Glycoconjugate Journal (1997) **14:** 439–447

Changes in the secretion and glycosylation of fibronectin by human skin fibroblasts associated with tuberous sclerosis

Hamdi Uysal, Janice Saxton and Frank W. Hemming‡

Department of Biochemistry, The University of Nottingham, Nottingham NG7 2UH, UK

Fibroblasts from skin and skin lesions of patients with tuberous sclerosis (TS) and from skin of normal individuals were grown in culture. ELISA showed that the spent medium of those derived from TS skin lesions contained significantly more fibronectin (FN) than spent medium from the other cells. Amino acid compositional analysis of the FN from TS and normal sources revealed no substantial differences. However the FN of fibroblasts from TS-skin lesions was shown by HPAEC to contain a two- to three-fold increased content of carbohydrate. The changed monosaccharide composition was consistent with an increased content of N- and O-linked glycans and with the former containing polylactosamine chains. Fibroblasts from a normal individual were shown to proliferate more slowly and to produce larger cells when grown on FN from a TS skin lesion compared to growth on FN from normal skin.

***Keywords*: Glycosylation of fibronectin, fibronectin, tuberous sclerosis, skin fibroblasts and tuberous sclerosis**

Introduction

Tuberous sclerosis (TS) is an autosomal dominant genetic condition causing a variety of neurological and physical symptoms including seizures, mental retardation, tumours and skin lesions in 1 in 5800 newly born [1, 2]. Genetic linkage studies have established locus heterogeneity involving chromosomes 9 [3] and 16 [4, 5]. Pathogenesis of the disease probably involves aberrant tuberin, the relevant gene product of the latter chromosome [5]. It is not yet clear how this relates molecularly to the earlier suggestion [6] that the condition involves disorder in embryonic cellular migration, and consequent proliferation and differentiation. However, altered cellular migration and the characteristic hardening of tissues are indicative of a disorder involving the extracellular matrix (ECM).

Fibronectin (FN) has been implicated as a glycoprotein involved in the assembly of the ECM, in the interaction of collagens and proteoglycans with the cell surface (through integrins) and in cell migration [7, 8]. It is synthesized by skin fibroblasts and can be recovered from the culture medium in which these cells have been grown. Insufficient tissue has been available from TS patients to carry out reliable analyses but we have been fortunate enough to be able to raise fibroblast cell cultures from small skin samples from patients at surgery. We have reported previously [9, 10] that metabolic labelling studies with these cultures indicate a small increase in formation of the protein chain of FN and a significantly larger incorporation of sugars. This paper confirms with direct analytical data the increased secretion of FN and increased carbohydrate content of FN purified from TS skin lesion fibroblasts.

Materials and methods

Materials

For cell culture, Dulbecco's Modification Eagle's Medium (DMEM), trypsin and L-glutamine were purchased from Gibco BRL, UK. Foetal calf serum (FCS) was from Advanced Protein Products, UK and cell culture dishes and plates were from Costar (Northumbria Biologicals Ltd), UK. Sigma Chemical Company, UK, provided the kanamycin. For purification of FN, Prosep-gelatin was obtained from Bioprocessing Ltd, UK. Rabbit derived antisera against human FN was from Telios Company, USA and horse radish peroxidase conjugated, swine-derived antisera against rabbit antibodies was from Dako, UK. All other chemicals were of analytical grade and obtained from BDH Chemicals Ltd, UK, from Fisons Scientific Apparatus Ltd,

*Current address: Department of Biochemistry, Faculty of Veterinary Medicine, University of Ankara, Turkey.
‡To whom correspondence should be addressed. Tel: 0115 970 9358; Fax: 0115 970 9926; E-mail: Frank.Hemming@nottingham.ac.uk.

0282–0080

UK, from FSH Laboratory Supplies, UK or from Sigma Chemical Company.

Cell culture

Fibroblast cultures were established from skin tissue from patients undergoing surgery with appropriate ethical permission. Six samples were derived from affected skin (skin lesions) of patients with tuberous sclerosis (TS): TSA 1 was from a forehead plaque of a 14-year-old female; TSA 2 was from a neck fibroma of a 13-year-old male; TSA 3, TSA 4 and TSA 5 were from ungual fibromas (toe nail lesions) of a 10-year-old male, a 31-year-old female and a 43-year-old female respectively; TSA 6 was from an affected patch of skin of the upper forearm of a 10-year-old male. One sample (TSU 1) was derived from unaffected skin of the lumbosacral area of a 65-year-old male. Samples of skin from normal individuals were derived at minor surgery: N1 from a 4-month-old male; N2 from an 8-year-old male; N3 from a 12-year-old female and N4 from a 6-year-old female. N2, 3 and 4 were kindly provided by Dr Bruce Middleton, University of Nottingham.

In each case, under sterile conditions, 8–10 pieces of tissue (each 1 mm^2) were cut from skin samples that had subcutaneous fat removed and these were fixed dermal side down to the surface of culture dishes (60 mm) using a serum clot [12]. These explants were incubated in FCS (15%) in DMEM (5 ml) containing kanamycin (100 $\mu g\,ml^{-1}$) at 37 °C under 5% CO_2 in air for at least 10 days without moving. When sufficient fibroblasts had grown from the edges of the explants they were recovered by trypsinization and centrifugation before resuspension in FCS (10%) in DMEM (10 ml) containing L-glutamine (2 mM) and kanamycin (50 $\mu g\,ml^{-1}$) and culture in a cell culture dish (100 mm) at 37 °C under 5% CO_2 in air. Spent medium was replaced by fresh medium every 3 days.

When confluent, fibroblasts were recovered by trypsinization and centrifugation and redistributed into 4 cell culture dishes (100 mm) to be grown on as before.

For recovery of FN from conditioned medium cell cultures between passage 10–15 were used. The layers of cells approaching confluence were washed three times with phosphate buffered saline (pH 7.4) (PBS) (5 ml) and growth was continued for a further 2–3 days in FN-depleted medium. Conditioned medium was then removed in the presence of protease inhibitors phenylmethylsulphonyl fluoride (PMSF) (2 mM) and ethylenediamine tetraacetic acid (EDTA) (5 mM) and stored at −80 °C for a maximum of a few weeks.

The growth of normal skin fibroblasts (N3) on FN derived from TS lesions (TSA 3) and on FN derived from normal skin (N2) was compared using a method based on that described by Freshney [11]. FN was dissolved in water and a small volume of the solution was transferred to the wells of tissue culture multi-well plates and dried at 40 °C to provide a range of FN-coats of 1.5, 3 and 6 $\mu g\,cm^{-2}$ well surface. Equal numbers (0.15×10^4) of N3 fibroblasts suspended in DMEM containing FN-depleted FCS (10%) and L-glutamine (2 mM) were then transferred to each well. After incubation at 37 °C in a humidified incubator with 5% CO_2 for 48 h the cultures were examined under a light microscope.

FN-depleted medium was prepared by replacing normal FCS by that which had been passed through a Prosep-Gelatin column in an analogous manner to that described (in the next section) for recovery of FN from conditioned medium.

Recovery and purification of FN from conditioned medium

FN was recovered by binding to gelatin immobilized on a support as described by Engvall and Ruoslahti [13]. Conditioned medium 500–1000 ml was filtered through paper (Whatman no. 50) and then mixed with Prosep-Gelatin (10 ml) prewashed with PBS containing EDTA (2 mM) and PMSF (2 mM). After shaking for 2 h at room temperature the mixture was poured into a column (12 × 1.5 cm, 20 ml bed volume). The recovered medium was again passed through the column which was then washed with PBS (10 column volumes) containing PMSF (2 mM) and EDTA (2 mM) followed by PBS (10 column volumes) containing NaCl (1 M) and urea (1 M) to remove contaminants. Bound FN was then eluted with cyclohexylaminopropane sulfonate (CAPS) buffer (20 mM, pH 11, 10 column volumes) containing urea (4 M), NaCl (0.15 M), PMFS (2 mM), and EDTA (2 mM). After extensive dialysis, desalting on a prepacked PD-10 column (Pharmacia) and freeze-drying the FN preparation was redissolved in a small volume (approx. 200 µl) of CAPS buffer and stored at −80° until further purification.

A column of Superose 12 (24 ml bed volume, Pharmacia) was used in conjunction with a fast protein liquid chromatography system (FPLC, Pharmacia) in the next purification step. The eluent was of CAPS buffer (20 mM, pH 11, 35 ml, 0.2 $ml\,min^{-1}$). Fractions, 1 ml, were collected and assayed for FN by ELISA and subjected to reductive sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Electrophoresis of FN

Electrophoresis (reductive SDS-PAGE) was carried out essentially according to Laemmli [14] using a Mighty Small II SE250 Vertical Slab Electrophoresis Unit (Hoeffer Scientific Instruments, USA) and a 5% resolving gel. Gels were stained for protein with Coomassie Brilliant Blue, R-250. Western blots of electrophoretograms were produced by transferring to nitrocellulose membranes using Semi Dry Electrophoretic Transfer Equipment (Hoeffer Scientific Instruments) as described by the manufacturer. The

membranes were blocked with bovine serum albumin and then stained with rabbit anti human FN, followed by peroxidase conjugated swine anti rabbit immunoglobulin and 3-3′-diaminobenzidine plus hydrogen peroxide. Detection of glycan associated with FN on nitrocellulose blots was by a Glycan Detection Kit (Boehringer Mannheim, Germany) which utilized metaperiodate, a digoxygenin hydrazide, an alkaline phosphatase-conjugated anti-digoxygenin and a mixture of 5-bromo-4-chloro-3-indolyl phosphate and 4-nitroblue tetrazolium chloride.

Assays

ELISA of FN was based on the method of Rennard *et al.* [15]. A standard curve was prepared by coating the wells of a 96 well microtitre plate (Costar) with human plasma FN (1–110 ng). After blocking remaining absorption sites with bovine serum albumin (BSA 0.5% w/v, containing 0.05% v/v Tween 20) incubation with rabbit anti human FN antiserum for 2 h at room temperature occurred. Further washing with PBS (twice) and BSA blocking solution (twice) was followed by treatment at room temperature for a further 2 h with peroxidase conjugated swine anti-rabbit IgG and 2,2′-azion-bis-3 ethylbenzthiazoline-6-sulphonic acid plus H_2O_2. Samples of FN for assay replaced the standard plasma FN in adjacent wells. The absorbance of each well was measured at 405 nm using a plate reader (Dynatech, MR5000).

Protein in samples was assayed by a micro version of the Lowry procedure [16] using BSA (5–20 μg) as standard and a plate reader at 620 nm.

The carbohydrate composition of FN was determined by liberating the monosaccharides by hydrolysis followed by separation and assay of these using high pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) [17]. For non-acidic sugar analysis hydrolysis of samples of FN was by heating with 2 M trifluoracetic acid (200 μl) at 100 °C for 5 h. They were then dried under nitrogen gas at 30 °C, dissolved in water and injected onto a Carbo Pac PA-1 resin column (4 × 250 mm, pre-equilibrated with 17 mM NaoH) of a HPAEC-PAD system (Dionex (UK) Ltd, Camberley, Surrey) using an autoinjector (Spectra Physics SP8880, Spectra Physics Inc., USA). Monosaccharides were chromatographed isocratically with 17 mM NaOH at 1 ml min^{-1} and 30 °C over a period of 15 min. Settings of the PAD gold electrode were $E_1 = 0.05$ V, $E_2 = 0.8$ V, $E_3 = -0.15$ V and pulse durations of $T_1 = 420$ ms, $T_2 = 180$ ms and $T_3 = 360$ ms with a response time of 1 s and a range of 1 μA. The method was precalibrated with fucose, galactosamine, galactose, glucosamine, glucose and mannose with respect to elution time, detector response and resistance to the trifluoroacetolysis regime.

For sialic acid assays FN samples were heated with 50 mM trifluoracetic acid (200 μl) for 1 h at 80 °C. The carbo Pac PA-1 column was pre-equilibrated with NaOH (100 mM) containing sodium acetate (NaAc, 50 mM). Elution was by a linear gradient of 50–150 mM NaAc (in 100 mM NaOH) over 7.5 min followed isocratically at 150 mM NaAc–100 mM NaOH over 7.5–15 min and a further linear gradient from 150 to 500 mM NaAc (in 100 mM NaOH) over 15–20 min all at 1 ml min^{-1} and at 30 °C. PAD settings were as before. The method was precalibrated with *N*-acetylneuraminic acid and *N*-glycoloylneuraminic acid for elution time, detector response and resistance to the trifluoracetolysis regime.

In both carbohydrate analyses data from the PAD was collected, stored and analysed using a RaChel (Lablogic, UK) software program that took account of the calibration of each method.

The amino acid composition of samples of FN was determined independently by the Biopolymer Synthesis and Analysis Unit (BSAU) of this department. This involved PAGE (5%) followed by blotting onto a polyvinylidene difluoride (PVDF) membrane using a CAPS buffer. The blots were stained with amido black and stained bands of FN were marked and, after destaining, were excised for analysis by an automated amino acid analysis system (Applied Biosystems model 420H, USA).

Several attempts were made to sequence the amino-terminal peptide of samples of FN (after blotting as above) by Edman degradation [18] in the BSAU using an automated pulsed liquid phase protein sequencer (Applied Biosystems model 473A). Each attempt failed to yield a sequence although control proteins gave the expected sequences.

Results

Presence of FN in fibroblast culture medium

The FN-content of conditioned culture medium from several fibroblast cultures was assayed by ELISA using a polyclonal antiserum from rabbit specifically against human FN as described by Rennard *et al.* [15]. The method was calibrated with pure human plasma-FN over the range 1 to 110 ng. Table 1 shows that all three TS-lesion-derived fibroblasts secreted more FN into the medium than did control fibroblasts. The increase was particularly marked in fibroblasts raised from an ungual fibroma (TSA 3).

Purification of FN

Fibroblasts from TS- and normal skin were grown at 37 °C until they reached confluence. At this stage the standard medium was replaced by FN-depleted medium and after further culture for 2–3 days the conditioned medium was harvested in the presence of protease inhibitors and stored at −80 °C for a maximum of a few weeks.

FN was isolated from conditioned medium by affinity chromatography on a Prosep-gelatin column by a method established by Engvall and Ruoslahti [13]. The elution of

Table 1. ELISA measurement of human FN in fibroblast culture medium after 72 h of growth. The mean of triplicate assays is given. Each assay was within 5% of the mean.

ng FN per 100 µl	
Normal cells	
N2	10.26
N3	10.44
TS cells	
TSA 1	13.01
TSA 2	15.21
TSA 3	45.40

protein from the column by PBS followed by 1 M NaCl and 1 M urea in PBS was monitored by absorption of light at 280 nm. When absorption had fallen from approximately 0.2 to close to baseline the bound FN was eluted by 4 M urea in 20 mM CAPS pH 11 containing 0.15 M NaCl, 2 mM PMFS and 2 mM EDTA. Assay by ELISA before and after chromatography indicated a recovery of FN routinely of above 85%. Reductive SDS-PAGE (4–15% gradient) of the FN-containing fractions indicated the presence of small amounts ($<10\%$) of several other proteins.

The partially-purified FN was then subjected to FPLC on Superose-12. The eluted fractions were again monitored for protein (280 nm) and FN (ELISA). FN-containing fractions were combined and shown to be pure by 5% SDS PAGE and staining with Coomassie Blue, silver (not shown) and antisera to human FN as shown in Figure 1. Recovery of samples of FN from the Superose-12 column was approximately 100–250 µg depending on the sample, approaching 70% of that applied.

FN from fibroblasts of TS-lesions, TS-unaffected skin and from normal skin fibroblasts had identical electrophoretic mobility to commercial FN running as a broad band with a mean size of 230 kDa in reducing SDS-PAGE. Typical gels are shown in Figure 1.

Amino acid analysis of FN

The amino acid composition of purified FN derived from fibroblasts of TS skin lesions and unaffected skin was compared with that of FN from fibroblasts from control individuals, with that of cellular FN obtained pure commercially and with published results [19]. No consistent and significant differences appeared between TS and control samples.

Attempts to sequence the N-terminus of samples of TS- and control FN failed, consistent with the N-terminus of each peptide chain being blocked as has been described previously for plasma FN [20].

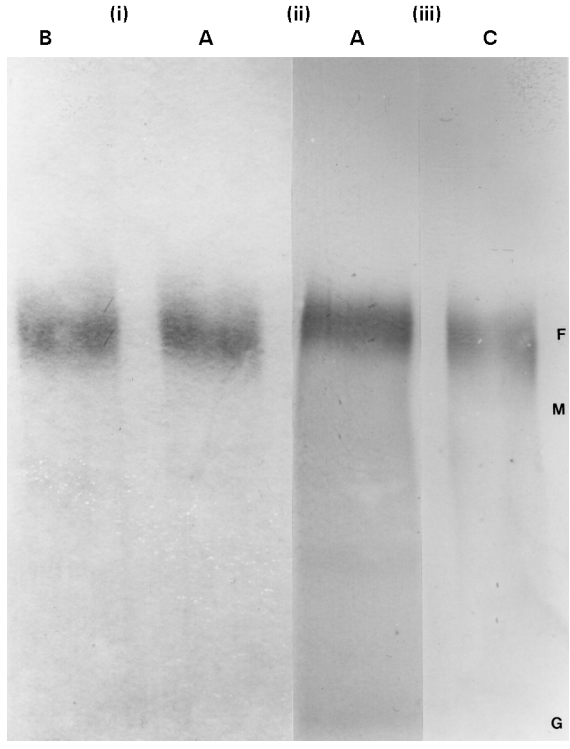

Figure 1. Reductive SDS-PAGE (5% gel) of FN purified from the culture medium of TSU(A) NI(B) and TSA 2(C) fibroblasts: (i) stained with Coomassie Brilliant Blue; (ii) Western blot stained using rabbit anti-human FN, peroxidase-conjugated swine anti-rabbit immunoglobulin and 33′diaminobenzidine; (iii) blot stained with a DIG-Glycan Detection Kit (Boehringer). The position of standards run on adjacent gels is also shown: F, commercial fibronectin; M, myosin (205 kDa); G, β-galactosidase (116 kDa).

Carbohydrate analysis of FN

All samples of purified FN stained positively with the DIG Glycan Detection Kit (see *eg* Figure 1). Each sample showed one broad band of glycan-positive material that correspond in electrophoretic mobility exactly to the protein-positive and anti-FN positive band.

Samples of FN were desalted, lyophylized and aliquots (25–100 µg) were hydrolysed with TFA as detailed in the Assays section. HPAEC was then used to separate individual neutral monosaccharides and sialic acid and to assay them by pulsed amperometric detection precalibrated for each individual sugar (Assays section). Typical results are shown in Figure 2. Figure 2(i) demonstrates clearly the presence of six neutral monosaccharides (it is assumed that the aminosugars were present on FN as *N*-acetyl derivatives) and Figure 2(ii) provides good evidence for the presence of *N*-acetylneuraminic acid. There was no evidence for the presence of *N*-glycolylneuraminic acid which in this HPAEC system has a retention time of 15 min.

Table 2 summarizes the results for assays of carbohydrate composition FN from TS- and control fibroblasts. The peak

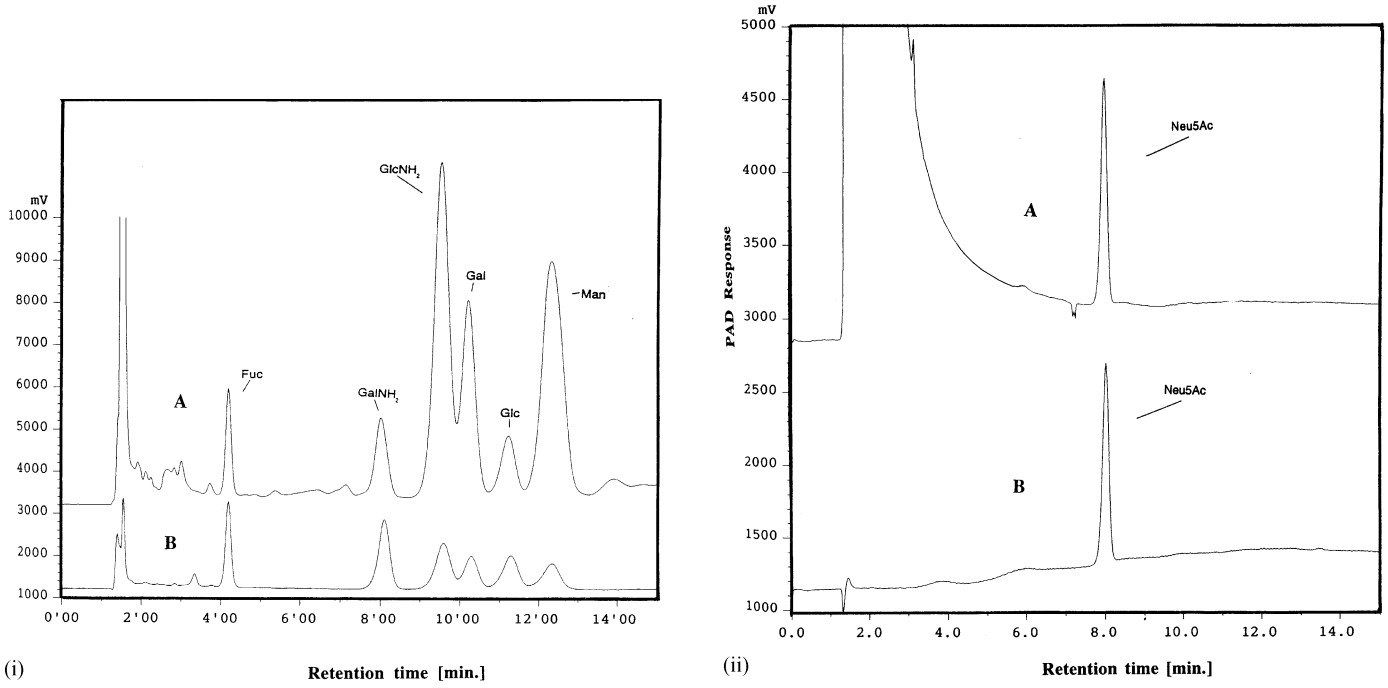

Figure 2. Elution profile from HPAEC-PAD (i) of neutral monosaccharide analysis of FN derived from TSA 2 fibroblasts (A) and also of a standard mixture of pure monosaccharides (B). The assignment of each peak is Fuc-fucose, $GalNH_2$-galactosamine, $GlcNH_2$-glucosamine, Gal-galactose, Glc-glucose and Man-mannose; (ii) of *N*-acetylneuraminic acid analysis of FN derived from TSA 2 fibroblasts (A) and also of authentic *N*-acetylneuraminic acid (Neu5Ac) (B) (see section 2.5 for detailed methodology).

Table 2. Monosaccharide composition of FN produced by skin fibroblasts of different sources. Figures are mol monosaccharide per mol (460 kDa) FN based on HPAEC-PAD analysis of duplicate samples of FN (variation between duplicates was less than 5%).

Monosaccharide	*N2 fibroblasts A*	*TSU fibroblasts B (B/A)*	*TSA 2 fibroblasts C (C/A)*	*TSA 4 fibroblasts D (D/A)*
Fucose	5.3	3.9 (0.7)	9.3 (1.8)	7.3 (1.4)
Galactosamine	3.1	2.3 (0.7)	7.6 (2.5)	5.2 (1.7)
Glucosamine	21.5	18.8 (0.9)	63.9 (3.0)	47.1 (2.2)
Galactose	14.1	15.9 (1.1)	31.6 (2.2)	36.8 (2.6)
Mannose	16.5	18.3 (1.1)	48.4 (2.9)	33.9 (2.1)
N-acetyl-neuraminic acid	7.3	9.4 (1.4)	23.0 (3.2)	24.4 (3.3)
Total	67.8	68.6 (1.0)	183.8 (2.7)	154.7 (2.3)

for glucose in all samples corresponded to a peak in the same position and of similar intensity in blank runs and was assumed to be a contaminant and therefore omitted from the table. Only results for those samples sufficiently large to allow duplicate assays are reported. A further analysis of one sample of FN from control fibroblasts (N3) confirmed the analysis for N2 in Table 2. Single analyses of the very small amounts of FN available from the other TSA samples gave results within the range set by TSA2 and TSA4. The analyses show that FN produced by fibroblasts from TS skin lesions contained two- to three-fold more carbohydrate than that from normal skin. Of the individual monosaccharides the change in sialic acid content (about threefold) was the greatest and that in fucose content (about 1.5-fold) was the smallest. Overall the carbohydrate content of FN produced by fibroblasts of unaffected TS-skin did not change although this included small increases in some monosaccharides offset by small decreases in others.

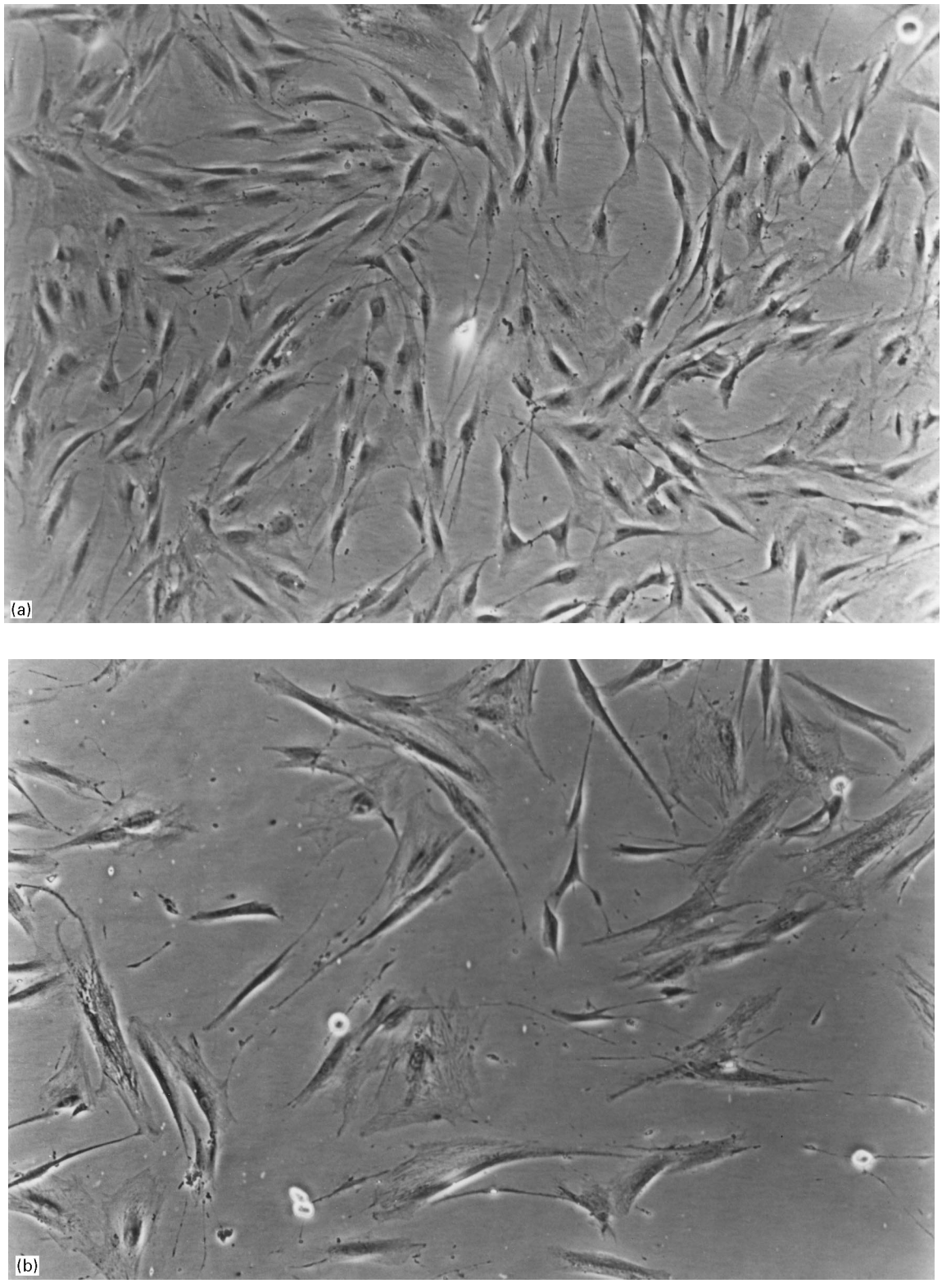

Figure 3. Normal skin fibroblasts (N-3) cultured on: (A) FN (3 μg cm^{-2}) purified from conditioned medium of normal skin fibroblasts (N-2); (B) FN (3 μg cm^{-2}) purified from conditioned medium of TS skin lesion fibroblasts (TSA 3) (magnification × 26).

Effects of FN on cell morphology and spreading

A preliminary comparison was made of TSA 3 derived FN with N2 derived FN with regard to their effect on the morphology and spreading of N3-fibroblasts. When coated on tissue culture multi well plates at both 3 and 6 $\mu g\,cm^{-2}$ they both promoted cell adhesion. However routinely proliferation on TSA 3 derived FN was slower (approximately 30–50%) and many of the cells were larger. Typical results can be seen in Figure 3. Insufficient TSA3-derived FN was available for further studies in this area. The morphology of N3-fibroblasts grown on human plasma FN was identical to that on N2 derived cellular FN (data not shown) although proliferation was slightly slower (80–90%).

Discussion

Table 1 demonstrates that all three lines of fibroblasts derived from skin lesions of TS patients release into the growth medium increased quantities of FN compared with control fibroblasts. This is in agreement with earlier preliminary observations [9] from this laboratory of an increased metabolic incorporation (120% $\pm$ 11, SD) by all seven lines of TS-skin lesion fibroblasts of [^{3}H] leucine and [^{35}S] methionine into material electrophoretically and immunochemically (by Western blotting) identical to FN. This confirms that this latter data was not the result of differences in pool sizes of endogenous (unlabelled) amino acids. The four- to five-fold increase in FN recovered from the culture medium of fibroblasts (TSA 3) derived from an ungual fibroma is particularly striking and not typical of other TS lesions. These differences alone might well be expected to contribute to aberrations in the assembly of the extracellular matrix if they also occur in tissue, and hence, contribute to the sclerosis of this condition.

Chromatographic purification on Prosep-gelatin and FPLC on Superose-12 yielded samples of pure FN from the culture medium of TS fibroblasts showing a single broad band of 230 kDa in reducing SDS-PAGE indistinguishable from that recovered from normal fibroblasts. Any differences in carbohydrate composition were apparently insufficient to affect electrophoretic mobility of the samples.

Amino acid compositional analysis of several samples of FN from TS and control fibroblasts revealed no gross consistent differences. It is also relevant that all samples of TS-FN could be stained on Western blots of electrophoretograms by anti human FN and that they could all be assayed by the ELISA technique suggesting no major changes in the epitopes present on the molecule. It is doubtful, however, if these procedures are sufficiently sensitive to rule out the possibility of differences resulting from alternative splicing, a phenomenon that has been described for FN in some other diseased conditions and in other species [7, 8]. Unfortunately it was not possible to determine the N-terminal sequence of any samples of FN, presumably because this was blocked [20].

The positive staining of all samples of FN on blots of electrophoretograms as a single broad band with the Glycan Detection Kit (see Figure 1) confirmed that they were glycoproteins in a pure state, as expected. A detailed monosaccharide analysis using HPAEC-PAD of several of these samples established that FN from fibroblasts of TS skin lesions were more heavily glycosylated than from those from normal skin and from unaffected skin (Figure 2, Table 2). This is consistent with the increased (154% $\pm$ 15 SD) incorporation of [^{3}H] glucosamine (relative to radioactive leucine) into the FN secreted by all of seven lines of TS-skin lesion fibroblasts studied previously [9]. In Table 2 only results for FNs available in sufficient quantities for duplicate assays are reported.

Cellular FN possesses seven or eight potential N-glycosylation sites [8]. The extent to which these carry N-linked glycans and the nature of the glycans varies with the species and cell type producing the FN and can be influenced by the developmental stage of the tissue and by oncogenic transformation. Variation in the extent of *O*-glycosylation with reports of up to five or six O-linked glycans per molecule has also been observed [21, 22].

Previous work [23] suggests an average occupancy of potential N-glycosylation sites of approximately five in FN recovered from the culture medium of normal adult human skin fibroblasts. The glycans present were primarily of the biantennary complex type, approximately half of which were neutral, one third were mono-sialylated and one sixth disialylated. Others [24] have suggested that cellular FN invariably has a fucose substituent on the *N*-acetylglucosamine adjacent to the asparagine. This would require a compositional analysis of $fucose_5$, $glucosamine_{20}$, $galactose_{10}$, $mannose_{15}$ and *N*-acetylneuraminic $acid_{3-4}$ for the N2 sample of FN. A further two to four residues each of galactosamine, galactose and *N*-acetylneuraminic acid in the form of O-linked glycans would be consistent with the analytical data for this sample of FN in Table 2. Relatively minor modification of this pattern of N- and O-glycosylation would also explain the result for the FN of fibroblasts from unaffected skin of a TS patient (TSU in Table 2).

A striking feature of Table 2 is the large increase in the carbohydrate content of both samples of FN produced by fibroblasts of TS skin lesions (TSA 2 and TSA 4). Some of this change may result from an increased occupancy of potential N-glycosylation sites and from increased O-glycosylation. However, several aspects of detail in Table 2 suggest also a major change in the nature of the glycan chains present. The high content of mannose would require the presence of two to four high mannose ($Man_9GlcNAc_2$) glycans presumably the result of escaping mannosidase processing.

The elevated levels of glucosamine, galactose and *N*-acetylneuraminic acid are consistent with the presence of

four to five tri- or tetraantennary N-glycans containing, in total, approximately 30 *N*-acetyllactosamine units. These changes are reminiscent (but more marked) of the situation for cellular FN of human placenta [25] and amniotic fluid [21]. In placental FN increases in glucosamine and galactose appear to be due partly to the presence of tri- and tetra-antennary N-glycans and in particular to the presence of linear polylactosaminyl chains containing between four and twenty-five *N*-acetyllactosamine units per N-glycan [25, 26]. The presence of a bisecting *N*-acetylglucosamine residue on the β-linked mannose has also been observed on some of these N-glycans [26]. The possibility of N-glycosylation of nine asparagine residues per molecule of FN is also raised in this paper. The presence of linear polylactosaminyl chains on a small proportion of the complex N-glycans of FN of human amniotic fluid has also been described and again a bisecting *N*-acetylglucosamine was common [26]. Amniotic FN has a galactosamine content of 8–9 mol^{-1} FN [24] with each molecule of FN carrying four to five O-glycans [26]. A similar extent of O-glycosylation on TSA FNs could account for the increased galactosamine and for part of the increased galactose and *N*-acetylneuraminc acid present.

Thus, most aspects of the increased glycosylation of FN of TS-skin lesion fibroblasts resemble features observed in cellular FNs from other human sources. Extensive structural analysis of the TSA-FNs will be necessary to establish just how closely related their structures are to the glycans of other human FNs. However, the small amounts of TSA-FNs available may make this a difficult task.

The preliminary observation that proliferation of normal skin fibroblasts on TSA-FN and their morphology is different from that on normal FN is not altogether surprising in view of earlier reports of the influence of changed glycosylation on the binding domains of the molecule. It has been suggested that an increased number of sugar chains and/or the presence of polylactosamine groups in N-linked glycans weakens the binding of FN to collagen groups [27–29]. Also, the binding of normal skin fibroblasts to FN is reported to be reduced by N-linked glycans [29]. The presence of polylactosamine units has been demonstrated to increase the resistance of FN to proteolysis [25]. Clearly this could have a bearing on the recovery of larger quantities of FN from the medium of TSA fibroblasts (Table 1). It appears that changes in glycosylation of FNs may well result in modulation of the interactions between fibroblasts and extracellular matrix to produce significant changes in cellular characteristics.

In conclusion, we have established by direct assay on purified samples of FN from fibroblasts from skin lesions of TS patients marked changes in the extent and pattern of glycosylation. These results confirm and extend preliminary observations [9, 10] based on metabolic labelling demonstrating that these latter results were not the result of changed endogenous pools of glycoprotein precursors. We suggest that these changes in glycosylation and expression of FN influence the interaction of cells with extracellular matrix in ways that may contribute to tissue hardening and possibly to embryonic migration of cells, both of which are associated with tuberous sclerosis.

Acknowledgements

We wish to acknowledge the University of Yuzuncu Yil, Turkey for a research scholarship to H.U.; the Tuberous Sclerosis Association of Great Britain for support of a tissue and cell culture bank and K. Bailey for the protein analysis in the BSAU, University of Nottingham.

References

1 Gomez MR (1979) *Tuberous Sclerosis*. New York: Raven Press.
2 Osborne JP, Fryer AE, Webb D (1991) *Ann NY Acad Sci* **615**: 125–27.
3 Fryer AE, Chalmers A, Connor JM, Fraser U, Povey S, Yates AD, Yates JRW, Osborne JP (1987) *Lancet* **1**: 659–61.
4 Kandt RS, Haines JL, Smith M, Northrup H, Gardner RJM, Short MP, Dumars K, Roach ES, Steingold S, Wall S, Blanton SH, Flodman P, Kwiatkowski DJ, Jewell A, Weber JL, Roses AD, Pericak-Vance MA (1992) *Nature Genet* **2**: 37–41.
5 Nellist M, Janssen B, Brook-Carter PH, Henselling-Janssen ALW, Maheshwar MM, Verhoef S, Van den Ouweland AMW, Lindhout D, Eussen B, Cordeiro I, Santos H, Halley DJJ, Sampson JR, Ward CJ, Peral B, Thomas S, Hughes J, Harris PC, Roelfsema JH, Saris JJ, Spruit L, Peters DJM, Dauwerse JG, Breuning MH (1993) *Cell* **75**: 1305–15.
6 Johnson WG, Gomez MR (1991) *Ann NY Acad Sci* **615**: xi-xii.
7 Hynes RO (1990) *Fibronectins*. New York: Springer-Verlag, series in Molecular Biology.
8 Ruoslahti E (1988) *Annu Rev Biochem* **57**: 375–413.
9 Ellis ZK, Hemming FW (1990) In *Tuberous Sclerosis and Neurofibromatosis*: *Epidemiology*, *Pathophysiology*, *Biology and Management* (Ishibashi Y, Hori Y, eds) Amsterdam: Elsevier p 123–34.
10 Ellis ZK, Hemming FW (1991) *Ann NY Acad Sci* **615**: 149–157.
11 Freshney RI (1992) *Animal Cell Culture*: *A Practical Approach*, 2nd edition, Oxford: Oxford University Press.
12 Sly WS, Grubb J (1979) *Methods Enzymol* **58**: 444–50.
13 Engvall E, Ruoslahti E (1977) *Int J Cancer* **20**: 1–5.
14 Laemmli UK (1970) *Nature* **227**: 680–85.
15 Rennard ST, Berg R, Martin GR, Foldart JM, Robey PG (1980) *Anal Biochem* **104**: 205–211.
16 Lowry OH, Rosebrough RJ, Farr AL, Randall RJ (1951) *J Biol Chem* **193**: 265–72.
17 Hardy MR, Townsend RR (1994) *Methods Enzymol* **230**: 208–25.
18 Edman P, Begg G (1967) *Eur J Biochem* **1**: 80–91.
19 Vuento M, Wrann M, Ruoslahti E (1977) *FEBS Lett* **82**: 227–31.
20 Furie MB, Rifkin DB (1980) *J Biol Chem* **255**: 3134–40.
21 Ruoslahti E, Engvall E, Hayman EG, Spiro RG (1981) *Biochem J* **193**: 295–99.

22 Krusius T, Fukuda M, Dell A, Ruoslahti E (1985) *J Biol Chem* **260**: 4110–16.
23 Ledger PW, Nishimoto SK, Hayashi S, Tanzer ML (1983) *J Biol Chem* **258**: 547–54.
24 Nichols EJ, Fenderson BA, Carter WG, Hakomori S (1986) *J Biol Chem* **261**: 11295–301.
25 Zhu BC-R, Fisher SF, Pande H, Calaycay J, Shively JE, Laine RA (1984) *J Biol Chem* **259**: 3962–70.
26 Takamoto M, Endo T, Isemura M, Kochibe N, Kobata A (1989) *J Biochem* **105**: 742–50.
27 Zhu BCR, Laine RA (1985) *J Biol Chem* **260**: 4041–45.
28 Yamaguchi Y, Isemura M, Yosizawa Z, Kan M, Sato A (1986) *Int J Biochem* **18**: 437–43.
29 Jones GE, Arumugham RG, Tanzer ML (1986) *J Cell Biol* **103**: 1663–70.

Received 16 July 1996, revised 30 September 1996