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# Comparative studies on amniotic fluid and plasma fibronectins

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Human fibronectin was isolated from second-trimester amniotic fluid, from amniotic fluid obtained at term and from adult plasma. The amniotic-fluid fibronectins had a slightly higher apparent molecular weight on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis than the plasma fibronectin. Early- and late-amniotic-fluid fibronectin had 9.5 and 9.6% carbohydrate respectively, whereas plasma fibronectin had 5.8%. The amniotic-fluid fibronectins had similar mannose and sialic acid contents to plasma fibronectin, but greater amounts of glucosamine, galactosamine, galactose and fucose. There were no detectable differences in the amino-acid composition of amniotic-fluid and plasma fibronectins, and the patterns of peptides obtained after tryptic digestion of fibronectin from the two sources showed extensive similarities. Fibronectins from plasma and amniotic fluid were equally active in promoting cell attachment and were immunologically indistinguishable. These results show that fibronectin from amniotic fluid is more heavily glycosylated than plasma fibronectin or previously analysed fibronectins from cultured fibroblasts. The observed differences in glycosylation may be related to cell type and/or stage of development.

Fibronectin, a 450000-mol.wt. glycoprotein, is present in soluble form in plasma of different vertebrate species and in the growth media of cells cultured *in vitro*. It is found in an insoluble form at the surface of normal cells and in the surrounding extracellular matrix, where it seems to be involved in cell attachment (for references, see Vaheri *et al.*, 1978). Fibronectin is also present in amniotic fluid (Chen *et al.*, 1976; Crouch *et al.*, 1978; Kuusela *et al.*, 1978). The amounts present far exceed the concentration that could be expected to reach amniotic fluid from the maternal circulation, the main source of proteins in amniotic fluid. Based on this and the fact that cells cultured from amniotic fluid produced fibronectin, it is assumed that fibronectin in amniotic fluid is produced locally (Chen *et al.*, 1976; Crouch *et al.*, 1978). A difference in the apparent molecular weights of fibronectin from plasma and amniotic fluid as detected by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Crouch *et al.*, 1978) supports this conclusion. Fibronectins obtained from cultured cells have also, in many instances, been found to be slightly larger than the plasma form (Keski-Oja *et al.*, 1977; Engvall *et al.*, 1978; Yamada & Kennedy, 1979).

As a step toward clarification of the structural and functional relationship of different fibronectins, we have compared the properties of fibronectin from plasma and amniotic fluid. We show that fibronectins from these sources differ by several criteria most notably by carbohydrate composition, but are immunologically indistinguishable when tested with conventional antisera and are equally active promoters of cell attachment.

## Experimental

### *Isolation of fibronectin and immunological methods*

Pooled adult human citrate plasma and pooled amniotic fluid collected during the second trimester or at term were used as sources of fibronectin. Chromatography on gelatin–Sepharose was used to isolate fibronectin (Engvall & Ruoslahti, 1977). To remove possible contaminants, fibronectin was further purified by column fractionation on DEAE-cellulose (Whatman DE-52; Whatman Inc., Clifton, NJ, U.S.A.). The column was equilibrated with



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4.5 M-urea in 0.01 M-Tris/HCl, pH 7.2. Fibronectin was eluted from the column by using a linear gradient of 10 column volumes of 0–0.5 M-NaCl in the same buffer. Fibronectin was eluted as a sharp u.v.-absorbance peak at 0.12 M-NaCl. Purification of fibronectin was monitored by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. For carbohydrate and amino-acid analysis and for proteolytic digestion, fibronectin was precipitated and washed with 70% ethanol, dissolved in 0.2 M-acetic acid and freeze-dried.

For tryptic digestion, the dry fibronectin was suspended in 0.5 M-$NH_4HCO_3$ and digested with 1% (w/w) of diphenylcarbamoylchloride-treated trypsin (Sigma Chemical Co., St. Louis, MO, U.S.A.) for 6 h at 37°C. The digests were fractionated on gelatin–Sepharose and concanavalin A–Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.) as described previously (Ruoslahti *et al.*, 1979).

Preparation and properties of the fibronectin antibodies and radioimmunoassay of fibronectin have been described in detail previously (Ruoslahti *et al.*, 1978). Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate was carried out as described by Laemmli (1970). Thyroglobulin (mol.wt. 330000), ferritin (mol.wt. 220000), albumin (mol.wt. 67000) and catalase (mol.wt. 60000) from Pharmacia and myosin (mol.wt. 200000) from Sigma were used as molecular-weight markers.

*Chemical analyses*

Neutral sugars were analysed by borate complex anion-exchange chromatography (Spiro *et al.*, 1976) after hydrolysis (1 M-HCl for 6 h at 100°C) and passage through coupled columns of Dowex 50 and Dowex 1 as previously described (Spiro, 1966). Amino sugars were resolved on the Technicon NC-2 amino-acid analyser with a program that utilized pH 5 buffers (Spiro *et al.*, 1976) after hydrolysis in 4 M-HCl at 100°C for 6 h. The thiobarbituric acid reaction (Warren, 1959) was employed to determine sialic acids after hydrolysis with 0.1 M-$H_2SO_4$ at 80°C for 60 min. Amino-acid analyses were performed on the Technicon NC-2 amino-acid analyser after hydrolysis of the samples in constant-boiling HCl in sealed tubes under $N_2$ at 105°C for 28 h.

*Cell-attachment assay*

To determine the capacity of fibronectin to mediate cell attachment, wells in non-treated polystyrene microtitre plates (Flow Laboratories, Inglewood, CA, U.S.A.) were coated with different amounts of fibronectin. Trypsin-treated normal rat kidney cells in serum-free medium containing 0.5 mg of soya-bean trypsin inhibitor/ml were added to the wells. The plate was washed after 1 h of incubation and attached cells were counted (Ruoslahti & Hayman, 1979).

**Results**

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis analysis showed that fibronectin from amniotic fluid moved as a diffuse band of lower mobility than the doublet band obtained with plasma fibronectin. Fibronectin from amniotic fluid collected during the second trimester and at term gave a similar band. Comparison with standards gave mol.wts. of approx. 220000 and 235000 for the subunits of plasma and amniotic-fluid fibronectins respectively.

It has been established previously that fibronectin from plasma and amniotic fluid are indistinguishable when tested for immunodiffusion against antisera to plasma fibronectin (Kuusela *et al.*, 1978). Competitive radioimmunoassays were performed by using both $^{125}$I-labelled fibronectin from plasma and antibodies against plasma fibronectin, and $^{125}$I-labelled amniotic-fluid fibronectin and antibodies against the fibronectin from amniotic fluid. Unlabelled fibronectin from the two sources gave identical inhibition curves in the two assays. A representative assay result is shown in Fig. 1.

When peptides from tryptic digests of fibronectin from plasma and early amniotic fluid were compared on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, the patterns showed much similarity, but also some slight differences (Fig. 2).

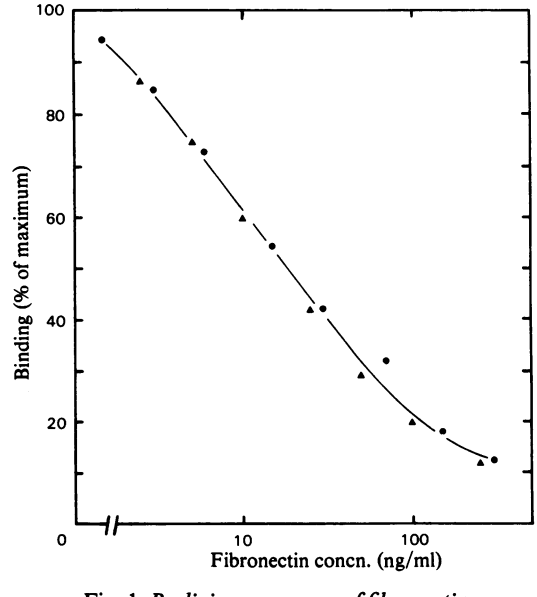


Fig. 1. *Radioimmunoassay of fibronectin*
Fibronectins from plasma (●) and amniotic fluid (▲) were used as inhibitors of the binding of labelled amniotic-fluid fibronectin to antibodies against amniotic-fluid fibronectin.

To increase the resolution, the fragments from tryptic digestion of fibronectin isolated from plasma and early amniotic fluid were fractionated on the basis of binding to gelatin–Sepharose and concanavalin A–Sepharose. Both digests showed a prominent band at mol.wt. 30000 in the fraction that was bound to gelatin (Fig. 2, lanes 5 and 6). This band co-migrated with the mol.wt.-30000 collagen-binding fragment we have described before (Ruoslahti *et al.*, 1979).

The amino-acid analyses obtained were in

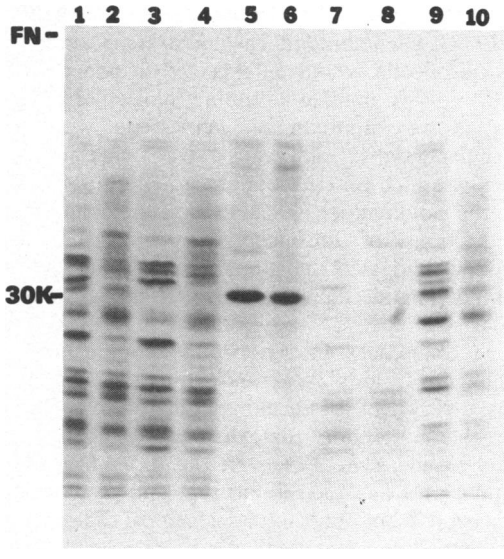


Fig. 2. *Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate of tryptic digests of plasma fibronectin (odd-numbered lanes) and of amniotic-fluid fibronectin (even-numbered lanes)*

Lanes (1) and (2) show total digests; (3) and (4), gelatin–Sepharose-non-binding fractions; (5) and (6), gelatin-bound fractions; (7) and (8), concanavalin A-non-binding fractions; (9) and (10), concanavalin A-bound fractions. The position of fibronectin (FN) and the mol.wt.-30000 gelatin-binding fragment of fibronectin (30K) are shown.

Table 1. *Amino-acid composition of human fibronectins*

Results for plasma are means for three preparations and those for second trimester and term amniotic fluid are means for two preparations.

| | Amino acid (mol %) | | |
|---|---|---|---|
| | | Amniotic fluid | |
| Amino acid* | Plasma | Second trimester | Term |
| Aspartic acid | 10.1 | 10.0 | 9.8 |
| Threonine | 11.8 | 9.2 | 11.9 |
| Serine | 8.5 | 8.1 | 8.2 |
| Glutamic acid | 10.5 | 9.8 | 11.2 |
| Proline | 8.7 | 9.4 | 7.6 |
| Glycine | 8.2 | 7.8 | 9.2 |
| Alanine | 4.4 | 4.7 | 4.4 |
| Valine | 7.3 | 8.3 | 7.6 |
| Half cystine | 2.3 | 2.8 | 2.6 |
| Methionine | 1.1 | 1.0 | 1.0 |
| Isoleucine | 4.4 | 4.3 | 4.3 |
| Leucine | 5.0 | 5.7 | 5.6 |
| Tyrosine | 4.6 | 4.9 | 4.2 |
| Phenylalanine | 2.4 | 2.7 | 2.1 |
| Lysine | 3.2 | 3.6 | 3.4 |
| Histidine | 2.0 | 2.2 | 2.1 |
| Arginine | 5.7 | 5.9 | 5.2 |

* Tryptophan analyses were not performed.

Table 2. *Carbohydrate composition of human fibronectins*

Values for composition in mg/100mg are given as means ± S.D. and are expressed as residue weight per 100mg of glycoprotein weight, which was determined from the sum of the amino-acid and saccharide residues; three preparations of plasma fibronectin and two each of second trimester and term amniotic-fluid fibronectins were analysed.

| | Composition (mg/100mg) | | | Composition (mol/mol of fibronectin)* | | |
|---|---|---|---|---|---|---|
| | | Amniotic fluid | | | Amniotic fluid | |
| Monosaccharide | Plasma | Second trimester | Term | Plasma | Second trimester | Term |
| Mannose | 1.07 ± 0.08 | 1.25 ± 0.03 | 0.95 ± 0.06 | 15 | 18 | 14 |
| Galactose | 0.86 ± 0.07 | 2.08 ± 0.11 | 2.70 ± 0.14 | 12 | 30 | 38 |
| Fucose | 0.07 ± 0.01 | 0.39 ± 0.02 | 0.44 ± 0.04 | 1 | 6 | 7 |
| Glucosamine† | 1.91 ± 0.12 | 2.92 ± 0.50 | 3.15 ± 0.49 | 22 | 33 | 36 |
| Galactosamine† | 0.21 ± 0.08 | 0.92 ± 0.14 | 0.72 ± 0.06 | 2 | 10 | 8 |
| Sialic acid | 1.73 ± 0.14 | 1.96 ± 0.36 | 1.67 ± 0.08 | 14 | 16 | 13 |
| Total | 5.85 | 9.52 | 9.63 | 66 | 113 | 116 |

* Expressed to the nearest integer per mol of fibronectin subunit ($M_r = 230000$).

† Assumed to be present in the *N*-acetyl form.

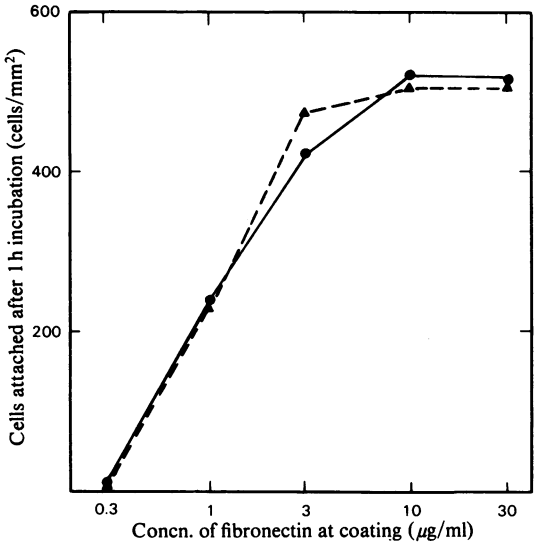


Fig. 3. *Attachment of normal rat kidney cells to wells of microtitre plates coated with solutions of plasma* (▲) *and amniotic fluid* (●) *fibronectin at different concentrations*

generally good agreement with previously published data. No substantial differences between the amino-acid composition of plasma and amniotic-fluid fibronectins were detected (Table 1). The carbohydrate compositions of fibronectins from the two sources, however, differed substantially (Table 2). Fibronectin from second trimester as well as that from term amniotic fluid was found to contain more total saccharide than the protein isolated from plasma. Galactose, fucose, glucosamine and galactosamine were all present in larger amounts in fibronectin from amniotic fluid, whereas the quantities of mannose and sialic acid were about the same as in plasma fibronectin.

Comparison of the ability of fibronectin from plasma and amniotic fluid to promote attachment of normal rat kidney cells showed them to be approximately equally active (Fig. 3).

## Discussion

Our results establish some substantial chemical differences between fibronectins from amniotic fluid and plasma. We found that these fibronectins differ in their carbohydrate compositions. The carbohydrate analyses suggest that both plasma and amniotic-fluid fibronectins contain the complex-type of asparagine-linked units (Spiro, 1973), such as have been described in fibronectin from hamster embryo fibroblasts (Fukuda & Hakomori, 1979) and from human (Wrann, 1978) and bovine (Takasaki *et al.*, 1979) plasma. However, the occurrence of larger amounts of galactose and glucosamine as well as substantial quantities of fucose and galactosamine in the amniotic-fluid fibronectin suggests that this protein may contain a second type of saccharide unit, perhaps similar to the newly described heteroglycan of erythrocyte membranes (Krusius *et al.*, 1978). The additional carbohydrate of amniotic-fluid fibronectin would be expected to add approx. 10000 mol.-wt. units to its subunit molecular weight. This is in approximate agreement with the molecular-weight difference we obtained in polyacrylamide-gel electrophoresis. This and the fact that the immunological reactivities and amino-acid compositions of fibronectins from plasma and amniotic fluid were indistinguishable suggests that their polypeptide chains could be identical or very similar.

The differences in the peptide composition of fibronectin from plasma and amniotic fluid after trypsin treatment are seemingly difficult to reconcile with the contention that their polypeptide parts would be identical. However, the differences were minor, and it has been found that non-glycosylated fibronectin from tunicamycin-treated cells is more susceptible to proteolysis than glycosylated fibronectin (Olden *et al.*, 1979). The additional carbohydrate residues in amniotic-fluid fibronectin could offer protection against proteolysis, resulting in peptide differences. Since this work was completed Alitalo *et al.* (1980) have reported on a fibronectin from cultured amniotic epithelial cells that migrates more slowly than plasma fibronectin in sodium dodecyl sulphate/polyacrylamide gels. Similar to our results, they found that this fibronectin, which seems to be a counterpart *in vitro* of fibronectin in amniotic fluid, shares with plasma fibronectin all but one of the peptides generated by digestion with Staphylococcal proteinase.

Since fibronectin from cultures of embryonic fibroblasts has been reported to move on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis with a mobility lower than that of plasma fibronectin (Yamada & Kennedy, 1979; Engvall *et al.*, 1978), and amniotic-fluid fibronectin shares this characteristic (Crouch *et al.*, 1978), it has been assumed that amniotic-fluid fibronectin represents a cellular fibronectin distinct from the circulating fibronectin. However, it has been shown previously that fibronectins from plasma and spent culture media of fibroblasts both have about 5% carbohydrate with closely similar compositions (Vuento *et al.*, 1977; Yamada *et al.*, 1977; Wrann, 1978; Fukuda & Hakomori, 1979; Takasaki *et al.*, 1979). The features that distinguish amniotic-fluid fibronectin from plasma fibronectin, therefore, do not seem to reflect general differences between cellular and circulating fibronectins. Instead, they could be

related to the different developmental stage and/or origin of the cell type that synthesizes these fibronectins.

There are other examples of a situation in which the glycosylation of a polypeptide depends on the phenotype and developmental stage of the cell synthesizing it. α-Foetoprotein synthesized by the mouse yolk sac does not bind to concanavalin A, but the same protein, when it originates from the liver, does so (Ruoslahti & Adamson, 1978). Several membrane proteins carry a larger oligosaccharide moiety when they come from transformed cells than when they come from normal cells (Warren *et al.*, 1978).

Whether the variations between fibronectins from different sources have functional significance needs to be further explored. It has been reported that fibronectin derived from cultured fibroblasts is more active in altering the morphology of transformed cells toward normal (Yamada & Kennedy, 1979). We found the plasma and amniotic-fluid forms approximately equally active in our assay for cell attachment. In our hands (E. G. Hayman & E. Ruoslahti, unpublished work), transformed cells have not assumed a normal morphology when treated with either plasma or amniotic-fluid fibronectin, even at concentrations well above those at which plasma fibronectin has been reported to be active. Whether or not fibronectins from different sources differ functionally, they could still provide useful markers for various cell types on the basis of their sugar compositions and/or electrophoretic mobility. We have recently found that fibronectin produced by human teratocarcinomas resembles that of amniotic fluid, suggesting that it could provide a new oncodevelopmental marker for such tumours (E. Ruoslahti & D. Raghavan, unpublished work).

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