ROLE OF GLYCOSYLATİON İN BİOLOGİCAL FUNCTİON OF FİBRONECTİNS

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Fibronectins are the group of large, multifunctional adhesive glycoproteins that play a significant role in promoting cell adhesion, cell shape and spreading, wound healing and particularly in extracellular matrix (ECM) formation. There are at least two major classes of fibronectins which are termed ‘‘cellular fibronectin’’ and ‘‘plasma fibronectin’’. Cellular fibronectin is produced mainly by fibroblasts and other different cell types. The plasma form of fibronectin circulates in the blood is mainly secreted in the liver by hepatocytes.

In this study, cellular fibronectin was purified from conditioned medium of established fibroblasts from human skin explants. Purity of fibronectin sample was confirmed by sodium dodecyl sulphate polyacrylamide-gel electrophoresis (SDS-PAGE). Carbohydrate compositions of human plasma and cellular fibronectins were analysed by high pH anion-exchange chromatography (HPAEC-PAD) using a Carbopac PA100 column. Results of the study demonsrated that the plasma and cellular fibronectins showed structural differences in terms of glycosylation. Cellular fibronectin has no fucose in response to the fucose of plasma fibronectin. Monosaccharide content (especially sialic acid content) in the plasma fibronectin (3.88%) is greater than cellular fibronectin (2.81%). It is well known that sialic acid (N-acetyl neuraminic acid) of plasma fibronectin is linked alpha (2-6) to galactose while sialic acid of cellular fibronectin is linked alpha (2-3) to galactose. Moreover, to investigate the effects of cellular fibronectin and plasma fibronectin on fibroblasts, cell culture plates were coated with different concentrations (1.5μg/cm^2, 3μg/cm^2 and 6μg/cm^2) of purified cellular and plasma fibronectins. We have observed that plasma and cellular fibronectins at different concentrations were equally active in promoting cell attachment. However, cellular fibronectin obtained from skin fibroblasts appeared more active in the promotion of cell spreading, proliferation and morphology of fibroblasts than plasma fibronectin. It can be concluded that functional differences between the cellular and plasma form of fibronectins may be due to the changes in glycosylation of fibronectin.

Key Words: *Glycosylation, biological role, cellular fibronectin, plasma fibronectin.*

Introduction

Fibronectins (FNs) are adhesive glycoproteins present on cell surfaces and in extracellular matrices. FNs play important roles in promoting cell adhesion, migration, growth and differentation, cell shape and spreading, wound healing and particularly in extracellular matrix (ECM) formation (Mosher, 1989; Carsons, 1989; Hynes, 1990; Yamada and Clark, 1996).

FNs are the "grandfather" of extracellular matrix proteins and are currently one of the most widely studied and best known matrix proteins, although much still remains to be learned about its structure and function.

FNs present on cell surfaces and in extracellular matrices, as well as in blood, amniotic fluid, and cerebrospinal fluid. There are at least two major classes of fibronectins which are termed ‘‘cellular fibronectin’’ and ‘‘plasma fibronectin’’. Cellular fibronectin is produced mainly by fibroblasts and other different cell types. The plasma form of fibronectin circulates in the blood is mainly secreted in the liver by hepatocytes (Hynes, 1990).

FNs are glycoproteins that contain 4-9% carbohydrate, depending on the cell source.

Glycosylation sites that are either N-linked or O-linked reside predominantly within type III repeats and the collagen-binding domain. The physiological role of the carbohydrates is not certain, although they appear to stabilize FN against hydrolysis and modulate its affinity to some substrates.

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 0- 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. 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 a 2-3 galactose in contrast to the a 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).

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).

Objective of this study was to determine and compare the glycosylation pattern of plasma and cellular fibronectins as well as discussing the possible biological functions of fibronectin glycosylation.

Materials and Methods

Fibroblast Cultures

In this study, fibroblasts were established from primary cultures of skin explants. A cell culture system has been employed to study the adhesive glycoprotein fibronectin synthesised by these established skin fibroblasts (see Fig. 1). Established fibroblasts from normal skin were grown at 37^{o} 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 skin fibroblasts was stored at -800C until sufficient medium was available for fibronectin purification.

Purification of Cellular Fibronectin

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

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).

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 (24m1 bed volume) was first equilibrated with 5 bed volumes of 20mM CAPS buffer, pH 11. After obtaining a flat baseline on the chart recorder, 200µ1 of fibronectin solution was injected into the column and fractions were collected either manually or using a fraction collector, at constant flow rate (0.2m1/minute). Proteins were detected by measuring the absorbance at 280nm. Thirty-two lml 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 SDS-PAGE. Coomassie blue, and antibody staining of the reduced purified fibronectin from skin fibroblasts analysed by 5% SDS-PAGE showed a single broad band with approximate molecular weight of 230 000 dalton.

Carbohydrate Analysis of Human Plasma and Cellular Fibronectins

Carbohydrate compositions of human plasma and cellular fibronectins were analysed by high pH anion-exchange chromatography (HPAEC-PAD) using a Carbopac PA100 column (Table 1).

Effects of plasma and cellular fibronectins on fibroblasts morphology and spreading

To investigate the effects of cellular fibronectins purified from conditioned medium of skin fibroblasts, and plasma fibronectin (Gibco) on fibroblasts, cell culture plates were coated with different concentrations (1.5µg/cm^2, 3µg/cm^2 and 6µg/cm^2) of purified cellular and plasma fibronectins. A procedure according to Freshney (1992) to coat tissue culture plastic plate with fibronectin was used.

Results

Fibroblast Cultures

Established fibroblasts from the skin explants were grown at 37^{o} C until they reached confluence (see fig. 1). 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 skin fibroblasts was stored at -80^{o} C until sufficient medium was available for fibronectin purification.

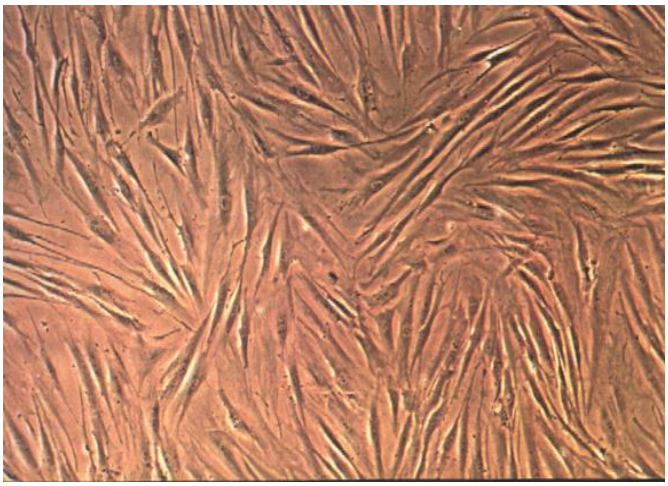

Figure 1. The figure shows the typical control fibroblasts established from human skin. 26x magnification.

Purification of Cellular Fibronectin

In this work, fibronectin was purified (see Fig.2) from culture medium of fibroblasts derived from the skin of 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.

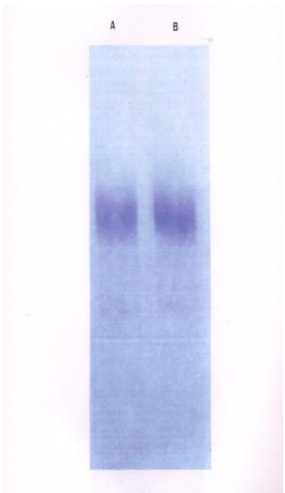

Figure 2. SDS-PAGE (5% gel) analysis of purified cellular fibronectin (A and B lines, 8µg

each) from skin fibroblasts using gelatin- affinity and size column chromatography (gel filtration) techniques. Purified fibronectin was detected by staining with Coomassie Brilliant Blue.

Carbohydrate Analysis of Human Plasma and Cellular Fibronectins

Fibronectin was purified from conditioned medium of established fibroblasts from human skin explants. The purity of each fibronectin sample was established by SDS-PAGE as demonstrated in Figure 2. Glycosylation of the purified fibronectin was confirmed using a Dig-Glycan detection kit (Boehringer) on blotted membranes (see Fig. 3). The pure fibronectin samples were de-salted using SephadexG -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 cellular fibronectin were subjected to acid hydrolysis in 2M trifluoroacetic acid (TFA) at 1000 C for 5 hours to release the neutral sugars, and 50mM TFA at 800 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 PA 100 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. Separated monosaccharides were detected by a pulsed amperometric detector (PAD).

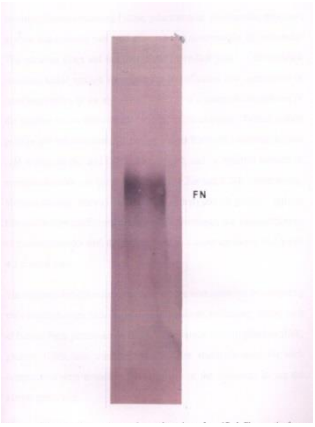

Figure 3. The figure shows glycan detection of purified fibronectin. 12µg of fibronectin sample mixed with sample buffer containing SDS and mercaptoethanol was separated by 5% SDS-PAGE. The gel was blotted onto nitrocellulose, and the fibronectin was detected with a DIG-Glycan Detection Kit (Boehringer).

Carbohydrate analysis showed that carbohydrate portion of the fibronectin molecule is made up of galactose, mannose, glucosamine, galactosamine, sialic acid, and fucose. Fibroblast-derived fibronectin contains fucose linked to the innermost N- acetylglucosamine, and its sialic acid is linked alpha 2-3 to galactose, whereas plasma fibronectin lacks fucose. 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 skin fibroblasts. Carbohydrates of cellular fibronectin from conditioned medium of skin fibroblasts were analysed and the results are presented in Table 1. The carbohydrate composition of plasma fibronectin (Gibco) was also analysed in duplicate and presented in Table 1.

In another analysis, sialic acid (Neu5Ac) was released from purified fibronectin samples in 50mM TFA for l hour at 80^{o}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 skin fibroblasts are presented in Table 1.

Monosaccharides	Human plasma fibronectin % Composition*	Purified fibronectin of fibroblasts derived from normal skin % Composition*
Fucose	Absent	0.19
Galctosamine	0.04	0.12
Glucosamine	1.12	0.82
Galactose	0.72	0.55
Mannose	0.94	0.64
Sialic acid	1.06	0.49
Total % sugar	3.88	2.81

Table 1. Carbohydrate compositions of human plasma (Gibco) and cellular fibronectins, which were purified from conditioned medium of fibroblasts grown from skin explants. Values for compositions are given as the mean of dublicate determinations from independent preparations and are expressed as sugar weight per 100 mg of glycoprotein weight. Variations within dublicates were always within ±5%. Glucose was excluded due to its presence in control samples.

Effects of plasma and cellular fibronectins on fibroblasts morphology and spreading

To investigate the effects of cellular fibronectins purified from conditioned medium of skin fibroblasts, and plasma fibronectin (Gibco) on fibroblasts, cell culture plates were coated with different concentrations of purified cellular and plasma fibronectins. Our results (Fig.4) showed that the appearance of the fibroblasts was very similar (and normal) on the cultured plates coated with plasma, and normal cellular fibronectin derived from healthy skin. However, cellular fibronectin coated fibroblast cultures (see Fig.4-A) were more confluent than plasma fibronectin coated culture plates (see Fig. 4-B). This indicated that cellular fibronectin is more active than plasma fibronectin in the promotion of the cell spreading, proliferation and morphology.

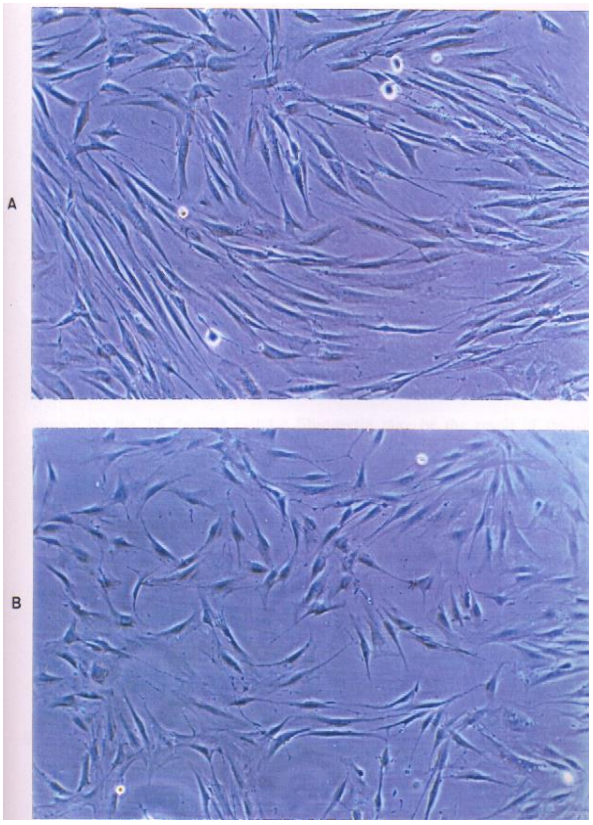

Figure 4. Morphological effects on normal skin fibroblasts of the purified cellular fibronectin from conditioned medium of normal skin fibroblasts (A), and plasma fibronectin purchased from Gibco (B). Photographs were taken at 26x magnification on 6gg/cm2 fibronectin-coated plates.

Discussion

The results of the carbohydrate analysis of purified fibronectin from conditioned medium of skin fibroblasts are presented and compared with plasma fibronectin in this study.

Monosaccharide analysis of purified cellular 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. However, 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. Nevertheless, the sialic is N-glycolyl neuraminic acid in hamster plasma fibronectin according to Fukuda and colleagues (1982).

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). Fibronectin glycosylation is involved in promotion of fibroblasts adhesion and spreading, and playing significant roles in

the extracellular matrix (ECM).

To observe the effects of cellular fibronectins purified from conditioned medium of skin 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 cellular fibronectin coated cell culture plates. Interestingly, the appearance of the fibroblasts was very similar (and normal) on the cultured plates coated with plasma, and normal cellular fibronectin derived from healthy 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.

It is concluded that changes in the glycosylation of fibronectin may well alter its association with components of the extracellular matrix and with cell surface. Hence, 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.

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