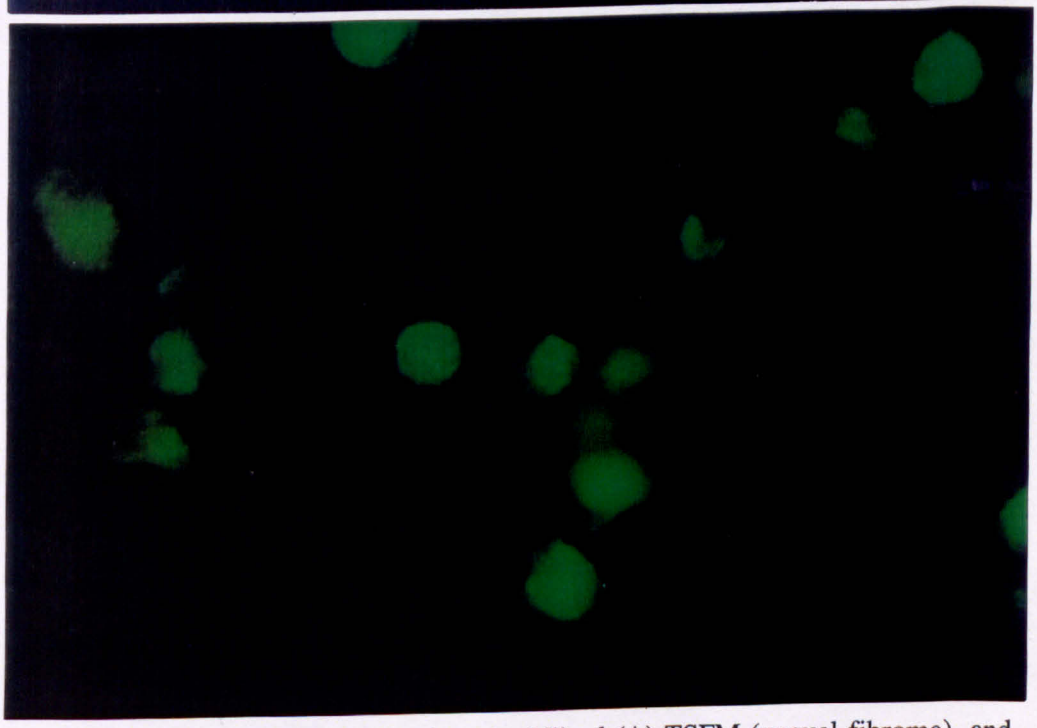


Figure 5.6. Trypsinised, fixed and permeabilised (A) TSFM (ungual fibroma), and (B) JPa (normal skin) rounded fibroblasts were stained with rabbit anti-fibronectin, and labelled with FITC-conjugated secondary (anti-rabbit) antibody, then suspended in 0.5 ml 1% BSA/PBS solution for flow cytometric analysis (see details in section 2.3.7.3.). Cells in suspension were cytospun and mounted in 1:1 (v/v) PBS/glycerol solution with 8 mg/ml (w/v) 1-4 diazabicyclo-2.2.2-octane (DABCO) to prevent photobleaching. The photographs of the cells were taken at 60X magnification using a Bio-Rad DVC-250 Confocal laser scanning microscope in Obstetrics and Gynaecology Department. For comparison, note the size and fluorescence intensity of TSFM fibroblasts (A) and normal JPa-fibroblasts (B).

Quantitatively, the fluorescence intensity of 2000 TS (TSFM and TSFL) and normal skin fibroblast (JPa) populations for fibronectin was measured as mean using a Becton Dickinson FACScan in the Department of Immunology. *Figure 5.5* shows that TSFM fibroblasts derived from an ungual fibroma have more dense staining of fibronectin than normal JPa skin fibroblasts. *Figure 5.6* confirm this observation and suggested that the large cells of TSFM fibroblasts posses the more dense fibronectin staining than normal JPa skin-fibroblasts. As discussed earlier, the distribution of intracellular fibronectin was patchy and predominantly perinuclear in the large TS fibroblasts (*Fig. 5.3*).

Previous indirect immunofluorescence studies have shown that the distribution and expression of fibronectin was only altered in TSFM ungual fibroma-derived skin fibroblasts of a 10 year old male TS patient. However, the distribution and expression of fibronectin was not changed in other fibroblasts derived from a forehead plaque lesion and neck fibroma of a 14 year old female and 10 year old male TS patient respectively, in comparison to control fibroblasts. Here, flow cytometry results confirmed and strengthened these earlier observations quantitatively.

The flow cytometric data of fibroblasts collected in Consort 30 program was gated using the FlowMATE program, and the mean (fluorescein intensity) was obtained quantitatively. An average mean of four analysis was used in *Figure 5.7*. In *Figure 5.7* we can observe that TSFM ungual fibroma-derived skin fibroblasts have a higher fluorescence intensity (mean) for fibronectin than TSFL neck fibroma-derived and JPa normal skin fibroblasts. Increased expression of fibronectin was observed in both cytoplasm (permeabilised) and cell surface (not-permeabilised) of TSFM ungual fibroma-derived skin fibroblasts in contrast to TSFL and JPa fibroblasts (see *Fig. 5.7*).

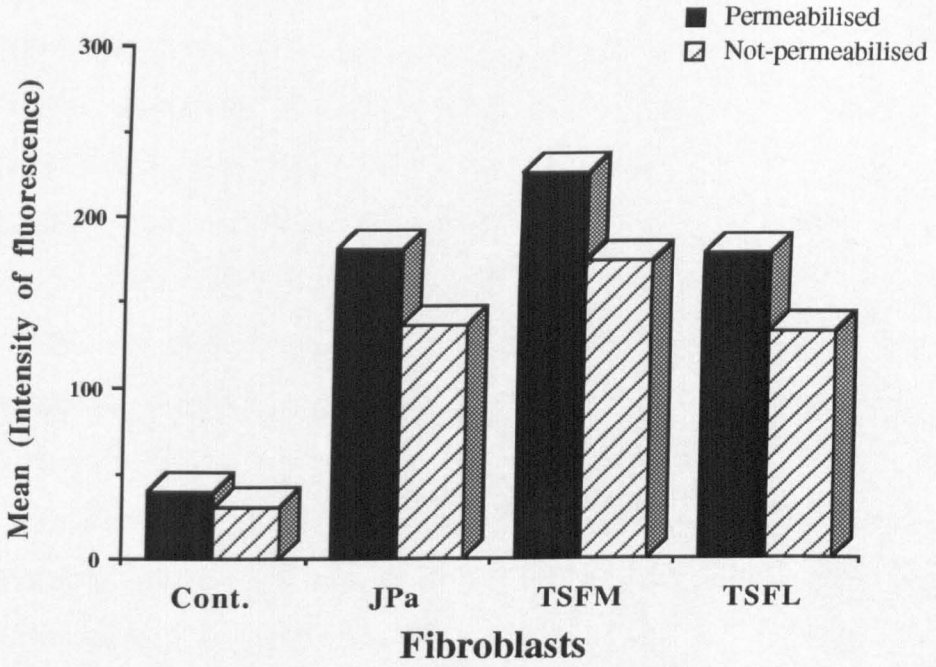


Figure 5.7. Quantitative analysis of fibronectin expressed by normal skin (JPa), and TS fibroblasts (TSFM, ungual fibroma; TSFL, neck fibroma) by flow cytometry. Cells were trypsinised, fixed and permeabilised for cytoplasmic staining or not-permeabilised for cell surface staining of fibronectin as described in detail in section 2.3.7.3. JPa, TSFM and TSFL fibroblasts were stained using primary polyclonal rabbit anti human fibronectin antibody and FITC-conjugated secondary (anti-rabbit) antibody. In control (cont.) normal skin fibroblasts primary anti-fibronectin was omitted, and cells were stained only with FITC-conjugated secondary (swine anti-rabbit) antibody as a negative control. 2000 events from each fibroblast line were analysed by flow cytometry. An average mean (fluorescence intensity) of four different flow cytometric analysis samples was plotted in this bar graph. Variations in mean within four different analysis of fibronectin were within ± 4%.

5.2.2.2 Enzyme-Linked Immunosorbent Assay (ELISA) for Fibroblasts Fibronectin Production

ELISAs were performed to quantitate the amount of fibronectin secreted into the cell culture medium by TS and normal skin fibroblasts. Briefly, near confluent fibroblasts cultures were set up by placing 1 X 10^5 freshly trypsinised fibroblasts in 1 ml of Dulbecco's Modification Eagle's Medium (DMEM) containing 10% foetal calf serum (FCS) into 24 well microculture plates (2 cm^2 surface area, Linbro; Flow laboratories). TS and normal skin fibroblasts were incubated for 24 hours at 37 °C in a humidified incubator with 5% CO_2. Medium was then removed and replaced with 1ml fresh DMEM with 10% FCS and incubated for 72 hours. For ELISA assay of fibronectin, supernatant medium was removed from 24 well microculture plates, and 100 µl of conditioned medium and 10 µl PBS were added per well to 96 well microtitre plates (Costar). The cells which had secreted the fibronectin into the supernatant medium were harvested with trypsin and counted using a hemocytometer. Fibronectin standard, diluted in the range 10 to 110ng/110 µl in increasing concentrations to give a standard curve, was also added to the first two lines of the 96 well microtitre plates. Control culture medium (DMEM with 10% FCS) which had been preincubated for 3 days along with the cells were used as the control. After 4 hours at room temperature, the 96 wells were emptied of samples and washed twice with PBS, and then blocking buffer (200 µl) made of PBS, pH 7.4 with 0.5% (w/v) BSA and 0.05% (v/v) Tween-20 was then applied to each microtitre well and left overnight at 4 °C. The following day, the ELISA method was performed exactly as described previously in section 2.3.7.2.

ELISA results of the fibronectin production into the cell culture medium by three TS (TSFL, TSFM and TSJH), and two normal skin fibroblasts (JPa and DAKO) is presented in *Table 5.1*.

Fibroblasts	Passage	Secreted Fibronectin* by 1 X 10^5 cell ng/100µl medium ± SD
TSFL	12-15	15.21 ± 0.64
TSFM	12-15	45.40 ± 0.85
JPa	12-15	10.26 ± 0.26
TSJH	12-15	13.01 ± 0.08
DAKO	12-15	10.44 ± 0.13

Table 5.1. Quantitative determination of fibronectin of TS (TSFL, neck fibroma, TSFM, ungual fibroma and TSJH, forehead plaque) and normal skin fibroblasts (JPa and DAKO) detected by ELISA. *Fibronectin production was assessed by measuring the quantity of fibronectin in fibroblast culture medium using ELISA and is expressed as ng/100µl medium of $0.25X10^5$ cells ± standard deviation (SD) of triplicate determinations.

ELISA assay of fibronectin suggested the increased production of fibronectin in TSFM fibroblasts derived from ungual fibroma of a TS patient in comparison to normal skin fibroblasts (JPa and DAKO) and other two TSFL and TSJH fibroblasts derived from neck fibroma and forehead plaque lesions of TS patients respectively. This more than 4 fold increase of fibronectin in TSFM fibroblasts also confirms previous findings of immunofluorescence and flow cytometry experiments of fibronectin. Production of fibronectin in TSFL and TSJH fibroblast cultures was also higher nearly 50% and 30% respectively in comparison to normal JPa and DAKO skin fibroblasts.

5.3 Expression and Distribution of Tenascin by Skin Fibroblasts from Patients with Tuberous Sclerosis and Normal Individuals

5.3.1 Immunoflourescence Microscopic Localisation of Tenascin

In this thesis, the expression of another extracellular matrix glycoprotein, tenascin, was studied in two normal human skin fibroblast cell lines (DAKO, JPa), and in four TS skin fibroblast cell lines (TSFL, TSFM, TSJH and TSAH) established from tissue explants as detailed in section 2.3.1.1.

By means of immunofluorescence, the distribution of tenascin was examined in the TS and normal fibroblast cell lines using polyclonal anti-tenascin. *Figures 5.8*, *5.9*, and *5.10* show the results of the indirect immunofluorescence studies of these established cell lines. The method used was as before, described in section 2.3.10.

Immunofluorescence experiments showed that tenascin was visible both at the cell surface and in intracellular granular structures. The cell surface distribution of tenascin in TSFM-fibroblast derived from ungual fibroma of a TS patient was uneven, and appeared more dense than in normal JPa-fibroblasts (*Fig. 5.8*). The fluorescence intensity of the intracellular tenascin staining was uneven and perinuclear in both TS and normal skin fibroblasts (*Fig.5.9*).

The presence of most of the tenascin was observed mainly in the extracellular matrix of both TS and normal skin fibroblasts, rather than at cell surface or within the cells. In confluent cultures from normal and TS skin fibroblasts, tenascin forms a meshwork of fibrils. These meshwork occurred in large (diffuse) patches in TSFM-fibroblast derived from ungual fibroma of a TS patient, while they were more ordered (linear) fibrillar structures in normal JPa-fibroblasts (*Fig. 5.10*).

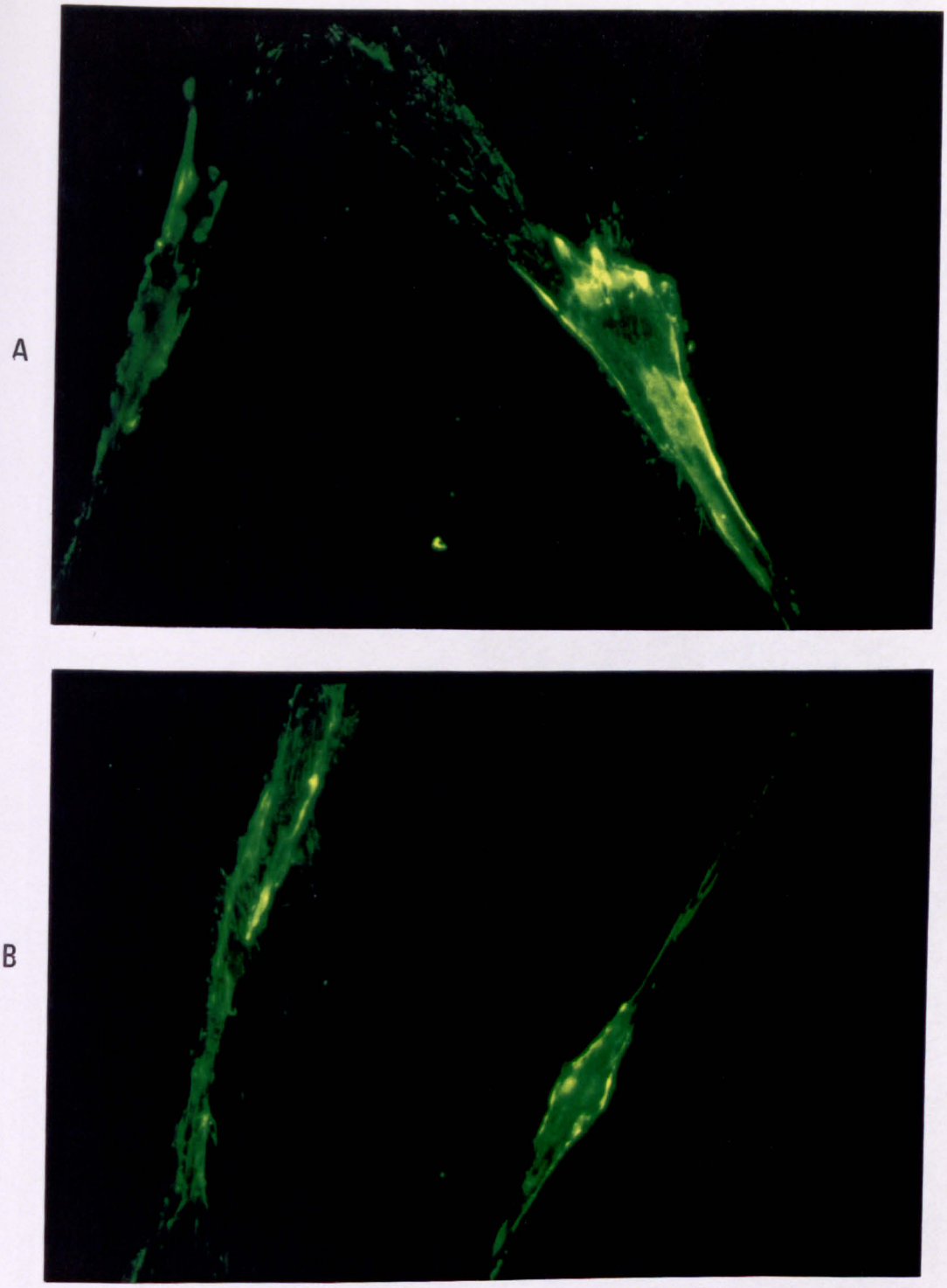
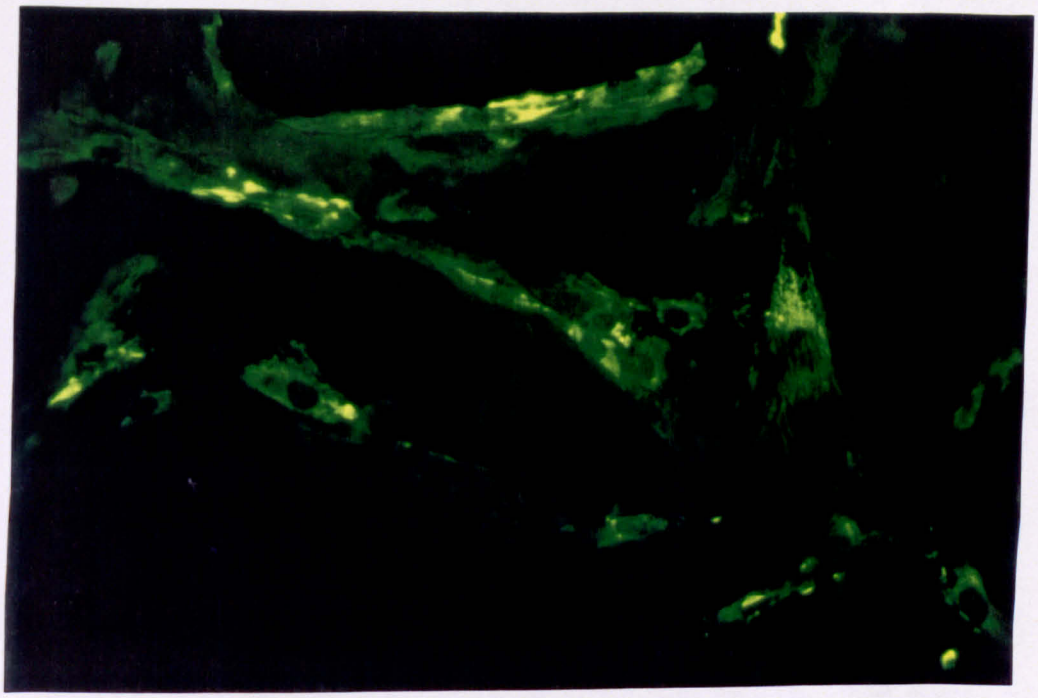


Figure 5.8. Indirect immunofluorescence localisation of cell-surface tenascin on TSFM-fibroblast derived from ungual fibroma of a TS patient (a), and JPa-fibroblasts derived from normal skin (b). Fibroblasts were fixed in formaldehyde and stained with a polyclonal rabbit anti-human tenascin antibody followed by a secondary antibody coupled to FITC. Photographs were taken at 40X magnification.

A



B

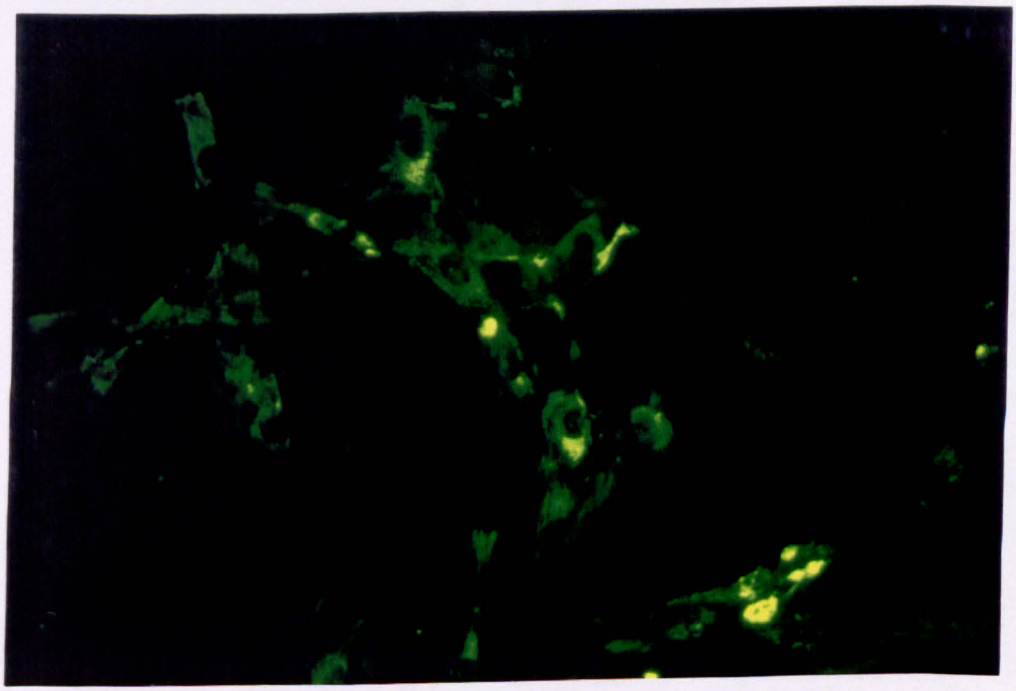


Figure 5.9. Indirect immunofluorescence localisation of intracellular tenascin on TSFM-fibroblasts derived from an ungual fibroma of a TS patient (a), and JPa-fibroblasts derived from normal skin (b). Both cell lines were fixed in formaldehyde and made permeable with Triton X-100, then stained with a polyclonal rabbit anti-human tenascin antibody followed by a secondary antibody (swine anti-rabbit) coupled to FITC. Photographs were taken at 20X magnification.

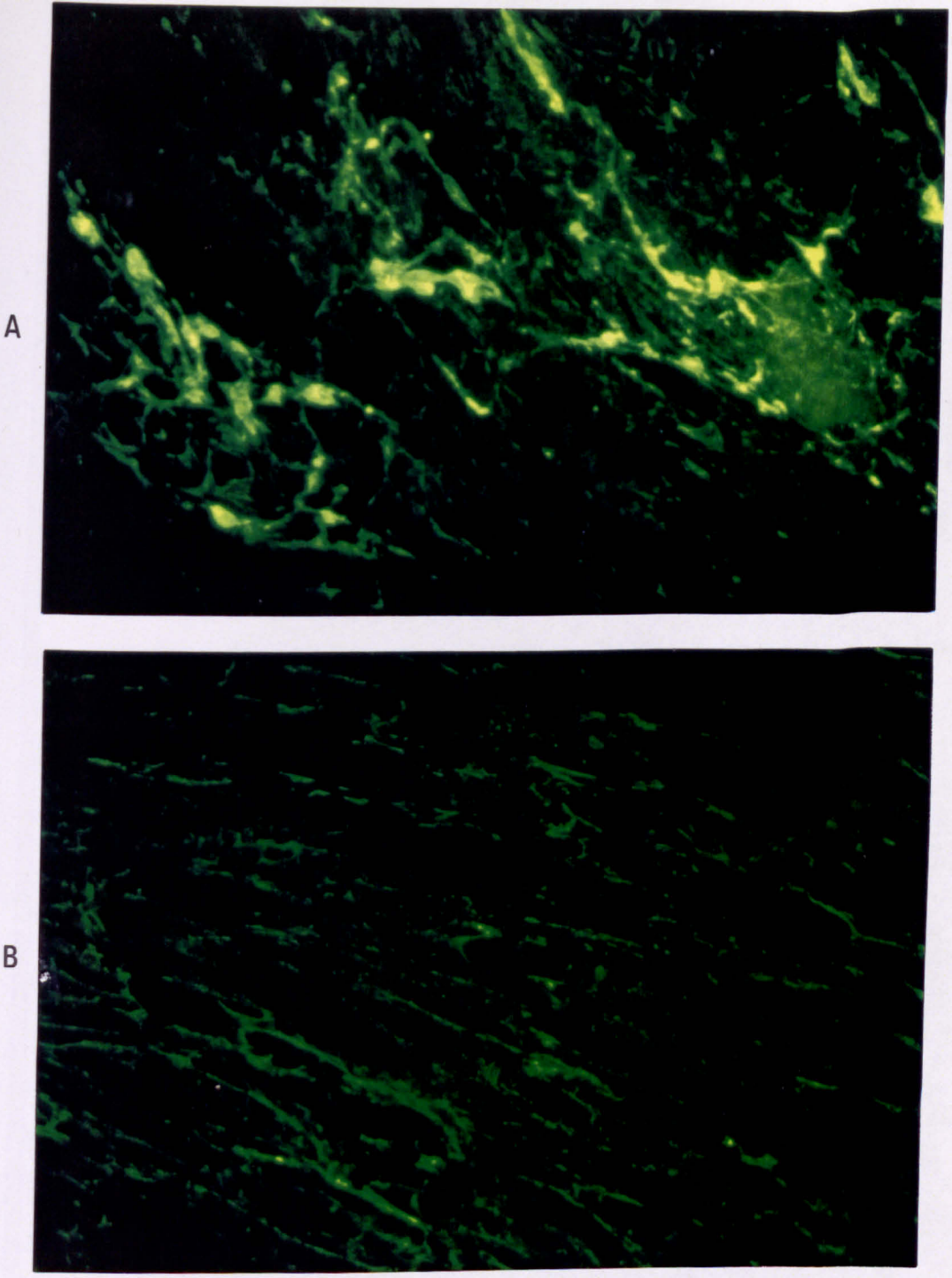


Figure 5.10. Indirect immunofluorescence localisation of tenascin in TSFM-fibroblast derived from ungual fibroma of a TS patient (a), and JPa-fibroblasts derived from normal skin (b). This sort of fibril network is typical of confluent cultures of fibroblasts. Both cell lines were fixed in formaldehyde, and stained with a polyclonal rabbit anti-human tenascin antibody followed by a secondary antibody coupled to FITC. Photographs were taken at 20X magnification.

Interestingly, as for fibronectin immunofluorescence studies showed that the distribution of tenascin was only changed in TSFM ungual fibroma-derived skin fibroblasts of a 10 year old male TS patient, as presented here. However, the distribution of tenascin was not changed in other fibroblasts (TSJH and TSFL) derived from a forehead plaque lesion, and a neck fibroma, of a 14 year old female and 13 year old male respectively in comparison to control fibroblasts. Unaffected skin fibroblasts (TSAH) derived from skin of the lumbosacral area of a TS patient also showed similar distribution and fluorescence intensity for tenascin in comparison to normal (control) skin fibroblasts (data not shown).

As a negative control, primary antibody (anti-tenascin) staining was omitted, and fibroblasts were incubated with a fluoresceinated secondary swine anti-rabbit antibody (at 1:30 dilution). There was no staining of secondary antibody in these negative control fibroblasts as expected (see *Fig. 5.4*).

5.3.2 Quantitative Analysis of Tenascin Expressed by TS and Normal Skin Fibroblasts

5.3.2.1 Flow Cytometric Analysis of Tenascin

In addition to immunofluorescence studies of tenascin, quantitative analysis of tenascin expression was performed using a Becton Dickinson FACScan in the Department of Immunology. 2000 events were collected using consort 30 program and standard fibroblasts settings. A logical fibroblasts gate was set before data analysis. FlowMATE (Dako) and Consort 30 (Becton Dickinson) programs as PC and FACScan software respectively were used to analyse the mean fluorescence intensity of dual labelled cells for tenascin.

The fluorescent intensity of tenascin was measured and compared on TS and normal skin fibroblasts as for fibronectin. In the example shown in *Figure 5.11* (A) forward (FSC) versus side scatter (SSC) light measurements (cytogram), and (B) the fluorescence of fibroblasts, is displayed on the gated

histogram. Using specific gating procedures in the computer program, a box was drawn around the fibroblast populations. This gate or window is used by the computer to select cells for accumulation of events into the fluorescence histogram.

TSFL (neck fibroma), TSFM (ungual fibroma) and JPa (normal skin) fibroblasts were subjected to the flow cytometric analysis of tenascin. An adapted flow cytometry protocol was described in detail in section 2.3.7.3. Quantitatively, the mean fluorescence intensity of 2000 TS (TSFM and TSFL) and normal skin fibroblast (JPa) populations for tenascin was measured using a Becton Dickinson FACScan.

The flow cytometric data of fibroblasts collected in Consort 30 program was gated using FlowMATE program (*Fig. 5.11*), and the mean (fluorescein intensity) was obtained quantitatively. An average mean of four analysis was used and shown graphically in *Figure 5.12*.

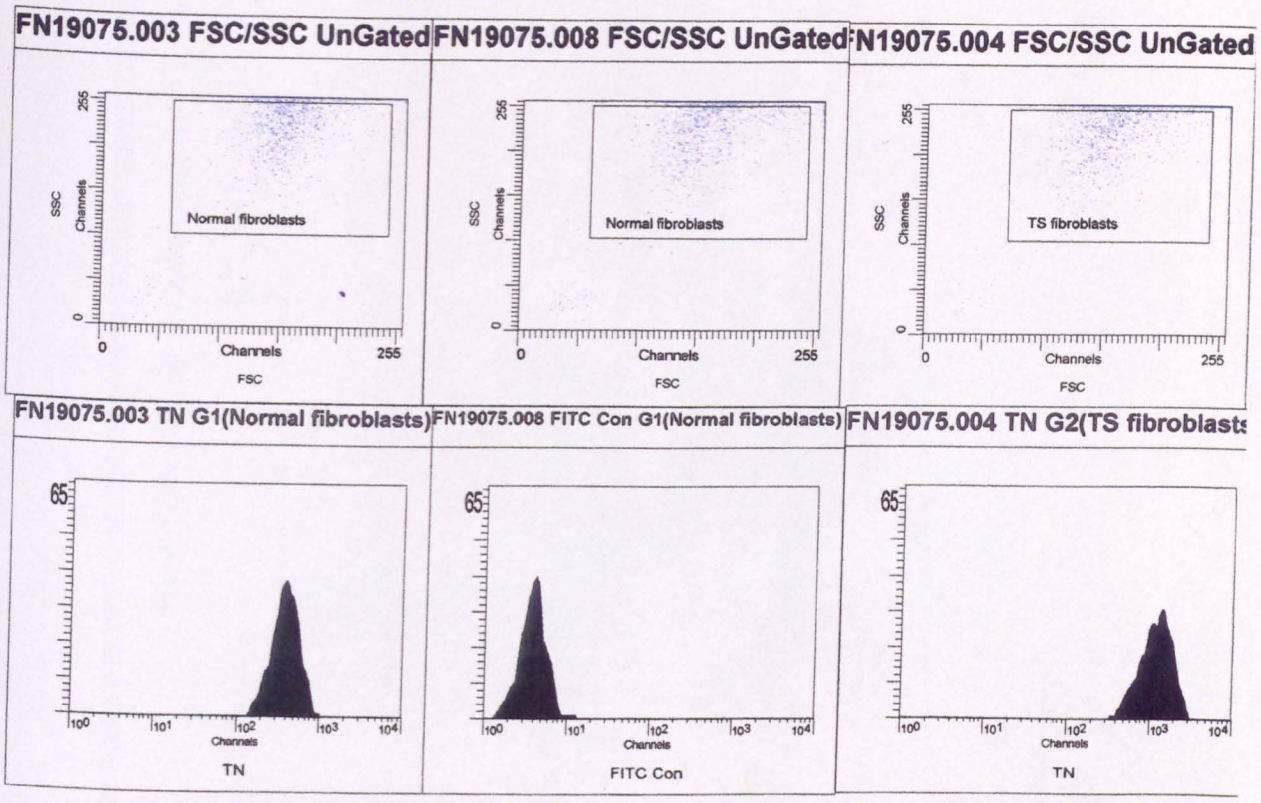


Figure 5.11. The figure shows the typical cytograms and histograms of flow cytometric analysis of tenascin (TN). Panel A shows the forward (FSC) versus side scatter (SSC) cytograms (dotplots of tenascin expression) of TS and normal skin fibroblasts. Panel B shows the fluorescence histograms of TS (TSFM) and normal skin fibroblasts (JPa). A box is drawn around the fibroblast cluster and used as a gate so that the fluorescence intensity of only the cells falling within this gate are displayed in the immunofluorescence profile shown in Panel B. For comparison, see panel B-1 and B-3 which represents fluorescence intensity of tenascin of JPa and TSFM fibroblasts respectively. Panel B-2 represents only FITC-conjugated secondary (anti-rabbit) antibody staining of normal JPa-fibroblasts (omitting primary anti-TN) as negative control staining.

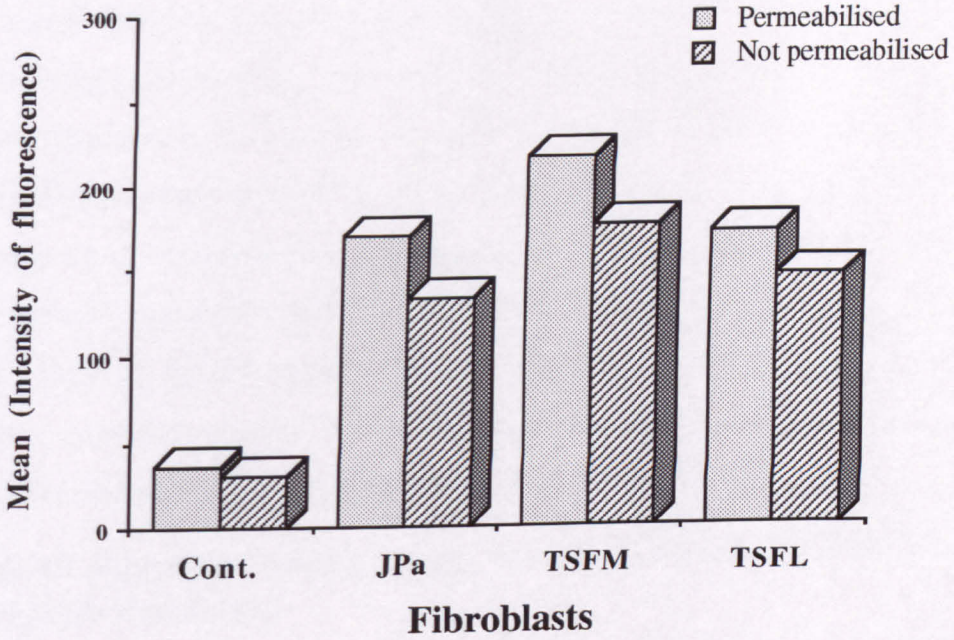


Figure 5.12. Quantitative analysis of tenascin expressed by normal skin (JPa), and TS fibroblasts (TSFM, ungual fibroma; TSFL, neck fibroma) by flow cytometry. Cells were trypsinised, fixed, and permeabilised for cytoplasmic staining or left intact for cell surface staining of tenascin as described in detail in section 2.3.7.3. JPa, TSFM and TSFL fibroblasts were stained using primary polyclonal rabbit anti-human tenascin antibody and FITC-conjugated secondary (anti-rabbit) antibody. In control (cont.) normal skin fibroblasts primary antibody was omitted, and cellls were stained only with FITC-conjugated secondary (swine anti-rabbit) antibody as a negative control. 2000 events from each fibroblast line were analysed by flow cytometry. An average mean (fluorescence intensity) of four different flow cytometric analysis samples was plotted in this bar graph. Variations in mean within four different analyses of tenascin were always within ± 5%.

Interestingly, similar to fibronectin expression, *Figure 5.12* show that TSFM ungual fibroma-derived skin fibroblasts have a higher fluorescence intensity (mean) for tenascin than TSFL neck fibroma-derived and JPa normal skin fibroblasts. Increased expression of tenascin was observed in both the cytoplasm (permeabilised) and cell surface (not-permeabilised) of TSFM ungual fibroma-derived skin fibroblasts in contrast to TSFL and JPa fibroblasts. Earlier, immunofluorescence studies showed that the distribution and expression of tenascin was altered only in TSFM ungual fibroma-derived skin fibroblasts of 10 year old male TS patient. The distribution and expression of tenascin was not changed in other fibroblasts derived from a forehead plaque lesion and neck fibroma of a 14 year old female and 10 year old male TS patients respectively in comparison to control fibroblasts. Here, flow cytometry results confirmed quantitatively, and strengthened these earlier findings of immunofluorescence studies of tenascin.

5.3.2.2 Enzyme-Linked Immunosorbent Assay (ELISA) for Fibroblasts Tenascin Production

Because of difficulties in obtaining standard tenascin commercially, ELISAs were performed to measure the absorbance of the tenascin secreted into the cell culture medium by TS and normal skin fibroblasts. As the amount of chromophore produced in ELISA is directly proportional to the original quantity of protein, absorbances of tenascin detected in conditioned medium would allow comparison of tenascin expression in both TS and normal skin fibroblasts. ELISA procedure for tenascin production was performed using anti-tenascin exactly as for fibronectin apart from using standard dose-response curves, (described in 5.2.2.2). ELISA absorbances at 405 nm were used to compare production of tenascin in TSFL, TSJH, TSFM and normal JPa and DAKO skin fibroblasts.

ELISA absorbances of tenascin produced into the cell culture medium by three TS, and two normal skin fibroblasts is presented in *Table 5.2.*

Fibroblasts	Passage	Secreted Tenascin* by 1 X 10^5 cell absorbances/200µl medium ± SD
TSFL	12-15	1.194 ± 0.014
TSFM	12-15	1.429 ± 0.026
TSJH	12-15	1.169 ± 0.015
JPa	12-15	0.972 ± 0.020
DAKO	12-15	0.931 ± 0.040

Table 5.2. Absorbances of tenascin produced into cell culture medium by TS (TSFL, neck fibroma, TSFM, ungual fibroma and TSJH, forehead plaque) and normal skin fibroblasts (JPa and DAKO) detected by ELISA. *Tenascin production was assessed by measuring the absorbance of tenascin in fibroblast culture medium using ELISA and is expressed as absorbance/200µl medium of $1X10^5$ cells ± standard deviation (SD) of triplicate determinations. Absorbances of control culture medium (DMEM with 10% FCS) which had been preincubated for 3 days along with the cells were subtracted from the data.

The most significant increased absorbances of tenascin production were seen in TSFM fibroblasts derived from ungual fibroma of a TS patient in comparison to normal skin fibroblasts (JPa and DAKO) and TSFL and TSJH fibroblasts derived from neck fibroma and forehead plaque lesions of TS patients respectively. As the amount of chromophore produced in ELISA is directly proportional to the original quantity of protein, these increased absorbances of tenascin (in TSFM fibroblasts reflect the elevated production of tenascin in TSFM fibroblasts. This increased tenascin production into cell cuture medium by TSFM fibroblasts (~50% of controls) also agrees with previous findings of immunofluorescence and flow cytometry experiments on tenascin. TSFL and TSJH fibroblast cultures was also showed higher absorbances (~23% of controls) in comparison to normal JPa and DAKO skin fibroblasts.

5.4 Expression and Distribution of Laminin by Skin Fibroblasts from Patients with Tuberous Sclerosis and Normal Individuals

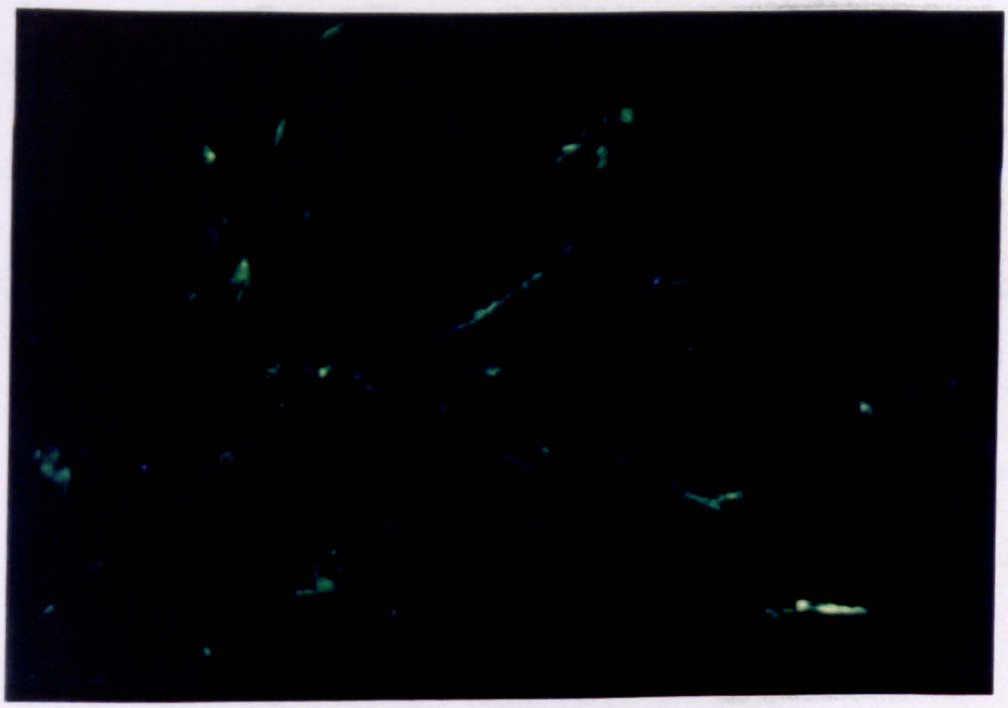
5.4.1 Immunofluorescence Microscopic Localisation of Laminin

In this thesis, the expression of laminin was also investigated in TS and normal skin fibroblasts by immunofluorescence as well as ELISA and flow cytometry techniques. As indicated earlier, the expression of laminin by fibroblasts was particularly important for this research, since there are contrary indications in previous published scientific papers about its expression by fibroblasts (Timpl *et al.*, 1982, Woodley *et al.*, 1988, and Cooper *et al.*, 1993). In chapter 3, the synthesis and secretion of laminin by skin fibroblasts was shown by western blotting.

Two normal human skin fibroblast cell lines (DAKO, JPa), and four TS skin fibroblast cell lines (TSFL, TSFM, TSJH and TSAH) established from tissue explants (see details of these cell lines in section 2.3.1.1) were used to analyse the distribution of laminin by means of immunofluorescence. The immunofluorescence method was performed using monoclonal (mouse) and polyclonal (rabbit) anti-human laminin antibodies followed by a secondary antibody coupled to FITC (see section 2.3.10).

The results of the indirect immunofluorescence localisation of laminin are presented in *Figures 5.13* (cell surface staining), *5.14*, *5.15*, and *5.16* (cytoplasmic staining) on TS and normal cell lines.

A



B

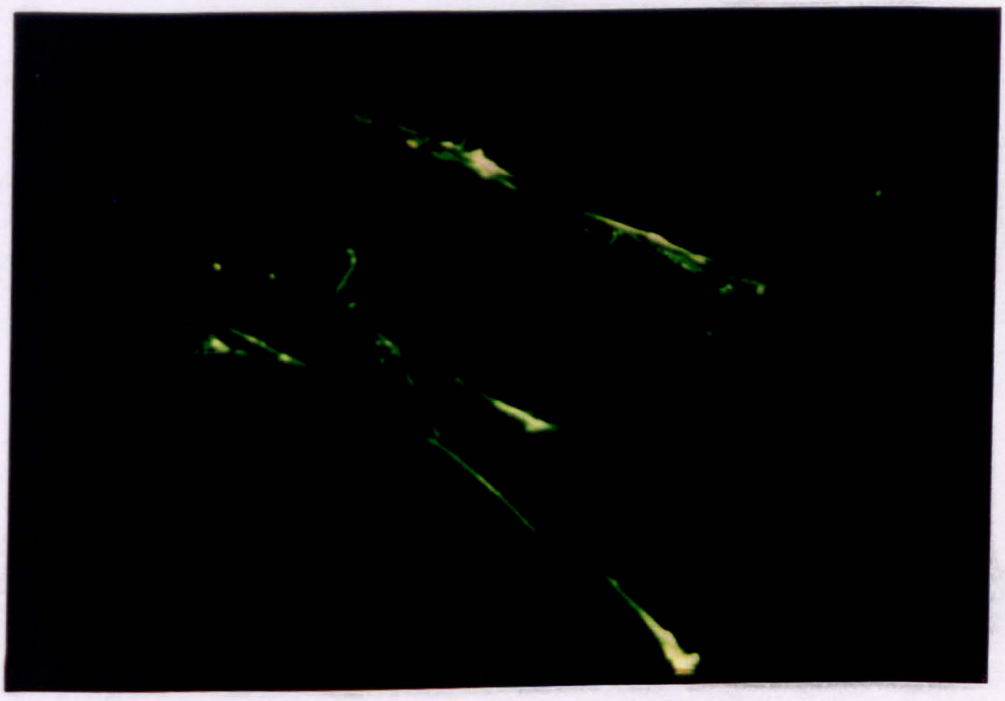
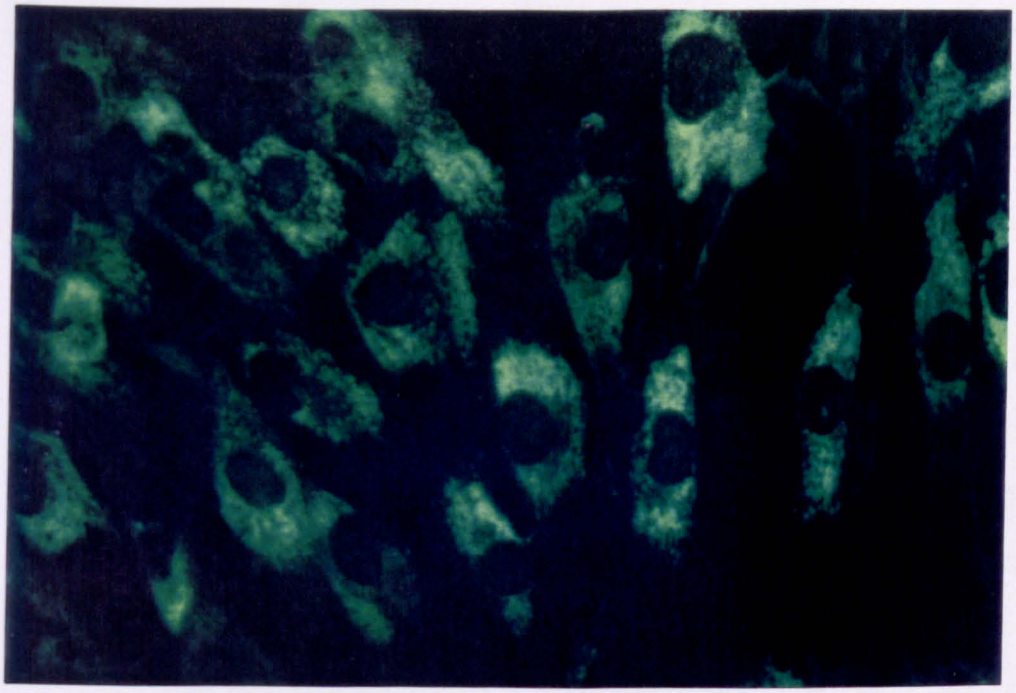


Figure 5.13. Indirect immunofluorescence localisation of laminin. Normal JPa-skin fibroblasts (a), and neck fibroma-derived TSFL-fibroblasts (b) were fixed with formaldehyde, and stained for cell-surface laminin with a polyclonal rabbit anti-human laminin antibody followed by a secondary antibody (swine anti-rabbit) coupled to FITC. Photographs were taken at 40X magnification.

A



B

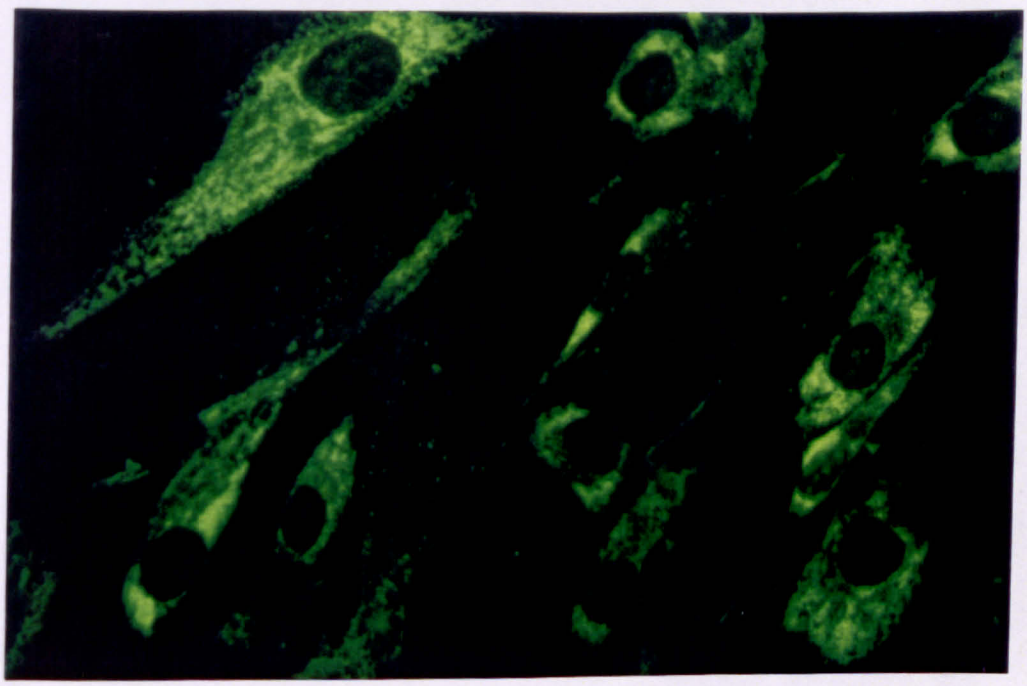


Figure 5.14. Indirect immunofluorescence localisation of intracellular laminin. Normal JPa-skin fibroblasts (a), and neck fibroma-derived TSFL-fibroblasts (b) were fixed with formaldehyde and made permeable with Triton X-100, and stained with a polyclonal rabbit anti-human laminin antibody followed by a secondary antibody (swine anti-rabbit) coupled to FITC. Photographs were taken at 40X magnification.

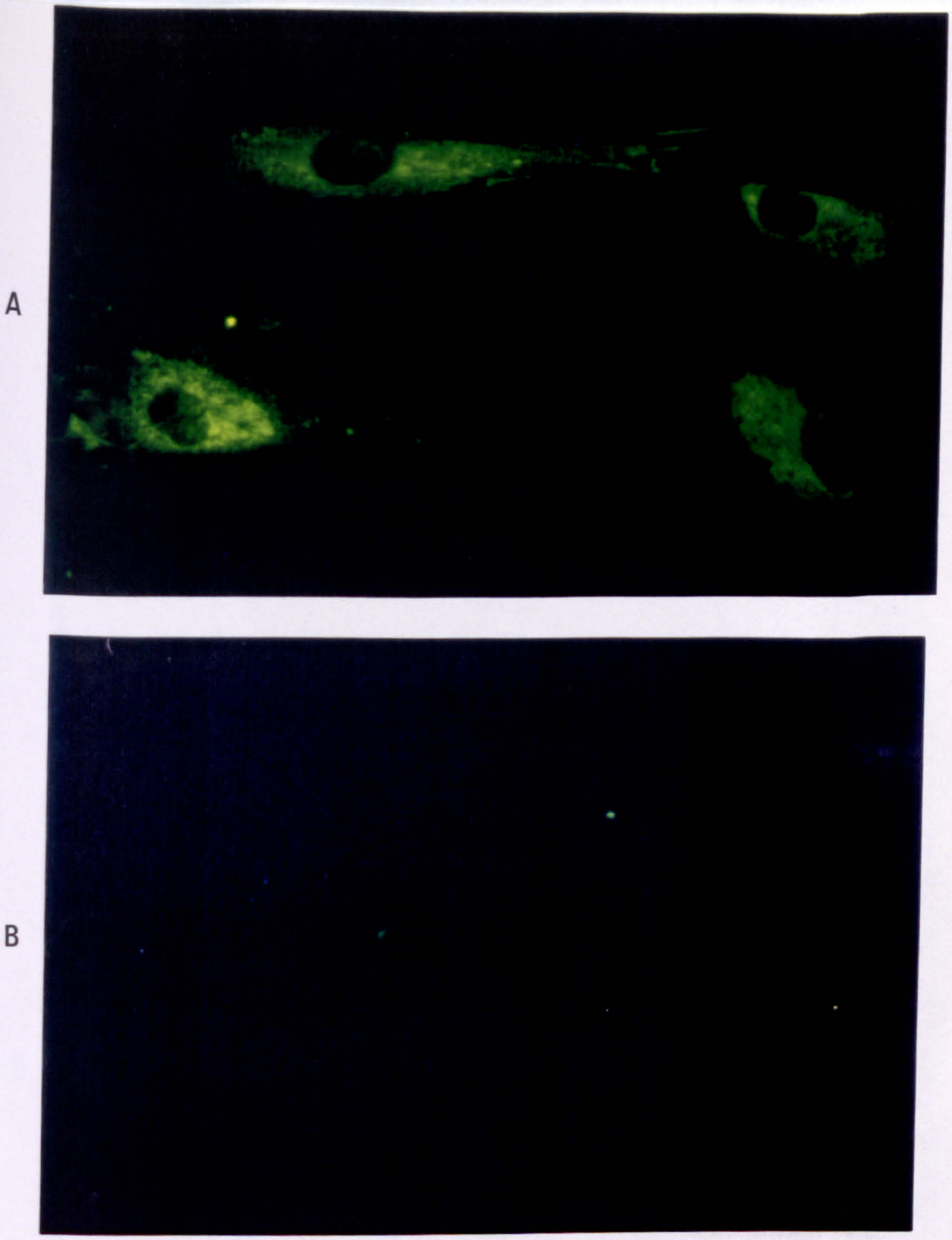
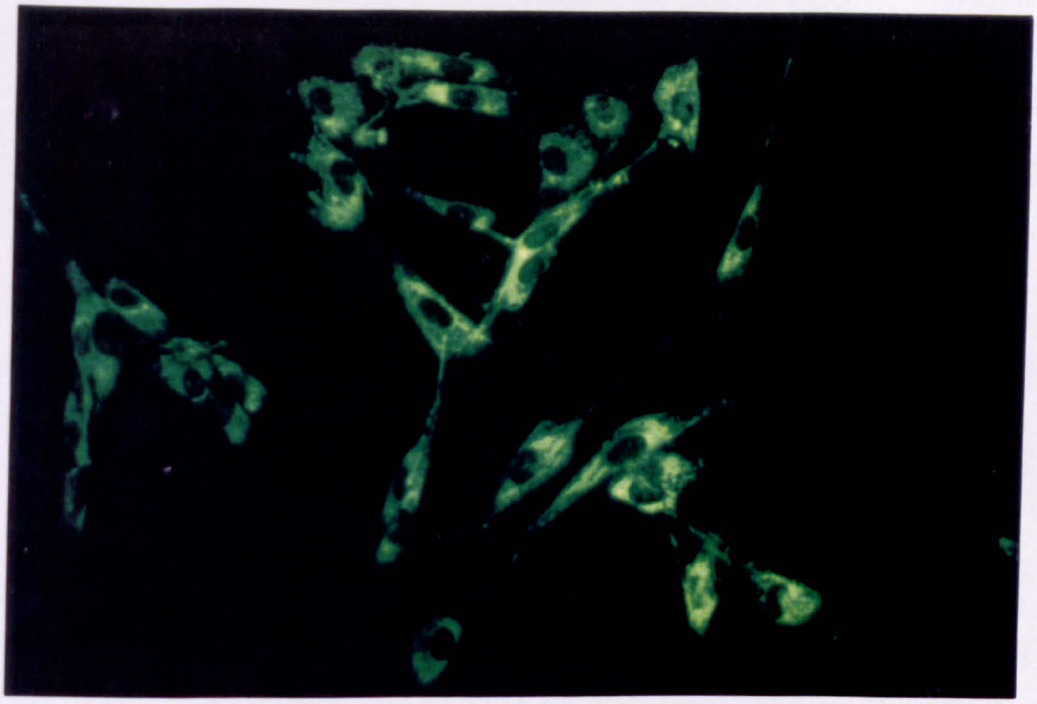


Figure 5.15. Indirect immunofluorescence localisation of intracellular laminin using monoclonal anti-laminin: (a) neck fibroma-derived TSFL-fibroblasts were fixed with formaldehyde and made permeable with Triton X-100, then stained with a monoclonal anti-human laminin (mouse) antibody followed by a secondary antibody (rabbit anti-mouse) coupled to FITC. (b) Negative control, ungual fibroma-derived TSFL-fibroblasts were fixed with formaldehyde and made permeable with Triton X-100, and stained only with a secondary antibody (rabbit anti-mouse) coupled to FITC. Photographs were taken at 40X magnification.

A



B

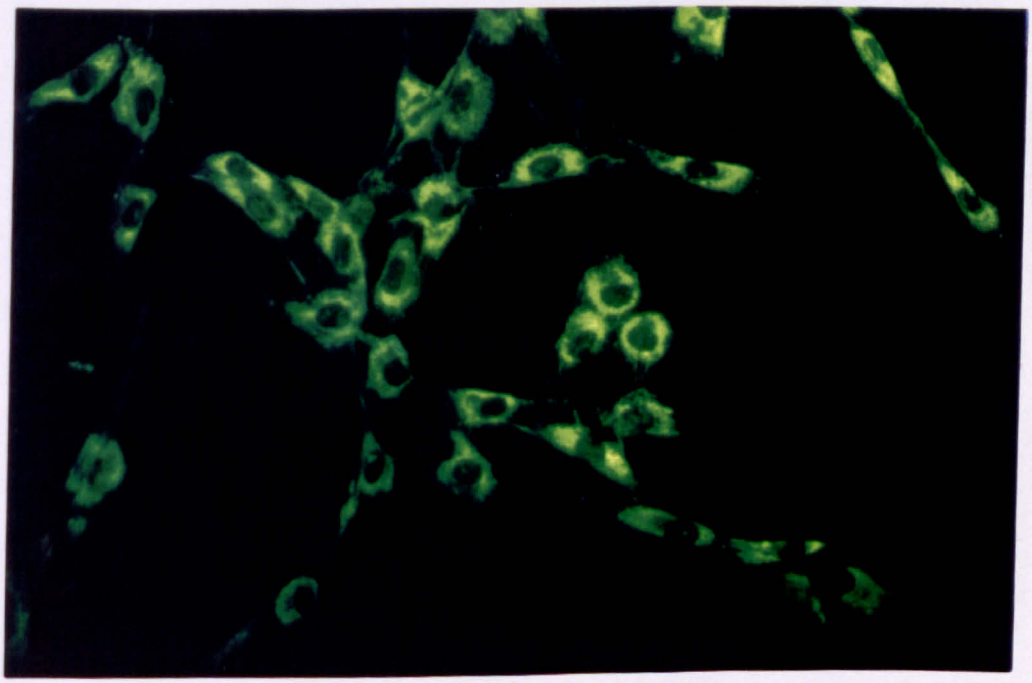


Figure 5.16. Indirect immunofluorescence localisation of intracellular laminin. Normal skin JPa-fibroblasts (a) and normal skin DAKO-fibroblasts (b) were fixed with formaldehyde and made permeable with Triton X-100 then stained with a polyclonal rabbit anti-human laminin antibody followed by a secondary antibody (swine anti-rabbit) coupled to FITC. Photographs were taken at 20X magnification.

Anti-laminin staining seen suggested that laminin is synthesed in detectable amounts by human skin fibroblasts. In *Figures 5.13* and *5.14*, laminin staining of TS fibroblasts derived from a neck fibroma is compared with that of normal skin fibroblasts. The increase in the former is apparent. In normal JPa skin fibroblasts, cell surface staining of laminin is not uniform, and laminin is hardly detectable in comparison to TSFL neck fibroma derived fibroblasts. In TSFL fibroblasts in particular, the laminin staining appears to be concentrated to produce intense patches. Intracellular laminin staining is bright, and diffuse in the cytoplasm of large cells derived from a neck fibroma of a TS patient, but this was usually seen around nuclei only in normal skin fibroblasts (*Fig. 5.16*).

Figure 5.15 confirmed more specifically the laminin synthesis by TS fibroblasts using monoclonal mouse anti-laminin as no staining occurred in control staining. Immunofluorescence stainings of two different normal fibroblasts (JPa and DAKO) derived from skin tissue of 8 year old male and 12 year old female showed similar perinuclear stainings of laminin (*Fig. 5.16*).

Interestingly, altered distribution of laminin was observed at the cell surface and in the cytoplasm of, particularly in large cells, TSFL-derived fibroblasts from neck fibroma of a 13 year old male. However, changes of fibronectin and tenascin staining were observed in TSFM-fibroblasts derived from an ungual fibroma of a 10 year old male TS patient, while TSFL-fibroblasts showed normal fibronectin and tenascin distribution. This suggested a different nature of extracellular matrix appears in neck and ungual fibromas of TS patients . The other TSFM, TSJH, and TSAH fibroblasts derived from an ungual fibroma, a forehead plaque and unaffacted skin of TS patients respectively showed normal and similar distribution of laminin in contrast to TSFL-fibroblasts. As a negative control, primary antibody (anti-tenascin) staining was omitted, and fibroblasts were incubated with a fluoresceinated

secondary swine anti-rabbit antibody (at 1:30 dilution). There was no staining of secondary antibody in these negative control fibroblasts as expected (see *Fig. 5.4*).

5.4.2 Quantitative Analysis of Laminin Expressed by TS and Normal Skin Fibroblasts

5.4.2.1 Flow Cytometric Analysis of Laminin

In addition to immunofluorescence studies of laminin, quantitative analysis of laminin expression was also performed using a Becton Dickinson FACScan as described in section 5.2.2.1 for fibronectin analyses.

The fluorescent intensity of laminin on the cytoplasma and cell surface of TS and normal skin fibroblasts was measured and compared as for fibronectin and tenascin. In the example shown in *Figure 5.17* (A) forward (FSC) versus side scatter (SSC) light measurements (cytogram), and (B) the fluorescence of fibroblasts is displayed on the gated histogram. Using specific gating procedures in the computer program, a box was drawn around the fibroblast populations. This gate or window is used by the computer to select cells for accumulation of events into the fluorescence histogram.

TSFL (neck fibroma), TSFM (ungual fibroma) and JPa (normal skin) fibroblasts were subjected to the flow cytometric analysis of laminin. An adapted flow cytometry protocol was described in detail in section 2.3.7.3. Quantitatively, the fluorescence intensity of 2000 TS (TSFM and TSFL) and normal skin fibroblast (JPa) populations for laminin was measured as a mean using a Becton Dickinson FACScan in the Department of Immunology. The flow cytometric data of fibroblasts collected in Consort 30 program was gated using FlowMATE program (*Fig. 5.17*), and the mean (fluorescein intensity) was obtained quantitatively. An average mean of four analysis was used and shown graphically in *Figure 5.18*.

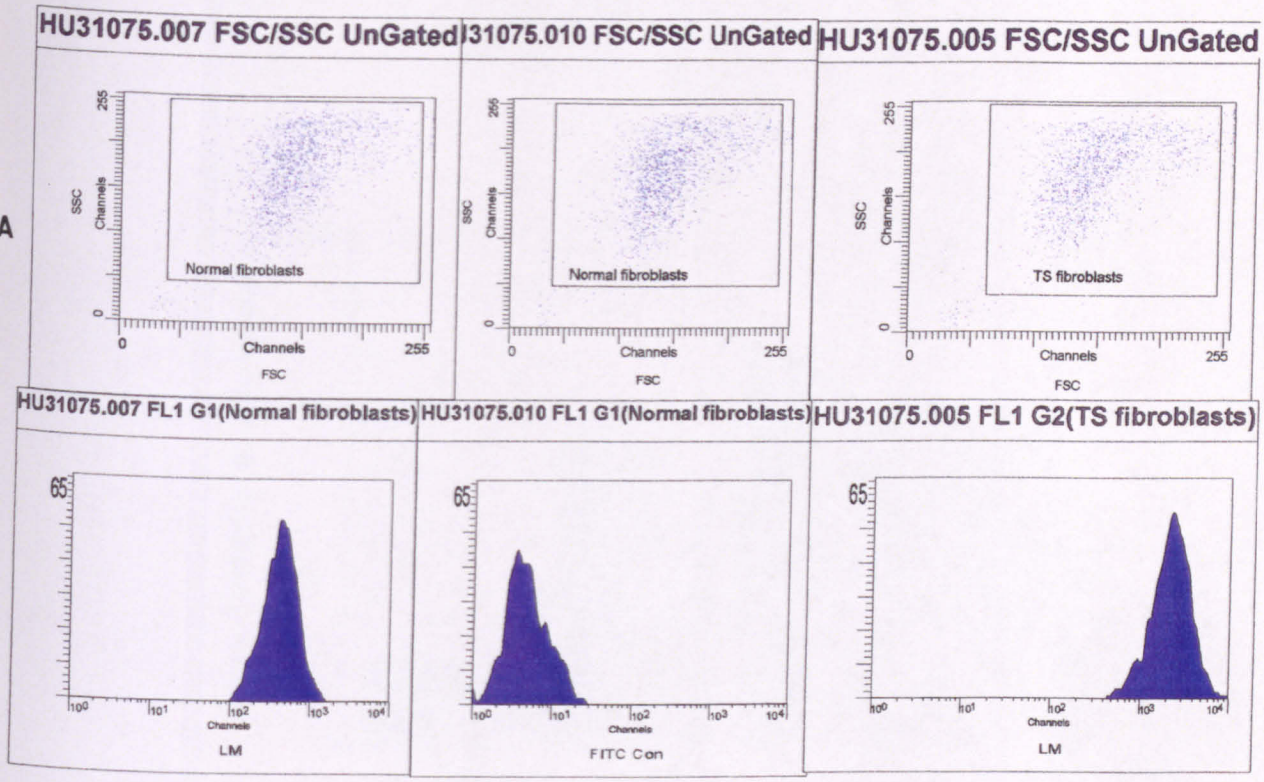


Figure 5.17. The figure shows the typical cytograms and histograms of flow cytometric analysis of laminin. Panel A shows the forward (FSC) versus side scatter (SSC) cytograms (dotplots of laminin expression) of TS and normal skin fibroblasts. Panel B shows the fluorescence histograms of TS (TSFL) and normal skin fibroblasts (JPa). A box is drawn around the fibroblast cluster and used as a gate so that the fluorescence intensity of only the cells falling within this gate are displayed in the immunofluorescence profile shown in Panel B. For comparison, see panel B-1 and B-3 which represents the fluorescence intensity of laminin of JPa and TSFL fibroblasts respectively. Panel B-2 represents only FITC-conjugated secondary (anti-rabbit) antibody staining of normal JPa-fibroblasts (omitting primary anti-LM) as negative control staining.

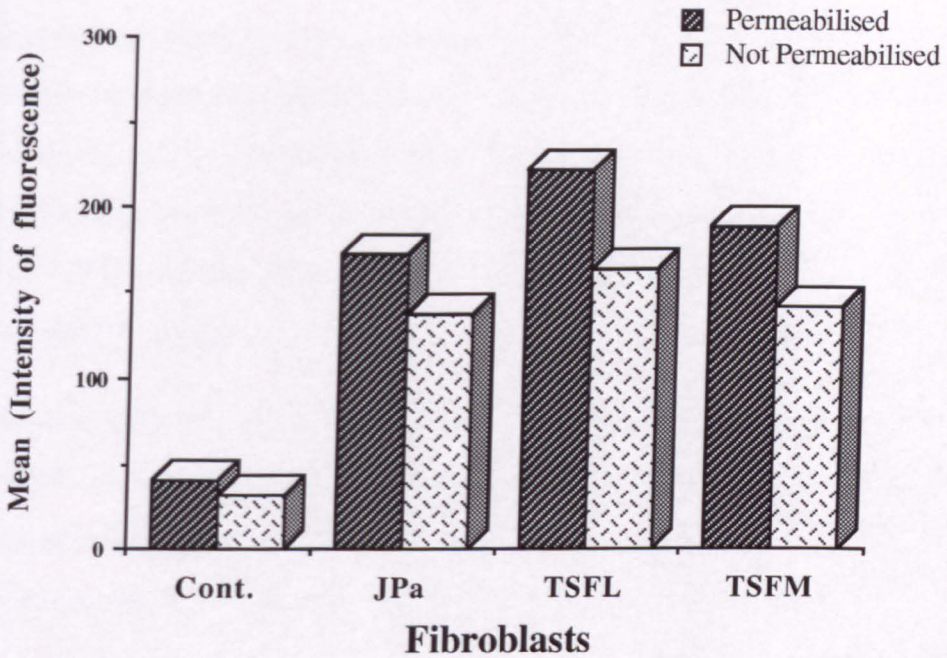


Figure 5.18. Quantitative analysis of laminin expressed by normal skin (JPa), and TS fibroblasts (TSFM, ungual fibroma; TSFL neck fibroma) by flow cytometry. Cells were trypsinised, fixed and permeabilised for cytoplasmic staining or not permeabilised for cell surface staining of fibronectin as described in detail in section 2.3.7.3. JPa, TSFM. and TSFL fibroblats were stained using primary polyclonal rabbit anti human laminin antibody and FITC-conjugated secondary (anti-rabbit) antibody. In control (cont.) normal skin fibroblats primary antibody, anti-laminin were omitted, and stained only with FITC-conjugated secondary (swine anti-rabbit) antibody as negative control. 2000 events from each fibroblast line were analysed by flow cytometry. An average mean (fluorescence intensity) of four different flow cytometric analysis samples was plotted in this bar graph. Variations in mean within four different analysis of laminin were always within ± 5%.

In contrast to fibronectin and tenascin expression, *Figure 5.18* showed that TSFL neck fibroma-derived skin fibroblasts have a higher fluorescence intensity (mean) for laminin than TSFM ungual fibroma-derived and JPa normal skin fibroblasts. Increased expression of laminin was observed in both cytoplasm (permeabilised) and cell surface (not-permeabilised) of TSFL neck fibroma-derived fibroblasts in contrast to TSFM and normal JPa skin fibroblasts. Earlier, immunofluorescence studies have shown that the distribution and expression of laminin was only changed in TSFL neck fibroma-derived skin fibroblasts of 13 year old male TS patient. However, the distribution and expression of laminin was not changed in other fibroblasts derived from a forehead plaque lesion and ungual fibroma of a 14 year old female and 10 year old male TS patient respectively in comparison to control fibroblasts.

Overall, these results suggest that the nature of the extracellular matrix (ECM) in the neck fibroma derived fibroblasts in terms of increased expression of laminin is different than ungual fibroma derived fibroblasts which posses the increased expression of fibronectin and tenascin.

5.4.2.2 Enzyme-Linked Immunosorbent Assay (ELISA) for Fibroblasts Laminin Production

As for fibronectin, ELISAs were performed to quantitate the amount of laminin secreted into the cell culture medium by TS and normal skin fibroblasts. ELISA assay for laminin production was performed as described previously in 5.2.2.2.

ELISA assay results of laminin production into the cell culture medium by three TS (TSFL, TSFM and TSJH), and two normal skin fibroblasts (JPa and DAKO) is presented in *Table 5.3*.

Fibroblasts	Passage	Secreted Laminin* by 1 X 10^5 cell ng/200µl medium ± SD
TSFL	12-15	13.58 ± 0.20
TSFM	12-15	3.16 ± 0.07
JPa	12-15	3.46 ± 0.29
TSJH	12-15	4.27 ± 0.05
DAKO	12-15	3.78 ± 0.28

Table 5.3. Production of laminin by TS (TSFL, neck fibroma, TSFM, ungual fibroma and TSJH, forehead plaque) and normal skin fibroblasts (JPa and DAKO) detected quantitatively by ELISA. *Laminin production was assessed by measuring the quantity of laminin in fibroblast culture medium using ELISA and is expressed as ng/200µl medium of $1X10^5$ cells ± standard deviation (SD) of triplicate determinations.

ELISA assay results of laminin in cell culture medium presented in *Table 5.3* showed the four fold increased production of laminin in TSFL fibroblasts derived from neck fibroma of a TS patient in comparison to normal skin fibroblasts (JPa and DAKO) and other two TSFM and TSJH fibroblasts derived from ungual fibroma and forehead plaque lesions of TS patients respectively. This increased production of laminin by TSFL fibroblast was also in agreement with the previous results of flow cytometry and immunofluorescence experiments. Laminin levels were not changed consistently in other two TS (TSFM and TSJH) and normal (JPa and DAKO) skin fibroblasts.

5.5 Discussion

In this chapter 5, expression and distribution of fibronectin, tenascin and laminin by established TS and normal skin fibroblasts using immunofluorescence, ELISA, and flow cytometry techniques were analysed and presented qualitative and quantitatively.

Indirect immunofluorescence studies revealed that the distribution of fibronectin compared to controls was only altered in TSFM ungual fibroma-derived skin fibroblasts of the 10 year old male TS patient. However, the distribution of fibronectin was not changed in other fibroblasts derived from a forehead plaque lesion and neck fibroma of a 14 year old female and 10 year old male TS patient respectively in comparison to control normal skin fibroblasts.

The intensity of the fluorescence of cell surface fibronectin was increased and showed uneven distribution in the TSFM-fibroblasts derived from ungual fibroma of a TS patient in comparison to normal JPa-fibroblasts. Intracellular staining of ungual fibroma derived TSFM-fibroblasts with anti-fibronectin was also elevated in comparison to normal (control) skin fibroblasts. In addition, the fluorescence intensity was evenly distributed and predominantly perinuclear in both TS and normal skin fibroblasts. However, the intracellular fluorescence was patchy and also predominantly perinuclear in large cells (N-cells) of TS fibroblasts.

In agreement with the literature (Hynes 1990), a cell surface associated fibrillar matrix of fibronectin was also observed in normal skin fibroblasts. However, the fibrillar arrays were better established in the ungual fibroma derived TSFM fibroblasts.

ELISA and flow cytometric analyses of fibronectin also showed increased quantities and increased fluorescence intensity (mean) respectively of

fibronectin in the TSFM-fibroblasts derived from an ungual fibroma of a TS patient in comparison to normal skin fibroblasts. Therefore, quantitative analysis of both TS and normal fibroblasts determined by flow cytometry and ELISA assays were confirmed and strengthened the increased expression of fibronectin in the TSFM-fibroblasts derived from ungual fibroma of a TS patient in comparison to normal skin fibroblasts, following earlier findings of immunofluorescence studies.

Similar to fibronectin, a meshwork of fibrils of tenascin occurred in large (diffuse) patches in TSFM-fibroblast derived from ungual fibroma of a TS patient, while they were more ordered fibrillar structures in normal JPa-fibroblasts.

Increased expression of tenascin was observed in both cytoplasmic and cell surface immunofluorescence staining of TSFM ungual fibroma-derived skin fibroblasts in contrast to other TS and normal skin fibroblasts. Flow cytometry and ELISA results confirmed quantitatively and strenghened these earlier findings of immunofluorescence studies of tenascin.

Interestingly, increased expression of tenascin was observed in a similar direction with fibronectin in TSFM fibroblasts derived from an ungual fibroma of a TS patient. This increase in TSFM fibroblasts may be the response of functions (adhesive and antiadhesive effects) of these two extracellular matrix glycoproteins to control the cell adhesion and spreading in ungual fibromas of TS patients, since it has been reported that tenascin inhibits cell adhesion to fibronectin (Chiquet-Ehrismann *et al.*, 1988).

Immunofluorescence studies showed that the distribution of laminin was altered in TSFL neck fibroma-derived fibroblasts, but not in ungual fibroma (TSFM), forehead plaque lesion (TSJH) or unaffacted skin (TSAH) derived fibroblasts of TS patients in comparison to control fibroblasts. Particularly. large cells of neck fibroma-derived TSFL fibroblasts populations showed

bright and diffuse staining of laminin in the cytoplasm, however laminin staining was usually observed around the nucleus (perinuclear) in normal (control) skin fibroblasts.

In contrast to increased expression of fibronectin and tenascin in ungual fibroma derived TSFM fibroblasts, quantitative analyses of laminin by flow cytometry and ELISA assay showed the increased expression of laminin only in neck fibroma-derived TSFL fibroblasts. Laminin expression was not changed in ungual fibroma-derived TSFM fibroblasts and remained at similar levels to normal skin fibroblasts. These results suggested the presence of altered composition of extracellular matrix in neck and ungual fibromas as well as forehead plaque lesions of TS patients.

Moreover, detection of laminin using monoclonal and polyclonal antibodies in immunofluorescence and flow cytometry studies of cells and in ELISA of cell culture medium also demonstrated once more the synthesis and secretion of this protein by human skin fibroblasts. However, laminin synthesis and secretion into cell culture medium by TS and normal skin fibroblast appeared to be less (three fold) than fibronectin synthesis as a general tendency.

So far, there have been few studies that examine the sythesis of laminin by human dermal fibroblasts. Since there are contrary indications in these previous published papers about laminin expression by dermal fibroblasts (Timpl *et al.*, 1982; Woodley *et al*, 1988; Cooper *et al.*, 1993), these results reported in this thesis may also be important in elucidating the question of whether or not human dermal fibroblasts synthesise laminin as well as in understanding the pathogenesis of TS tissues.

Chapter 6

Chapter 6

General Discussion and Conclusions

In this project, studies were carried out

i) *to characterise the amino acid composition and compare the glycosylation of purified cellular fibronectin from the conditioned medium of cultured skin fibroblasts derived from patients with tuberous sclerosis (TS) and from normal individuals, and*

ii) *to establish the expression, distribution, and localisation of extracellular matrix (ECM) glycoproteins fibronectin, laminin and tenascin in cultures of skin fibroblasts derived from patients with TS and normal individuals.*

6.1 Studies on purification, carbohydrate and peptide analysis of fibronectin from the conditioned medium of fibroblast cultures established from skin explants of patients with TS and normal individuals

The macromolecules that constitute the extracellular matrix (ECM) are mainly secreted locally by cells in the matrix. In most connective tissues these macromolecules are secreted by fibroblasts. Fibroblasts are the principal cells of the dermis and responsible for the production and maintenance of the connective tissue matrix. Therefore, cultured fibroblasts were used in this research of skin lesions of TS patients to study the production of the adhesive glycoproteins of the ECM.

Fibroblasts were established from primary cultures of skin explants of patients with tuberous sclerosis. In some cases this was from skin lesions and in other cases from unaffected skin. Control fibroblasts, for comparison, have been grown from explants of people who are not known to be suffering from TS nor any other disease or disorder

Fibroblasts secrete much of the fibronectin they synthesise into the culture medium in preference to retaining it on the cell surface. Therefore, cellular fibronectin was purified from conditioned medium of confluent fibroblasts by affinity chromatography on a Prosep-gelatin (Bioprocessing) column as a first step purification procedure, by a method established by Engvall and Ruoslahti (1977). However, purified fibronectin by this method was only partially pure. Therefore, final purification of cellular fibronectin was achieved from conditioned medium of established TS and control fibroblasts using gel filtration chromatography for carbohydrate analysis. Foetal calf serum previously depleted of fibronectin was used in the preparation of the medium of cell cultures to ensure that the purified product is pure cellular fibronectin.

In the final purification step of the fibronectin, partially-pure fibronectin from the Prosep-gelatin column was applied on to Superose 12 gel filtration column (sizing column) connected with FPLC. Coomassie blue, silver and antibody staining of the reduced purified cellular fibronectin from TS and normal fibroblasts analysed by 5% SDS-PAGE showed a single broad band with approximate molecular weight of 230 000. The mobility of fibronectin isolated from fibroblasts grown from skin lesions of TS patients was identical to that from fibroblasts of unaffected skin. Typically, the final yield of fibronectin from the Superose-12 column was 70% of that applied from Prosep-gelatin.

Solubility of fibronectin was sometimes a problem at neutral pH after purification. Fibronectin was also sensitive to proteolysis. The dissolution of fibronectin was achieved by using cyclohexylaminopropane sulfonic (CAPS) buffer, pH 11, and protection from proteolysis using PMSF and EDTA. The solution was stored at -80° C in plastic vials for further analysis. The majority of studies of fibronectin in the scientific literature were carried out with plasma fibronectin rather than cellular fibronectin of fibroblast origin. This is because of the higher cost and complexity of cell culture and purification of cellular fibronectin.

Analysis of purified fibronectin samples by high pH anion-exchange chromatography (HPAEC-PAD) revealed that the carbohydrate portion of the fibronectin molecule was made up of galactose, mannose, glucosamine, galactosamine, sialic acid and fucose. Oligosaccharides of TSFC-purified fibronectin from fibroblasts of TS skin lesion and plasma fibronectin appeared basically as mono and disialylated biantenary structures respectively. The major type of side chain in fibronectin glycosylation is of the biantennary complex asparagine-linked type. As expected, fucose was not detected in human plasma fibronectin. In human plasma and fibroblasts derived cellular fibronectin, the most common form of sialic acid was found to be N-acetyl neuraminic acid. Moreover, there was altered monosaccharide composition of fibronectin purified from conditioned medium of fibroblasts grown from skin lesions of different TS patients in comparison to carbohydrates of fibronectin derived from normal fibroblasts. An increased concentration of sugars was observed in sialic acid, galactosamine, glucosamine, galactose and mannose of purified fibronectin from neck and ungual fibromas of patients with TS. The total increase of carbohydrates of TS fibroblasts fibronectin was more than two fold in comparison to normal fibroblasts-derived fibronectin.

Several oligosaccharide-dependent functional alterations of fibronectin have previously been described. It was shown that glycosylation can modulate the interaction of fibronectin with collagen and with the cell membrane. Jones *et al.* (1986) compared glycosylated and non-glycosylated human skin fibroblast fibronectins and found that the deglycosylated form had an increased affinity for gelatin and enhanced adhesion-promoting properties. An important function of the carbohydrate moieties of the fibronectin is also protection of the protein from proteolytic attack. Bernard and colleagues demonstrated that the heavily glycosylated collagen-binding domain of chick embryo fibroblast fibronectin was selectively resistant to a broad variety of proteases (Bernard *et al.*, 1982).

Harper *et al.*, (1990) previously demonstrated that human tumors of neuroectodermal origin secrete fibronectin that contains the cell-adhesion-related HNK-1 epitope. In this study, the glycoproteins including fibronectin derived from TS skin fibroblasts and normal fibroblasts were found not to express the HNK-1 carbohydrate epitope. This result is not surprising, because the epitope was reported to be a marker for neural cell adhesion molecules involved in cell-cell and and cell substrate interactions during neural development.

Since fibronectin glycosylation is involved in promotion of fibroblasts adhesion and spreading, and playing significant roles in the ECM, the abnormal glycosylation of fibronectin found in skin lesions of TS patients may effect the cell-cell adhesion and cell-ECM interaction during the early and later embryonic development.

In general, the amino acid composition of the purified fibronectin from normal fibroblasts (JPa) was very similar to that of purified fibronectins from TS fibroblasts as well as standard plasma (pFN) and commercial cellular fibronectin (cFN). Amino acid composition of these fibronectins was also

consistent with the scientific literature. This suggests, as expected, no major differences in the primary structure of the fibronectin in TS.

Amino acid sequencing of fibronectin purified from TS and normal fibroblasts was not satisfactorily completed because of the N-terminal blocking and low expression of these fibroblast derived cellular fibronectin. However, N-terminal amino acid sequencing (Edman degradation) was demonstrated on the purified gelatin-binding domain of chymotrypsin digested human plasma fibronectin. The results of sequence analysis showed the N-terminal sequence glutamine-proline-glutamine-proline-histidine (Q-P-Q-P-H) in the chymotryptic 45 kDa collagen-binding domain in agreement with earlier reports by other workers (Zhu *et al.*, 1984).

To investigate the effects of cellular fibronectins purified from conditioned medium of TS and control fibroblasts, and plasma fibronectin on fibroblasts, cell culture plates were coated with different concentrations of purified cellular and plasma fibronectins. Adhesion of fibroblasts was promoted in all concentrations of fibronectins coated cell culture plates. Altered morphology and less confluence were observed in the culture plates coated with cellular fibronectin derived from TS skin lesions. This altered morphology may be the consequence of the effects of altered (increased) glycosylation of the fibronectin as a ECM macromolecule. The appearance of the fibroblasts was very similar (and normal) on the cultured plates coated with plasma, and normal cellular fibronectin derived from normal skin. However, cellular fibronectin coated fibroblast cultures were more confluent than plasma fibronectin coated culture plates. This indicated that cellular fibronectin is more active than plasma fibronectin in the promotion of the cell spreading, proliferation and morphology.

6.2 Expression, distribution, and localisation of ECM glycoproteins fibronectin, laminin and tenascin in cultures of skin fibroblasts derived from patients with TS and normal individuals.

There have been few studies that examine the synthesis of laminin by human dermal fibroblasts. There were contrary indications in these previous published scientific papers about its expression by fibroblasts (Timpl *et al.*, 1982, Woodley *et al.*, 1988, and Cooper *et al.*, 1993). In this study, the synthesis and secretion of tenascin and laminin into cell culture medium in addition to fibronectin by TS and control skin fibroblasts were demonstrated by western blotting. Partial purification of laminin and tenascin were achieved by ammonium sulphate precipitation and Mono-Q anion exchange chromatography techniques.

Expression and distribution of fibronectin, tenascin and laminin by established TS and normal skin fibroblasts using immunofluorescence, ELISA, and flow cytometry techniques were analysed and presented qualitative and quantitatively.

Indirect immunofluorescence studies revealed that the distribution and expression of fibronectin compared to controls was only altered in TSFM ungual fibroma-derived skin fibroblasts of the 10 year old male TS patient. However, the distribution and expression of fibronectin was not changed in other fibroblasts derived from a forehead plaque lesion and neck fibroma of a 14 year old female and 10 year old male TS patient respectively in comparison to control fibroblasts. Unaffected skin fibroblasts (TSAH) derived from the skin of the lumbosacral area of a TS patient also showed similar distribution and fluorescence intensity for fibronectin in comparison to normal (control) skin fibroblasts. Cell surface staining of ungual fibroma derived TSFM-fibroblasts with anti-fibronectin was found to be different

than normal (control) skin fibroblasts. Moreover, the cell surface distribution of fibronectin was uneven and appeared more dense on the TSFM-fibroblasts derived from ungual fibroma of a TS patient, while it is evenly distributed on control JPa-fibroblasts. The intensity of the fluorescence of intracellular fibronectin was increased in the TSFM-fibroblasts derived from ungual fibroma of a TS patient in comparison to normal JPa-fibroblasts. Cells from an ungual fibroma of a TS patient appeared larger than normal fibroblasts in size. In addition, the fluorescence intensity for fibronectin was evenly distributed and predominantly perinuclear in both TS and normal skin fibroblasts. However, the intracellular staining of fibronectin was patchy and also predominantly perinuclear in large cells (called N-cells by others) of TS fibroblasts. In general, fibrillar matrix of fibronectin were better established around the large TS cells derived from ungual fibroma than normal skin fibroblasts.

Similar to fibronectin, immunofluorescence studies showed that the distribution and expression of tenascin was only altered in TSFM ungual fibroma-derived skin fibroblasts of 10 year old male TS patient. However, the distribution and expression of tenascin was not changed in other fibroblasts derived from a forehead plaque lesion and neck fibroma of a 14 year old female and 10 year old male TS patients respectively in comparison to control fibroblasts. Increased expression of tenascin was observed in both cytoplasm and cell surface of TSFM ungual fibroma-derived skin fibroblasts compared to neck fibroma derived TSFL fibroblasts and normal skin JPa fibroblasts.

Immunofluorescence studies with mono and polyclonal antibodies on both TS and normal fibroblasts also showed laminin synthesis by dermal skin fibroblasts. Interestingly, altered distribution of laminin was observed at the cell surface and in the cytoplasm of, particularly in large cells, TSFL-derived

fibroblasts from neck fibroma of a 13 year old male. However, changes in the immunofluorescent staining of fibronectin and tenascin were observed in TSFM-fibroblasts derived from an ungual fibroma of a 10 year old male TS patient. In normal JPa skin fibroblasts, cell surface staining of laminin was hardly detectable compared to TSFL neck fibroma derived fibroblasts. Intracellular laminin staining is bright, and diffuse in the cytoplasm of large cells derived from a neck fibroma of a TS patient, but this was usually seen around nuclei only in normal skin fibroblasts.

Increased expression of laminin in neck fibroma-derived TSFL fibroblasts was also confirmed by ELISA and flow cytometry quantitatively . In contrast to TSFL fibroblasts, ELISA and flow cytometry results confirmed quantitatively and strenghened the earlier findings of immunofluorescent studies of fibronectin and tenascin in TSFM fibroblasts derived from an ungual fibroma. Flow cytometry and ELISA results showed quantitatively that laminin exression was increased in cytoplasm, cell surface and in the conditioned medium of TSFL fibroblasts derived from neck fibroma. These two quantitative experiments also proved that fibronectin and tenascin were increased in cytoplasm, cell surface and in the conditioned medium of TSFM fibroblasts.

Cell-matrix interactions via ECM macromolecules play many critical roles in embryonic development and disease processes. Lallier (1991) proposed a model for tuberous sclerosis mutation effects on neural crest cell migration (see *Fig. 1.2*). According to Lallier "normal neural crest cells may migrate through a series of extracellular matrix molecules, causing them to differentiate into a specific cell type. Tuberous sclerosis mutant cells may lack the ability to properly interact with the matrix molecules they encounter, leading to misplaced cells or cells that differentiate into abnormal cell types."

Altered expression and distribution of fibronectin and laminin were well observed particularly in large cells of ungual fibroma and neck fibroma of TS patients respectively. All these results suggest the abnormal assembly of ECM in different TS skin lesions. It is also clear that there are some differences between TS fibroblasts from different sources. This may be important in understanding the nature of the skin lesions which occur in various locations of TS patients bodies.

Therefore, altered expression and arrangements of fibronectin, laminin and tenascin on ungual fibroma and neck fibroma derived fibroblasts reported in this thesis may effect the interactions of ECM with cells during embryonic development, and therefore that may result with misplaced cells or cells that differentiate into abnormal cell types (for example large, N-cells observed in this research) in different TS lesions. Moreover, functional distruption between ECM macromolecules and the cells may result in disease states, hardness of tissues and alterations in the behavior and shape of the cells in TS tissues during development. As reported earlier in chapter 4 (see *Figs. 4.15 and 4.16*), altered morphology and less confluence were observed in the culture plates coated with cellular fibronectin derived from TS skin lesions. This altered morphology of fibroblasts may be the consequence of the effects of increased glycosylation of the fibronectin as a ECM macromolecule, since fibronectin glycosylation modulates fibroblast adhesion and spreading according to Jones and his colleagues (1986).

Overall, these results suggest that abnormal assembly of the ECM demonstrates defects in the biosynthesis or deposition of fibronectin, laminin and tenascin which might result in hardened tissues of TS. These findings of this thesis may be a good evidence to support the idea that changes in ECM composition and organisation may have profound effects on cell spreading, adhesion, proliferation, cell shape and behaviour during the development of

hardened TS tissues. Studies of these changes in the ECM components may contribute to the understanding of the mechanisms involved in the aetiology of hardened tissues of TS.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J.D. (**1989**). *Molecular Biology of the Cell.* Second Edition, Garland Publishing, New York.

Arumugham, R.G., Hsieh, T.C.Y., Tanzer, M.L. and Laine, R.A. (**1986**). Structures of other asparagine-linked sugar chains of laminin. *Biochim. Biophys. Acta,* **883**: 112-126.

Balian, G., Click, E.M., Crouch, E., Davidson, J.M. and Bornstein, P. (**1979**). Isolation of a collagen-binding fragment from fibronectin and cold-insoluble globulin. *J. Biol. Chem.* **254**: 1429-1432.

Bernard, B.A., Yamada, K.M. and Olden, K. (**1982**). Carbohydrates selectively protect a specific domain of fibronectin against proteases. *J. Biol. Chem.* **257**: 8549-8554.

Borsi, L., Carnemolla, B., Castellani, P., Rosellini, C., Vecchio, D., Allemanni, G., Chang, S.E., Taylor-Papadimitriou, J., Pande, H. and Zardi, L. (**1987**). Monoclonal antibodies in the analysis of fibronectin isoforms generated by alternative splicing of mRNA precursors in normal and transformed human cells. *J. Cell Biol.* **104**: 595-600.

Bosman, F.T. (**1993**). Integrins: cell adhesive and modulators of cell function. *Histochemical Journal.* **25**: 469-477.

Bourdon, M.A. and Ruoslahti, E. (**1989**). Tenascin mediates cell attachment through an RGD-dependent receptor. *J. Cell Biol.* **108**: 1149-1155.

Bourdon, M.A., Wikstrand, C.J., Furthmayr, H., Matthews, T.J. and Bigner,D.D. (**1983**). Human glioma-mesenchymal extracellular matrix antigen defined by monoclonal antibody. *Cancer Res.* **43**: 2796-2805.

Bourneville, D. M. and Brissaud, E. (**1881**): Encephalite ou sclerose tubereuse des circonvolutions cerebrales. *Arch. Neurol.* (Paris) **1**: 390-412.

Bristow, J., Tee, M.K., Gitelman, S.E., Mellon, S.H. and Miller, W.L. (**1993**). Tenascin-X: A novel extracellular matrix protein encoded by the human XB gene overlapping p450c21B. *J.Cell Biol.* **122**: 265-278.

Buck, C.A., and Horwitz, A.F. (**1987**). Cell surface receptors for extracellular matrix molecules. *Annu. Rev. Cell Biol.* **3**: 179-205.

Carsons, S.E. (**1989**). The structure and function of fibronectins in Carsons, S.E (ed.) *Fibronectin in Health and Disease.* CRC Press inc.. Boca Raton, Florida, pages 1-21.

Carter, W.G. and Hakomori, S.I. (**1979**). Isolation of galactoprotein a from hamster embryo fibroblasts and characterization of the carbohydrate unit. *Biochemistry*, **18**: 730-738.

Chandrasekaran, S., Dean, J.W., Griniger, M.S. and Tanzer, M.L. (**1991**). Laminin carbohydrates are implicated in cell signalling. *J.Cell Bichem.* **46**: 115-124.

Chiquet, M. (**1989**). Tenascin/J1/cytotactin: the potential function of hexabrachion proteins in neural development. *Dev. Neuroscience.* **11**: 266-275.

Chiquet-Ehrismann, R. (**1990**). What distinguishes tenascin from fibronectin? *FASEB J.* **4**: 2598-2604.

Chiquet-Ehrismann, R. (**1991**). Anti-adhesive molecules of the extracellular matrix. *Current Opinion in Cell Biology.* **3**: 800-804.

Chiquet-Ehrismann, R., Kalla, P. Pearson, C.A. (**1989**). Participation of tenascin and transforming growth factor-β in reciprocal epithelial-masenchymal interactions of MCF7 cells and fibroblasts. *Cancer Res.* **49**: 4322-4325.

Chiquet-Ehrismann, R., Kalla, P. Pearson, C.A., Beck, K. and Chiquet, M. (**1988**). Tenascin interferes with fibronectin action. *Cell.* **53**: 383-390.

Chiquet-Ehrismann, R., Mackie, E.J., Pearson, C.A. and Sakakura, T. (**1986**). Tenascin: an extracellular matrix protein involved in tissue interactions during fetal development and oncogenesis. *Cell.* **47**: 131-139.

Chou, D.K.H., Ilyas, A.A., Evans, J.E., Costello, C., Quarles, R.H. and Jungalwala. F.B. (**1986**). Structure of sulfated glucuronyl glycolipides in the nervous system reacting with HNK-1 antibody and some IgM paraproteins in neuropathy. *J. Biol. Chem.* **261** (**25**): 11717-11725.

Chuong, C.M., Crossin, K.L. and Edelman, G.M. (**1987**). Sequential expression and differential function of multiple adhesion molecules during the formation of cerebellar cortical layers. *J. .Cell Biol.* **104**: 331-342.

Conneally, M.P. (**1991**). Locating disease genes through linkage in Johnson W.G. and Gomez, M.R. (eds) Tuberous Sclerosis and Allied Disorders: Clinical, Cellular, and Molecular Studies, *Annals of the New Academy of Sciences.* **615**: 252-255.

Cooper, M.L., Andre, C., Hansbrough, J.F., and Zapata-Sirvent, R.L., (**1993**). Direct comparison of a cultured composite skin substitute containing human keratinocytes and fibroblasts to an epidermal sheet graft containing human keratinocytes on athymic mice. *The Journal of Investigative Dermatology.* **101** (**6**): 811-819.

Crossin, K.L. (**1991**). Cytotactin binding: Inhibition of stimulated proliferation and intracellular alkalinization in fibroblasts. *Proc. Natl. Acad. Sci. USA.* **88**: 11403-11407.

Cunningham, B.A., Hemperly, J.J.,Murray, B,A., Prediger, E.A., Brackenbury, R. and Edelman, G.M. (**1987**). Neuronal cell adhesion molecule: Structure, immunoglobulin-like domains, cell surface modulation and alternative RNA splicing. *Science.* **236**: 799-806.

Dean, J.W., Chandrasekaran, S. and Tanzer, M.L. (**1990**). A biological role of the carbohydrate moieties of laminin. *J.Biol. Chem.* **265**: 12553-12556.

Edward, P. and Richardson, J.R. (**1991**). Pathology of tuberous sclerosis: neuropathologic aspects. in Johnson, W.G., and Gomez, M. R. (eds.)

Tuberous Sclerosis and Allied Disorders: Clinical, Cellular, and Molecular Studies, *Annals of the New York Academy of Sciences* **615**: 128-139. The New York Academy of Sciences, New York.

Ehrig, K., Leivo, I., Argraves, W.S., Ruoslahti, E. and Engval, E. (**1990**). Merosin, a tissue-specific basement membrane protein, is a laminin-like protein. *Proc. Natl. Acad. Sci. USA.* **87**: 3264-3268.

Ellis, Z. K. and Hemming, F. W. (**1989**). Altered synthesis of fibronectin by skin fibroblasts grown from tuberous sclerotic patients. *Biochemical Society Transactions.* **17**: 190-191.

Ellis, Z. K. and Hemming, F. W. (**1990**). Glycosylation of fibronectin and other proteins in tissues of patients with tuberous sclerosis, in Y. Ishabashi and Y. Hori, (eds.) *Tuberous Sclerosis and Neurofibromatosis.* 123-134. Elsevier. Amsterdam.

Ellis, Z. K., Henderson, K. and Hemming.F.W. (**1991**) Glycoprotein and proteoglycan alterations in tuberous sclerosis, in Johnson, W.G., and Gomez, M. R. (eds.) Tuberous Sclerosis and Allied Disorders: Clinical, Cellular, and Molecular Studies, *Annals of the New York Academy of Sciences.* **615**: 149-157. The New York Academy of Sciences, New York.

End, P., Panayotou, G., Entwistle, A., Waterfield,M.D. and Chiquet, M. (**1992**). Tenascin: a modulator of cell growth. *Eur. J.Biochem.* **209**: 1041-1051.

Engel, J. (**1989**). EGF-like domains in extracellular matrix proteins: localised signals for growth and differentiation. *FEBS Letters.* **251 (1, 2)**: 1-7.

Engel, J. (**1992**). Laminins and other strange proteins. *Biochemistry.* **31**: 10643-10651.

Engel, J. (**1993**). Structure and function of laminin. In Rohrbach, D.H. and Timpl, R. (eds.), *Molecular and Cellular Aaspects of Basement Membranes.* Academic Press, inc, San Diego, pages 147-176.

Englund, P.T. (**1993**). The Structure and Biosynthesis of Glycosyl Phosphatidylinositol Protein Anchors, *Annu. Rev. Biochem.* **62**: 121-138.

Engvall, E. and Ruoslahti, E. (**1977**). Binding of soluble form of fibroblast surface protein, fibronectin, to collagen. *Int. J. Cancer.* **20**: 1-5.

Erickson, H.P. (**1993**). Tenasscin-C, tenascin-R and tenascin-X: A family of talented proteins in search of functions. *Current Opinion in Cell biology.* **5**: 869-876.

Erickson, H.P. and Bourdon, M.A. (**1989**). Tenascin: an extracellular matrix protein protein prominent in specialized embryonic tissues and tumors. *Ann. Rev. Cell Biol.* **5**: 71-79.

Erickson, H.P. and Iglesias, J.L. (**1984**). A six-armed oligomer isolated from cell surface fibronectin preparations. *Nature.* **311**: 267-269.

Erickson, H.P. and Lightner, V.A. (**1988**). Hexabrachion protein (tenascin, cytotactin, brachionectin) in connective tissues, embryonic brain, and tumors. In K.R. Miller (ed.), *Advances in Cell Biology.* **2**: 55-90, ed. London, JAI Press.

Erickson, H.P. and Taylor, H.C. (**1987**). Hexabrachion proteins in embryonic chicken tissues and human tumors. *The Journal of Cell Biology* **105**: 1387-1394.

Fabing, H. (**1934**). Tuberous sclerosis with epilepsy ("epiloia") in identical twins. *Brain.* **57**: 227-238.

Ferguson, M.A.J. (**1991**), Lipid anchors on membrane proteins. *Current Opinion in Structural Biology.* **1**: 522-529.

Ferraro, A. and Doolittle, G. J. (**1936**). Tuberous sclerosis (diffuse Neurospongioblastosis). *Psychiatr. Q.* **10**: 365-416.

Ffrench-Constant, C., Van De Water, L., Dvorak, H.F. and Hynes, R.O. (**1989**). Reappearance of an embryonic pattern of fibronectin splicing during wound healing in the adult rat. *J. Cell Biol.* **109**: 903-914.

Fischer, M. H., Fortune, S. J., Foster, S. H. and Gilbert, E. F. (**1977**). Chemical analysis of an angiofibroma from a patient with tuberous sclerosis. *J. Ment. Defic. Res.* **21**: 251-261.

Fisher, S.J. and Laine, R.A. (**1979**). Carbohydrate structure of the major glycopeptide from human cold-insoluble globulin. *J.Supramol. Struct.* **11**: 271-279.

Foidart, J.M. Bere. E. W., Yaar, M., Rennard, S.I., Gullino, M., et al., (**1980**). Distribution and immunoelectron microscopic localisation of laminin, a non-collagenous basement membrane glycoprotein. *Lab. Invest.* **42**: 336-342.

Freshney, R.I. (**1992**). *Animal Cell Culture: a practical approach*, Second Edition, Oxford University Press.

Fryer, A.E., Chalmers, A., Connor, J. M., Fraser, I., Povey, S., Yates, A. D., Yates, J. R.W. and Osborne, j. P. (**1987**). Evidence that the gene for tuberous sclerosis is on chromosome 9. *Lancet.* **1**: 659-661.

Fujiwara, S., Shinkai, H., Deutzmann, R., Paulsson, M. and Timpl, R. (**1988**). Structure and distrubution of N-linked oligosaccharide chains on various domains of mouse tumour laminin. *Biochem. J.* **252**: 453-461.

Fukuda, M., and Hakomori, S. (**1979**). Carbohydrate structure of galactoprotein a, a major transformation sensitive glycoprotein released from hamster embryo fibroblasts. *J.Biol. Chem.* **254**: 5442-5450.

Fukuda, M., Levery, S.B. and Hakomori, S.I. (**1982**). Carbohydrate structure of hamster plasma fibronectin. Evidence for chemical diversity between cellular and plasma fibronectins. *J. Biol. Chem.* **257**: 6856-6860.

Furie, M.B. and Rifkin, D.B. (**1980**). Proteolytically derived fragments of human plasma fibronectin and their localization within the intact molecule. *J.Biol. Chem.* **255**: 3134-3140.

Furie., M.B, and Rifkin, D.B. (**1980**). Proteolytically derived fragments of human plasma fibronectin and their localisation within the intact molecule. *J. Biol. Chemistry.* **255**:**7**: 3134-3140.

Fuss,B., Wintergerst, E.S., Bartsch, U. and Schachner, M. (**1993**). Molecular characterization and in situ mRNA localization of the neural recognition molecule J1-160/180: a modular structure similar to tenascin. *J. Cell Biol.* **120**: 1237-1249.

Givan, A.L. (**1992**). *Flow Cytometry; first principles*. Wiley-Liss, Inc. New York.

Gomez, M. R., Kuntz, N. L. and Westmoreland, B. F. (**1982**). Tuberous sclerosis, early onset of seizures, and mental subnormality: study of discordant monozygous twins. *Neurology*. **32**: **6**: 604-611.

Gomez, M.R. (**1979**). *Tuberous Sclerosis*, Raven Press, New York.

Gomez, M.R. (**1988**). *Tuberous Sclerosis*, Second Edition, Raven Press, New York.

Gomez, M.R. (**1991**). Phenotypes of the tuberous sclerosis, in Johnson, W.G., and Gomez, M. R. (eds.), Tuberous Sclerosis and Allied Disorders: Clinical, Cellular, and Molecular Studies, *Annals of the New York Academy of Sciences* **615**: 1-7. The New York Academy of Sciences, New York.

Gowda, D.C., Margolis, R.U. and Margolis, R.K. (**1989**). Presence of the HNK-1 epitope on poly (N-acetyllactosaminyl) oligosaccharides and identification of multiple core proteins in the chondroitin sulfate proteoglycans of brain. *Biochemistry*. **28**: 4468-4474.

Graf, J., Iwamoto, Y., Sasaki, M., Martin, G.R., Kleinman, H.K., Robey, F.A. and Yamada, Y. (**1987**). Identification of an amido acid sequence in laminin mediating cell attachment, chemotaxis and receptor binding.*Cell* **48**: 989-996.

Graham, J.M. (**1992**). The composition and structure of membranes. In Dealtry, G.B., and Rickwood, D., Bios (eds.), *Cell Biology-Labfax* scientific publishers limited, pp. 77.

Gunther, M. and Penrose, L.S. (**1935**). The genetics of epiloia. *J. Genet.* **31**: 413-430.

Hardy, M.R. and Townsend, R.R. (**1994**). High-pH anion-exchange chromatography of glycoprotein-derived carbohydrates. *Methods in Enzymology*. **230**: 208-225.

Harlow, E. and Lane, D.P. (**1988**). *Antibodies: A laboratory Manual,* Cold Spring Harbor Laboratory, New York.

Harper, J.R., Perry, S.K. Davis, R.M. and Laufer, D.M. (**1990**). Human neuroectoderm-derived cell line secretes fibronectin that shares the HNK-1/10C5 carbohydrate epitope with neural cell adhesion molecules, *Journal of Neurochemistry*. **54**: 395-401.

Hay, E. D. (**1991**). *Cell Biology of Extracellular Matrix*, Second Edition, Plenum Press, New York.

Hayashi, M. and Yamada, K.M. (**1983**). Domain structure of the carboxyl-terminal half of human plasma fibronectin. *J. Biol. Chem.* **258**: 3332-3340.

Hemming, F.W. (**1991**). Glycoproteins of Mammalian and Avian Cells, in Harding, J.J. and Crabbe, M.J.J. (eds.) *Post-Translational Modifications of Proteins*. 217-253.

Henderson, K. J. (**1991**). *Glycosaminoglycans of skin fibroblasts from tuberous sclerosis patients*, Thesis for Ph.D., Department of Biochemistry, The University of Nottingham.

Hirschberg, C. and Snider,M. D. (**1987**). Topography of Glycosylation in The Rough Endoplasmic Reticulum and Golgi Apparatus. *Ann. Rev. Biochemistry*. **56**: 63-87.

Homandberg, G.A. and Erickson, J.W. (**1986**). Model of fibronectin tertiary structure based on studies of interactions between fragments. *Biochemistry* **25**: 6917-6925.

Homandberg, G.A. and Kramer-Bjerke, J. (**1987**). Thrombospondin binds to amino-terminal fragments of plasma fibronectin. *Thromb. Res.* **48**: 329-335.

Hormann, H. and Seidl, M. (**1980**). Affinity chromatography on immobilized fibrin monomer III; the fibrin affinity center of fibronectin. *Hoppe-Seyler's Z. Physiol. Chem.* **365**: 517-524.

Hughes, R.C. (**1992**). Role of glycosylation in cell interactions with extracellular matrix. *Biochemical Society Transactions*. **20** (**2**): 279-284.

Humphries, M.J., Akiyama, S.K., Komoriya,A., Olden, K. and Yamada, K.M. (**1986**). Identification of an alternatively spliced site in human plasma fibronectin that mediates cell type-specific adhesion. *J Cell Biol.* **103**: 2637-2647.

Humphries, M.J., Komoriya,A., Akiyama, S.K., Olden, K. and Yamada, K.M. (**1987**). Identification of two distinct regions of the type III connecting segment of human plasma fibronectin that promote cell type-specific adhesion. *J. Biol. Chem.* **262**: 6886-6892.

Hunter, I., Schulthess, T., Bruch, M., Beck, K. and Engel, J. (**1990**). Evidence for a specific mechanism of laminin assembly. *Eur. J. Biochem.* **188**: 205-211.

Hynes, R.O. (**1986**). Fibronectins, *Sci.Am.*, **254**: 42-51.

Hynes, R.O. (**1987**). Integrins: a family of cell surface receptors. *Cell.* **48**: 549-554.

Hynes, R.O. (**1990**). *Fibronectins*, Springer -Verlag series in Molecular Biology, New York.

Inglis, K. (**1950**). Neurilemmoblastosis : The influence of intrinsic factors in disease when development of the body is abnormal. *Am. J. Pathology.* **26**: 521-536.

Ishibashi, Y., Nogita, T. and Onodera, K. (**1990**). Biological aspects of tuberous sclerosis, in Y. Ishibashi, Y., Hori (eds.) *Tuberous Sclrosis and Neurofibromatosis; Epidemiology, Pathology, Biology and Management*, Elsevier Science Publishers, Amsterdam. 65-75.

Iwamoto, Y., Robey, F.A., Graf, J., Sasaki, M., Kleinman, H.K., Yamada, Y. and Martin, G.R. (**1987**). YIGSR a pentapeptide from the B1 chain of laminin inhibits tumor cell metastases. *Science.* **328**: 1132-1134.

Jaffe, E.A., Ruggiero J.T., Leung, L.L.K., Doyle, M.J., McKeown-Longo, P.J. and Mosher, D.F., (**1983**). Cultured Human Fibroblasts synthesize and Secrete Thrombospondin and Incorporate It into Extracellular Matrix. *Proc. Natl. Acad. Sci. USA.* **80**: 998-1002.

Johnson, W.G., Yoshidome, H., Stenroos, E.S., and Davidson, M.M., (**1991**). Origin of the neuron -like cells in tuberous sclerosis tissues, in Johnson, W.G., and Gomez, M.R., (eds.), Tuberous Sclerosis and Allied Disorders; clinical, cellular and Molecular Studies, *Annals of the New York Academy of Sciences.* **615:** 211-219. The New York Academy of Sciences .

Jones, F.S., Burgoon, M.P., Hoffman, S., Crossin, K.L., Cunningham, B.A. and Edelman, G.M. (**1988**). A cDNA clone for cytotactin contains sequences similar to epidermal growth factor-like repeats and segments of fibronectin and fibrinogen. *Proc. Natl. Acad. Sci. U.S.A.* **85**: 2186-2190.

Jones, F.S., Hoffman, S., Cunningham, B.A. and Edelman, G.M. (**1989**). A detailed structural model of cytotactin: Protein homologies, alternative RNA splicing, and binding regions. *Proc. Natl. Acad. Sci.* USA **86**: 1905-1909.

Jones, G.E., Arumugham, R. and Tanzer, M.L. (**1986**). Fibronectin glycosylation modulates fibroblast adhesion and spreaading. *J. Cell Biol.* **103**: 1663-1670.

Kandt, R.S., Haines, J. L., Smith, M., Northrup, H., Gardner, R. J. M., Short, M. P., Dumars, K., Roach, E. S., Steingold, S., Wall, S., Blanton, S. H., Flodman, P., Kwiatkowski, D. J., Jewell, A., Weber, J. L., Roses, A. D. and Pericak-Vance, M. A. (**1992**). Linkage of an important gene locus for tuberous sclerosis to a chromosome 16 marker for polycystic kidney disease. *Nature Genet.* **2**: 37-41.

Kanemoto, T., Reich, R., Greatorex, D., Adler, S.H. Yamada, Y. and Kleinman, H.K. (**1990**). Identification, of an amino acid sequence from the laminin A chain which stimulates metastases formation and collagenase IV production. *Proc. Natl. Acad. Sci. USA* **87**: 2279-2283.

Kleinman, H.K. and Weeks, B.S. (**1989**). Laminin: structure, functions and receptors. *Current Opinion in Cell Biology.* **1**: 964-967.

Kornblihtt, A.R., Umezawa, K., Vibe-Pedersen, K. and Barelle, F.E. (**1985**). Primary structure of human fibronectin: Differential splicing may generate at least 10 polypeptides from a single gene. *EMBO, J.* **4** (**7**): 1755-1759.

Kornblihtt, A.R., Vibe-Pedersen, K. and Barelle, F.E. (**1984**). Human fibronectin: Molecular cloning evidence for two mRNA species differing by an internal segment coding for a structural domain. *EMBO J.* **3**: 221-226.

Kornfeld, R. and Kornfeld, S. (**1985**). Assembly of Asparagine-Linked Oligosaccharides. *Ann. Rev Biochem.* **54**: 631-664.

Koukoulis, G.K., Gould, V.E. Bhattacharyya, A., Gould, J.E., Howeedy, A.A. and Virtanen, I. (**1991**). Tenascin in normal, reactive, hyperplastic, and neoplastic tissues: Biologic and pathologic implications. *Human Pathol.* **22**: 636-643.

Kruse, J., Keilhauer, G., Faissner, A., Timpl, R. and Schachner, M. (**1985**). The J1 glycoprotein-a novel nervous system cell adhesion molecule of the L2/HNK-1 family. *Nature.* **316**: 146-148.

Kruse, J., Mailhammer, R., Wernecke, H., Faissner, A., Sommer, I., Goridis, C. and Schachner, M. (**1985**). Neural cell adhesion molecules and myelin-associated glycoprotein share a common carbohydrate moiety recognised by monoclonal antibodies L2 and HNK-1. *Nature.* **311**: 153-155.

Kruse, J., Mailhammer, R., Wernecke, H., Faissner, A., Sommer, I., Goridis, C., and Schachner, M., (**1984**). Neural cell adhesion molecules and myelin-associated glycoprotein share a common carbohydrate moiety recognised by monoclonal antibodies L2 and HNK-1. *Nature.* **311**, 153-155.

Krusius, T., Fukuda, M., Dell, A. and Ruoslahti, E. (**1985**). Structure of the carbohydrate units of human amniotic fluid fibronectin, *J.Biol. Chem.* **260**: 4110.

Kubota, S., Tashiro, K. and Yamada, Y. (**1992**). Signaling site of laminin with mitogenic activity. *J. Biol. Chem.* **267**: 4285-4288.

Kunemund, V., Jungalwala, F.B., Fischer, G., Chou, D.K.H., Keilhauer, G. and Schachner, M. (**1988**). The L2/HNK-1 carbohydrate of neural cell adhesion molecules is involved in cell interactions *J. Cell Biol.* **106**: 213-223.

Kuntz, N. (**1988**) Population studies, in Gomez, M.R. (ed.) *Tuberous Sclerosis*, Second Edition, Raven Press, New York. 213-215.

Kurkinen, M., Vartio, T. and Vaheri, A. (**1980**). Polypeptides of human fibronectin are similar but not identical. *Biochim. Biophys. Acta.* **624**: 490-498.

Kwiatkowski, D.J. and Priscilla, S. (**1994**). Tuberous sclerosis. *Arch. Dermatol.* **130**: 348-354.

Laemmli, U.K. (**1970**). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227**: 680-685.

Lallier, T. E. (**1991**). Cell lineage and cell migration in the neural crest. In Johnson, W.G., and Gomez, M.R. (eds.), Tuberous Sclerosis and Allied Disorders; clinical, cellular, and Molecular Studies, *Annals of the Newyork Academy of Sciences* **615:** 158-171. The New York Academy of Sciences .

Ledger, P.W., Nishimoto, S.K., Hayashi, S. and Tanzer, M.L. (**1983**). Abnormal glycosylation of human fibronectin secreted in the presence of monensin. *J.Biol. Chem.* **258**: 547-554.

Lehninger, A. L., Nelson, D. L. and Cox, M. M. (1993) *Principles of Biochemistry*, Second edition, New York, Worth Publishers.

Lightner, V.A., Slemp, C.A. and Erickson, H.P. (**1990**). Localisation and quantitation of hexabrachion (tenascin) in skin, embryonic brain, tumors, and plasma. *Annals of New York Academy of Science* **580**: 260-275.

Lis, H. and Sharon, N. (**1993**). Protein glycosylation-structural and functional aspects. *Eur.J. Biochem.* **218**: 1-27.

Lochter, A., Vaughan, L., Kaplony, A., Prochiantz, A., Schachner, M. and Faissner, A. (**1991**). J1/Tenascin in substrate-bound and soluble form displays contrary effects on neurite outgrowth. *J. Cell Biol.* **113**: 1159-1172.

Lotz, M.M., Burdsal, C.A., Erickson, H.P. and McClay, D.R. (**1989**). Cell adhesion to fibronectin and tenascin: quantitative measurements of initial binding and subsequent strengthening response. *J.Cell Biol.* **109**: 1795-1805.

Mackie, E.J., Thesleff, I. and Chiquet-Ehrismann, R. (**1987**). Tenascin is associated with chondrogenic and osteogenic differentiation in vivo and promotes chondrogenesis in vitro. *J. Cell Biol.* **105**: 2569-2579.

Martin, G.R. and Timpl, R. (**1987**). Laminin and other basement membrane components. *Ann. Rev. Cell Biol.* **3**: 57-85.

Mathews, C.K. and van Holde, K.E. (**1990**). *Biochemistry*, The Benjamin/Cummings Company, Inc. California.

McConville, M.J. and Ferguson, M.A.J. (**1993**). The Structure, biosynthesis and fuction of glycosylated phosphatidylinositols in the parasitic protozoa and higher eukaryotes. *Biochemical Journal.* **295** (**2**): 305-324.

Mecham, R.P. (**1991**). Receptors for laminin on mammalian cells. *FASEB J.* **5**: 2538-2546.

Moolten, S.E. (**1942**). Hamartial nature of the tuberous sclerosis complex and its bearing on the tumor problem. Report of case with tumor anomaly of the kidney and adenoma sebaceum. *Arch. Int. Med.* **69**: 589-623.

Moos, M., Tacke, R., Scherer, H., Teplow, D., Fruh, K. and Schachner, M. (**1988**). Neural adhesion molecule L1 as a member of the immunoglobulin superfamily with binding domains similar to fibronectin. *Nature* **334**: 701-703.

Mosher, D.F. and Proctor, R.A. (**1980**). Binding and factor XIIIa-mediated cross-linking of a 27-kilodalton fragment of fibronectin to Staphylococcus aureus. *Science.* **209**: 927-929.

Murata, J., Saiki, I., Azuma, I. and Nishi, N. (**1989**). Inhibitory effect of a synthetic polypeptide, poly (Tyr-Ile-Gly-Ser-Arg), on the metastatic formation of malignant tumor cells. *Int. J. Biol. Macromol.* **11**: 92-99.

Murray, R.K. (**1993**) Glycoproteins. in Murray, R. K., Granner, D. K., Mayes, P. A., Rodwell, V.W. (eds.) *Harper's Biochemistry*. 23rd edition. 622-633.

Nellist, M., Janssen, B., Brook-Carter, P.T., Henselling-Janssen, A.L.W., Maheshwar,M.M., Verhoef, S., Van den Ouweland,A.M.W., Lindhout, D., Eussen, B., Cordeiro, I., Santos, H., Halley, D.J.J., Sampson, J.R., Ward, C.J., Peral, B., Thomas, S., Hughes, J., Harris, P.C., Roelfsema, J.H., Saris, J.J.,Spruit, L., Peters, D.J.M., Dauwerse, J.G. and Breuning, M.H. (**1993**). Identification and characterization of the tuberous sclerosis gene on chromosome 16. *Cell.* **75**: 1305-1315.

Nevin, N.C. and Pearce, W. G. (**1968**). Diagnostic and genetical aspects of tuberous sclerosis. *J. Med. Genet.* **5**: 273-280.

Norenberg, U., Wille, H., Wolff, J.M., Frank, R. and Rathjen, F.G. (**1992**). The chicken neural extracellular matrix molecule restrictin: Similarity with EGF-, fibronectin type III-, and fibronogen-like motifs. *Neuron* **8**: 849-863.

Norton, P.A. and Hynes, R.0. (**1987**). Alternative splicing of chicken fibronectin in emmryos and in normal and transformed cells. *Mol. Cell. Biol.* **7**: 4297-4307.

Oikarinen, A., Palatsi, R., Linna, S.L. and Peltonen, L. (**1982**). Types I and III collagens and the activities of prolyl hydroxylase in skin lesions of Tuberous Sclerosis and Galactosylhydroxylysyl glucosyltransferase in skin lesions of tuberous sclerosis. *British Journal of Dermatology* **107**: 659-664.

Olden, K., Pratt, R.M. and Yamada, K.M. (**1978**). Role of carbohydrates in protein secretion and turnover: Effects of tunicamycin on the major cell surface glycoprotein of chick embryo fibroblasts. *Cell* **13**: 461-473.

Olden, K., Pratt, R.M. and Yamada, K.M. (**1979**). Role of carbohydrate in biological function of the adhesive glycoprotein fibronectin. *Proc. Natl. Acad. Sci. U.S.A.* **76**: 3343-3347.

Osborne, J.P., Fryer, A. and Webb, D. (**1991**). Epidemiology of tuberous sclerosis. *Ann. N.Y. Acad. Sci.* **615**: 125-127.

Pande, H., Corkill, J., Sailor, R., and Shively, J.E. (**1981**). Comparative structural studies of human plasma and amniotic fibronectins. *Biochemistry. Biophys. Res. Commun.* **101**: 265-272.

Patel, R.S., Odermatt, E., Schwarzbauer, J.E. and Hynes, R.O. (**1987**). Organization of the fibronectin gene provides evidence for exon shuffling during evolution. *EMBO J.* **6**: 2565-2572.

Paulsson, M. and Saladin, K. (**1989**). Mouse heart laminin: Purification of the mature protein and structural comparison with EHS tumor laminin. *J. Biol. Chem.* **264** (**18**): 726-732.

Pearson, C.A., Pearson, D., Shibahara, S., Hofsteenge, J. and Chiquet-Ehrismann, R. (**1988**). Tenascin: cDNA cloning and induction by TGF-beta. *EMBO J.* **7**: 2677-2681.

Petersen, T.E., Skorstengaard, K. and Vibe-Pedersen, K. (**1989**). Primary structure of fibronectin, in Mosher, D.F. (ed.), *Fibronectin*, Academic Press, San Diego,1-24.

Potts, J.R. and Campbell, I.D. (**1994**). Fibronectin structure and assembly. *Current Opinion in Cell Biology.* **6**(**5**): 648-655.

Quade, B.J. and McDonald, J.A. (**1988**). Fibronectin's amino-terminal matrix assembly site is located within the 29kDa amino-terminal domain containing five type 1 repeats. *J. Biol. Chem.* **263**: 19602-19609.

Ranscht, B. (**1988**). Sequence of contactin, a 130-kD glycoprotein concentrated in areas of interneuronal contact, defines a new member of the immunoglobulin supergene family in the nervous system. *J. Cell. Biol.* **107**: 1561-1573.

Rennard, S.T., Berg, R., Martin, G.R., Foldart, J.M. and Robey, P.G. (**1980**). Enzyme-linked immunoassay (ELISA) for connective tissue components. *Anal. Biochem.* **104**: 205-211.

Richter, H. and Hormann, H. (**1982**). Early and late cathepsin D-derived fragments of fibronectin containing the C-terminal interchain disulfide cross-link. *Hoppe Seyler's Z. Physiol.* Chem. **363**: 351-364.

Richter, H., Seidl, M.and Hormann, H. (**1981**). Location of heparin-binding sites of fibronectin. Detection of a hitherto unrecognized transamidase sensitive site. *Hoppe Seyler's Z. Physiol.* Chem.**362**: 399-408.

Ruegg, C.R., Chiquet-Ehrismann, R. and Alkan, S.S. (**1989**). Tenascin, an extracellular matrix, exerts immuno-modulatory activities. *Proc. Natl. Acad. Sci. USA.* **86**: 7437-7441.

Rundle, A. T., Atkin, J. and Dollimore, J. (**1975**). Serum and tissue proteins in tuberous sclerosis: immunoglobulin levels. *Human Genetic* **28**: 147-151.

Rundle, A.T. (**1976**). Plasma acute-phase reactant proteins in tuberous sclerosis. *J, Ment. Def. Res.* **20**: 237-242.

Ruoslahti, E. (**1988**). Fibronectin and its receptors. *Ann. Rev. Biochem.* 1988. **57**: 375-413.

Ruoslahti, E. and Pierschbacher, M.D. (**1987**). New perspectives in cell adhesion RGD and integrin. *Science.* **238**: 491-497.

Ruoslahti, E., Engvall, E., Hayman, E. G., and Spiro, R.G. (**1981**). Comparative studies on amniotic fluid and plasma fibronectins. *Biochem. J.* **193**: 295

Sage, E.H. and Bornstein, P. (**1991**). Extracellular proteins that modulate cell-matrix interactions. *J. Biol. Chem.* **266**: 14831-14834.

Sakakura, T. and Kusano I. (**1991**). Tenascin in tissue perturbation repair. *Acta Pathologica Japonica* . **41** (**4**): 247-258.

Sasaki, M., Kleinman, H.K., Huber, H., Deutzmann, R. and Yamada, Y. (**1988**). Laminin, a multidomainprotein: The A chain has a unique globular domain and homology with the basement membrane proteoglycan and the laminin B chains. *J. Biol. Chem.* **263** (**16**): 536-544.

Schachner, M. (**1989**). Families of neural adhesion molecules in *Carbohydrate recognition in cellular function*, Wiley, Chichester (Ciba Foundation Symposium 145) pp156-172.

Schachter, H., and Brockhausen, I. (**1992**). The biosynthesis of serine (threonine)-N-acetylgalactosamine-linked carbohydrate moieties. in Allen, H.J., and Kisailus, E.C. (eds.) *Glycoconjugates: Composition, Structure, and Function*, pp. 263-332.

Schreiner, R., Schnabel, E. and Wieland, F. (**1994**). Novel N-Glycosylation in Eukaryotes: Laminin contains the linkage unit β-glucosylasparagine. *The Journal of Cell Biology*. **124** (**6**): 1071-1081.

Schwarzbauer, J.E., Tamkun, J.W., Lemischka, I.R. and Hynes, R.O. (**1983**). Three different fibronectin mRNAs arise by alternative splicing within the coding region. *Cell*. **35**: 421-431.

Sekiguchi, K. and Hakamori, S. (**1983**). Topological arrangement of four unctionally distinct domains in hamster plasma fibronectin: A study with combination of S-cyanylation and limited proteolysis. *Biochemistry*. **22**: 1415-1422.

Sekiguchi, K. and Hakamori, S.I. (**1980**). Functional domain structure of fibronectin. *Proc. Natl. Acad. Sci. U.S.A.* **77**: 2661-1665.

Sekiguchi, K., Fukuda, M. and Hakomori, S. (**1981**). Domain structure of hamster plasma fibronectin. Isolation and characterization of four functionally distinct domains and their unequal distribution between two subunit polypeptides. *J. Biol. Chem.* **256**: 6452-6462.

Skorstengaard, K., Jensen, M.S., Petersen, T.E. and Magnusson, S. (**1986b**). Complete primary structure of bovine plasma fibronectin. *Eur. J. Biochem.* **161**: 441-453.

Skorstengaard, K., Jensen, M.S., Sahl, P., Petersen, T.E. and Magnusson, S. (**1986a**). Purification and complete primary structures of the heparin-, cell-, and DNA-binding domains of bovine plasma fibronectin. *Eur. J. Biochem.* **154**: 15-29.

Skubitz, A.P.N., McCarthy, J.B., Zhoa, Q., Yi, X. and Furcht, L.T. (**1990**). Definition of a sequence RYVVLPR, vithin laminin F-9 that mediates metastatic fibrosarcoma cell adhesion and spreading. *Cancer Res.* **50**: 7612-7622.

Sly, W.S. and Grubb, J. (**1979**). Isolation of fibroblasts from patients. *Methods In Enzymology*. **LVIII**: 444-450.

Spring, J., Beck, K. and Chiquet-Ehrismann, R. (**1989**). Two contrary functions of tenascin: Dissection of the active sites by recombinant tenascin fragments. *Cell* **59**: 325-334.

Stack, S., Gray, R.D. and Pizzo, S.V. (**1990**). Modulation of plasminogen activation and type IV collagenase activity by a synthetic peptide derived from the laminin A chain. *Biochemistry.* **30**: 2073-2077.

Tamkun, J.W. and Hynes, R.O. (**1983**). Plasma fibronectin is synthesised and secreted by hepatocytes. *J.Biol. Chem.* **258**: 4641-4647.

Tanaka, H., Nakazawa, K., Arima, M., Morooka, K., Suzuki, F., Aoki, T. and Kohno, Y. (**1983**). Tuberous sclerosis: proline and hydroxyproline contents in serum. *Brain Dev.* **5**: 450-456

Tanzer, M.L., Ginigier, M.S., Dean, J.W. and Chandrasekaran, S. (**1993**). The role of glycosylation in adhesive protein function. In Jones, G., Wigley, C. V. and Warn, R., (eds.) Cell Behaviour: adhesion and motility Cambridge, Publiched for The Society for Experimental Biology by The Company of Biologist Ltd., 271-308.

Thompson, L.K., Horowitz, P.M., Bentley, K.L., Thomas, D.D. Alderete, J.F. and Klebe, R.J. (**1986**). Lokalization of the ganglioside-binding site of fibronectin. *J. Biol. Chem.* **261**: 5209-5214.

Timpl, R., Rohde, H., Robey, P.G., Rennard, S.I., Foidart, J.M. and Martin, G.R. (**1979**). Laminin-A glycoprotein from basement membranes. *The Journal of Biological Chemistry.* **254** (**19**): 9933-9937.

Timple, R., Rohde, H., Risteli, L., Ott, U., Robey, P.G. and Martin, G.R. (**1982**). Laminin , *Methods in Enzymology.* **82**: 831-838.

Towbin, H., Staehelin, T., and Gordon, J. (**1979**). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. *Proc. Natl. Acad. Sci., USA*, **76**:4350.

Tryggvason, K. (**1993**). The laminin family. *Current Opinion in Cell Biology.* **5**: 877-882.

Vuento, M. Wrann, M. and Ruoslahti, E. (**1977**). Similarity of fibronectins isolated from human plasma and spent fibroblast culture medium. *FEBS Lett.* **82**: 227-231.

Wagner, D.D. and Hynes, R.O. (**1979**). Domain structure of fibronectin and its relation to function. *J. Biol. Chem.* **254**: 6746-6754.

Watt, F.M., (**1986**). The extracellular matrix and cell shape. *TIPS 11*, Elsveir Science Publishers B.V., Amsterdam, 482-485.

Weber, P., Zimmermann, D.R., Winterhalter, K.H. and Vaughan, L. (**1995**). Tenascin-C binds heparin by its fibronectin type III domain five. The *Journal of Biological Chemistry.* **270** (**9**): 4619-4623.

Wiederholt, W. C., Gomez, M.R. and Kurland, L. T. (**1985**). Incedence and prevalence of tuberous sclerosis in Rochester, Minnesota, 1950 through 1982. *Neurology.* **35**: 600-603.

Woodley, D.T., Stanley, J.S., Reese, B.S., and O'Keefe, E.J., (**1988**). Human dermal fibroblasts synthesize laminin. *The Journal of Investigative Dermatology.* **90** (**5**): 679-683.

Yamada, K.M. (**1991**). Adhesive recognition sequences. *J. Biol. Chem.* **266**: 12809-12912.

Yamada, K.M. and Kennedy, D.W. (**1979**). Fibroblasts cellular and plasma fibronectins are similar but not identical. *J. Cell Biol.* **80**: 492-498.

Yamada, K.M. and Olden, K. (**1978**). Fibronectins-adhesive glycoproteins of cell surface and blood. *Nature.* **275**: 179-184.

Yamada, K.M., Yamada, S.S. and Pastan, I. (**1975**). The major cell surface glycoprotein of chick embryo fibroblasts in an agglutinin. *Proc. Natl. Acad.Sci. U.S.A.* **72**: 3158-3162.

Yamada, Y. and Kleinman, H.K. (**1992**). Functional domains of cell adhesion molecules. *Current Opinion in Cell Biology.* **4**: 819-823.

Zardi, L., Carnemolla, B., Siri, A., Petersen, T.E., Paolella, G., Sebastio, G. and Baralle, F.E. (**1987**). Transformed human cells produce a new fibronectin isoform by preferential alternative splicing of a previously unobserved exon. *EMBO J.* **6**: 2337-2342.

Zhu, B. C., Fisher, S.F., Pande, H., Calaycay, J., Shively, J.E. and Laine, R.A. (**1984**). Human placental (fetal) fibronectin: increased glycosylation and higher pretease resistance than plasma fibronectin. *J. Biol. Chem.* **259**: 3962-3970.

Zhu, B.C. and Laine, R.A. (**1985**). Polylactosamine glycosylation on human placental fibroneectin weakens the binding affinity of fibronectin to gelatin. *J. Biol. Chem.* **260**: 4041-4045.

Zhu, B.C. and Laine, R.A. (**1987**). Developmental study of human fetal placental fibronectin: Alterations in carbohydrates of tissue fibronectin during gestation. *Arch. Biochem. Biophys.* **252**: 1-6.