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COMPARATIVE STRUCTURAL STUDIES OF HUMAN PLASMA AND AMNIOTIC FLUID FIBRONECTINS

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Summary

Human fibronectins from plasma and amniotic fluid were compared in terms of their migrations on SDS and isoelectric focusing gels, immunological reactions, amino acid and carbohydrate compositions, tryptic maps NH_2-terminal sequence analysis, and methylation analyses. Both plasma and amniotic fluid fibronectins had the following NH_2-terminal sequence: pGlu-Ala-Gln-Gln-Met-Val-Gln-(Cys)-?-?-Ser-Tyr-Ser. It is concluded that the fibronectins studied have closely similar polypeptide chains, but differ significantly in percent total carbohydrate and the relative amounts and types of sugar linkages. Plasma fibronectin has 4.8% carbohydrate with predominantly 2- and 3,6-linked mannose, 4- and 6-linked galactose, 4-linked N-acetylglucosamine, and terminal sialic acid. Amniotic fibronectin has 6.9% carbohydrate with 2,4-linked mannose in addition to 2- and 3,6-linked mannose, 3- and 4-linked galactose (but no 6-linked galactose), 4-linked N-acetylglucosamine, and terminal fucose, galactose, mannose, and N-acetylglucosamine in addition to sialic acid.

Fibronectin is a high molecular weight glycoprotein found on the surface of fibroblasts (1), in plasma (2), and in amniotic fluid (3). It is composed of two polypeptide chains (Mr = 220,000) covalently linked by disulfide bonds. The ability of fibronectin to bind to collagen or to collagen together with glycosaminoglycans may be important in the adhesion of fibroblasts to substrata, cell to cell interactions, the maintenance of normal morphology, cellular migration, and phagocytosis of gelatinized particles by Kupffer cells in the liver (4). Studies on fibronectin isolated from different sources (cells, plasma, or amniotic fluid) have suggested that differences in molecular forms may exist; however, the various forms of fibronectins are immunologically indistinguishable and have similar amino acid compositions (5,6).

[1]Abbreviations used are: SDS, sodium dodecyl sulfate; PFN, plasma fibronectin; AFN, amniotic fluid fibronectin; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediamine tetraacetic acid; DTT, dithiothreitol.

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This report compares the carbohydrate structural units and NH_2-terminal sequence analysis of the plasma and amniotic fluid forms of human fibronectin. Evidence is presented that suggests that the major difference between the two forms of fibronectin resides in the carbohydrate portion of the molecules.

Experimental Procedures

Isolation and Compositional Analysis of Fibronectins - Fibronectin was purified from pooled human plasma or amniotic fluid by affinity chromatography on gelatin-Sepharose (7). SDS[1]-polyacrylamide gel electrophoresis was performed according to Laemmli (8) using 6% or 12% gels. Proteins were stained with Coomassie brilliant blue. Amino acid analyses were performed as previously described (9). Duplicate samples (20 µg) were hydrolyzed *in vacuo* at 110°C for 48 hr with 0.5 ml of 3M p-toluenesulfonic acid containing 0.2% 3-(2-aminoethyl)indole and analyzed on a Beckman 121MB amino acid analyzer. Carbohydrate analyses were performed according to Clamp *et al.* (10) as modified by Pritchard and Todd (11). Duplicate samples (500 µg) were treated with 1.68 N methanolic HCl under nitrogen at 80°C for 24 hr. The resulting methylglycosides were analyzed as their trimethylsilyl derivatives by gas chromatography on a 6-ft glass column (2 mm ID) packed with 3.8% UCW-98 on Gas-Chrom Q (100/200 mesh).

Methylation Procedures - Samples (1 mg) were permethylated according to Hakomori (12), extensively dialyzed versus water, and hydrolyzed, reduced, and acetylated according to Stellner *et al.* (13). Analyses of the partially methylated alditol acetates were performed on a Dupont 321 mass spectrometer using 6 ft glass columns (2 mm ID) packed with either 3% ECNSS-M on Gas Chrom Q (100/120 mesh), temperature programmed from 100 - 190°C at 1°/min, or 3% OV-17 on Gas Chrom Q (100/120 mesh), temperature programmed from 100 - 200°C at 1°/min. Quantitation was performed by integration of selected ion peaks for a given derivative, compared to the response of a known derivative, and normalized to the percent composition obtained from compositional analysis. Standards used for the production of derivatives of known composition were α_1-acid glycoprotein (obtained from Dr. Yu-Lee Hao of the American National Red Cross Blood Research Laboratory, Bethesda, Md.), ovalbumin, and fetuin.

Deblocking With Pyroglutamyl Amino Peptidase - NH_2-terminal deblocking of fibronectins was performed as described by Podell *et al.* (14) using calf liver pyroglutamyl amino peptidase (Boehringer Mannheim Biochemicals). Fibronectin (5-10 nmoles) was dialysed at 4°C vs deblocking buffer (0.1 M disodium phosphate adjusted to pH 8.0 with 0.1 M monosodium phosphate, 5 mM DTT, 10 mM disodium EDTA, 5% glycerine (v/v) and finally adjusted to pH 8.0 with 1N NaOH). The protein preparation was treated with the enzyme (protein to lyophilized enzyme ratio of 20:1; protein to active enzyme ratio of 400:1) under N_2 at 4° with occasional stirring. After 9 hr the mixture was brought to room temperature and was treated with the same amount of enzyme for an additional 14 hr. The deblocked protein was then dialyzed vs 0.05 M acetic acid, 10^{-4} M PMSF and utilized for microsequence analyses.

Microsequence analysis - One to three nmoles of the sample was subjected to automatic Edman degradation on a modified Beckman 890C sequencer. The modifications which are similar to those described by Wittman-Liebold (15) and Hunkapiller and Hood (16) include an improved reagent/solvent delivery system, improved vacuum system, reagent and solvent purification, and automated converson of anilinothiazolinone to phenylthiohydantoin (PTH) derivatives of amino acids (17). The protein was retained in the spinning cup with the addition of 6 mg of polybrene which was precycled together with 100 nmoles of glycylglycine for 7 cycles (16). PTH-Amino acids were separated and

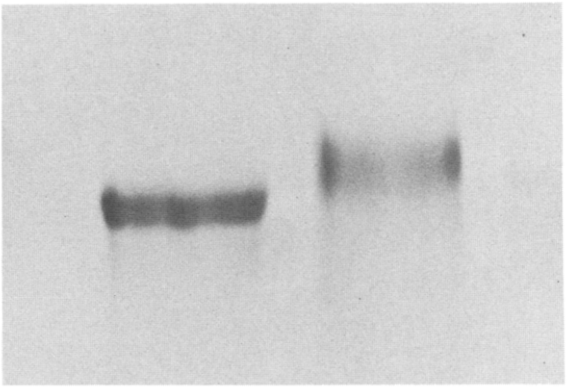

Fig. 1. SDS-polyacrylamide gel electrophoresis of human fibronectins run on 6% gels under reducing conditions. Only the upper portions of the gel are shown in order to demonstrate the differences in migration and banding patterns. Left, plasma fibronectin (molecular weight: 220,000 daltons); right, amniotic fluid fibronectin (molecular weight: 240,000 daltons).

identified by high performance liquid chromatography (HPLC) on either DuPont Zorbax ODS or Ultrasphere ODS columns. HPLC was performed on a Waters Associates chromotagraph equipped with 254 and 313 nm detectors. Peaks were integrated and gradient elution was controlled by a Spectra Physics 4000 integator system.

Results

Fibronectins were isolated by affinity chromatography on gelatin insolubilized on Sepharose 4B (7). When analyzed by SDS-gels under reducing conditions plasma fibronectin (PFN) gave a closely spaced doublet at Mr = 220,000, and amniotic fluid fibronectin (AFN) a single diffuse band at Mr = 240,000 (Fig. 1). When run on SDS-gels under nonreducing conditions their respective molecular weights were estimated as 440,000 and 480,000 daltons (data not shown). The two forms of fibronectin give lines of identity on double immunodiffusion when tested against antisera raised against purified PFN (data not shown). Preliminary attempts to perform isoelectric focusing of PFN and AFN on polyacrylamide gels in the presence of 8M urea or Triton X100 were unsuccessful; however, SDS-treated fibronectins migrated to identical positions (apparent pI of 5.0) on isoelectric focusing gels even when applied at different starting positions, and showed no differences between the two

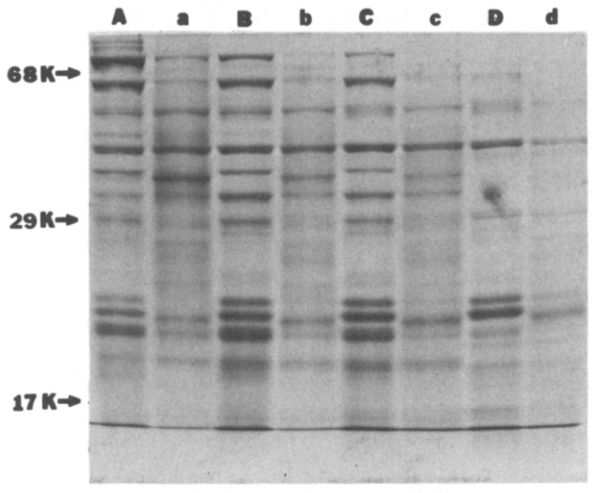

Fig. 2. Comparison of time course of trypsin digestion of plasma and amniotic fluid fibronectins. Lanes A, B, C, and D are 1 hr, 3 hr, 6 hr and 22 hr respectively of trypsin digestion of plasma fibronectin. Lanes a, b, c and d are identical times for amniotic fluid fibronectin. Twenty µg of protein were loaded per lane on 12% gels and run under reducing conditions.

forms tested. Tryptic maps of PFN and AFN on SDS-gels for a number of time points are shown in Fig. 2. AFN was digested more slowly than PFN and gave poorly staining, diffuse bands. After 22 hr of digestion, PFN and AFN showed considerable peptide homology with the exception of several prominent bands which appear to be unique to either PFN or AFN.

The amino acid compositions of the two fibronectins are closely similar (data not shown, see ref. 5), but PFN and AFN differ substantially in total carbohydrate and in the presence of fucose in AFN but not in PFN. PFN and AFN contain 4.8% and 6.8% carbohydrate by weight respectively. The methylation analysis of PFN (Table I) is consistent with simple biantennary asparagine-linked carbohydrate chains with branching predominately at 3,6-linked mannose and with an equal distribution of 4- and 6-linked intrachain galactose. AFN has a more complicated structure, which unlike PFN, has significant amounts of both 3,6- and 2,4-linked mannose and 3- and 4-linked but no 6-linked galactose PFN has sialic acid as its predominant terminal sugar, but AFN has terminal fucose, mannose, galactose, and N-acetylglucosamine, in addition to terminal sialic acid.

TABLE 1
Methylation Analysis of Human Fibronectins

Structural Unit	Glycosidic Linkage	Residue/Mol. Wgt. PFN	AFN
N-Acetylneuraminic Acid	Terminal	7.7	7.9
Fucose	Terminal	-	6.0
Mannose	Terminal	-	3.9
	2	11.6	10.6
	2,4	0.9	2.4
	3,6	4.1	4.3
Total		16.6	21.2
Galactose	Terminal	0.9	5.3
	3	-	5.8
	4	6.9	16.4
	6	6.9	-
Total		14.7	27.5
N-Acetylglucosamine	Terminal	-	4.0
	4	14.0	22.9
	3,4	1.6	1.4
Total		15.6	28.3

These results were consistent for a number of separate preparations of both PFN and AFN with one exception. One batch of AFN gave an unusually high carbohydrate content (18%), which included substantial amounts of N-acetyl-galactosamine and glucuronic acid. The presence of these sugars suggested that this preparation was contaminated with a glycosaminoglucuronan.

The NH_2-terminal sequence analysis of the two fibronectins after deblocking with pyroglutamyl aminopeptidase is shown below:

1 5 10
pGlu-Ala-Glu-Gln-Met-Val-Gln-(Cys)-?-?-Ser-Tyr-Ser

Discussion

The similarity of the amino acid compositions, NH_2-terminal sequences, isoelectric points, immunological reactivity, and tryptic maps of PFN and AFN suggests that these two glycoproteins have nearly identical polypeptide chains. The differences in carbohydrate compositions and linkage analysis appear to account for the slightly different migrations and Coomassie blue staining behavior of the two glycoproteins on SDS-gels. In addition, the higher carbohydrate content of AFN compared to PFN may protect portions of

AFN from trypsin digestion and thus lead to the observed differences in tryptic maps.

The carbohydrate compositions for PFN and AFN reported here are similar to those obtained by Balian et al. (5), who also showed limited peptide homology by CNBr and trypsin cleavage. The NH_2-terminal sequences reported here are similar to and extend those reported by Rifkin and Furie et al. (18) for human PFN. In our hands PFN showed an increased amount of peptide bond hydrolysis with increased number of Edman degradation cycles, as determined by running SDS gels on sequenced material after 10 cycles. The following distinct secondary sequence for PFN beginning at cycle 2 was obtained: Val-Val-Tyr-Gln-Lys-Asn. It is likely that it arises from hydrolysis of an acid labile peptide bond within PFN. In contrast, AFN shows a much lesser tendency to fragment under ordinary sequencing conditions.

The total carbohydrate content reported for PFN and AFN by Balian et al. (5) was 4.6% and 7.0% respectively, in agreement with our values of 4.8% and 6.9%. Also in agreement with our results, fucose was found in AFN only. The report by Ruoslahti (19) shows a much higher carbohydrate content for amniotic fluid fibronectin and the presence of substantial amount of N-acetylgalactosamine which could be due to contamination of the sample with glycosaminoglucuronan. The methylation analysis of PFN shown here is similar to that reported by Wrann (20), Fisher and Laine (21), and Takasaki et al. (22). Based on these data, PFN appears to have 4-6 biantennary, asparagine-linked carbohydrate chains per polypeptide chain: NeuAcα2 → 4/6Galβ1 → 4GlcNAcβ1 → 2 → Manα1 → 3 (NeuAcα2 → 4/6Galβ1 → 4GlcNAcβ1 → 2Manα1 → 6) Manβ1 → 4GlcNAcβ1 → 4GlcNAc. The relative amounts of terminal sialic acid and ratios of 4- and 6-linked galactose appear to vary depending on the source of the glycoprotein. The most striking differences between the carbohydrate units of PFN and AFN are the increased amounts of terminal sugars in AFN, the lack of 6-linked galactose in AFN, and the additional 2,4-branched mannose in AFN. These results suggest that AFN has significant amounts of triantennary (or

tetraantennary) carbohydrate chains, and that most of the chains are dissimilar from those found in PFN. The ratio of terminal sugars to branching sugars in AFN indicate that there are a higher number of carbohydrate chains per polypeptide chain than in PFN. It is likely that AFN contains Gal/Fuc → GlcNAc → Man sequences in addition to the NeuNAc → Gal → GlcNAc → Man sequence found in PFN.

The striking changes in carbohydrate structures between the similar glycoproteins PFN and AFN may play a role in their binding to cell surface receptors. It is likely that the differences in these carbohydrate moieties will result in different lectin binding specificities and affinities. Since PFN is presumably produced by fibroblasts (23,24) and AFN by amniotic epithelial cells (25), it is possible that the different cell types will have different binding affinities for the two forms of fibronectin. The apparent correlation of different glycosylation patterns with the production of glycoproteins by different cell types (or tissues) may be a central theme. For example, the correlation of different glycosylation patterns with production of similar polypeptides in different tissues has been observed for other glycoproteins such as α-fetoprotein (26) and carcinoembryonic antigen (27). A generalization which may be made from these observations is that different cell types producing similar polypeptides may imprint them with with different glycosylation patterns in order to exploit differential recognition patterns for glycoproteins which otherwise have similar or identical biological activities.

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