Enhanced Expression of a Peanut Agglutinin Reactive O Linked Oligosaccharide on Fibronectins from the Synovial Fluid of Patients with Rheumatic Disease: Quantitation, Domain Localization, and Functional Significance

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ABSTRACT. ***Objective.*** To characterize the domain localization, quantitation, and functional binding conse-quences of an O linked oligosaccharide expressed on synovial fluid (SF) fibronectin (Fn).
Methods. Identification and localization of the O linked oligosaccharide was performed by limited digestion of isolated SF Fn with a series of proteolytic enzymes followed by Western blotting with peroxidase labeled peanut agglutinin. Binding affinity to denatured collagen was performed utilizing a solid phase gelatin-binding assay. Quantitation was performed by measuring purified Fn in an anti-body-lectin sandwich binding assay.
Results. A desialyated O linked oligosaccharide was identified on the C-terminal 18 kDa segment of the SF Fn collagen-binding domain. These SF collagen-binding Fn fragments were more basic and had higher gelatin-binding affinities than corresponding plasma fibronectin fragments. Expression of this O linked oligosaccharide was highest on Fn isolated from osteoarthritic SF, followed by Fn isolated from rheumatoid arthritis SF, and finally normal human plasma.
Conclusion. Fn isolated from SF have glycosylation alterations that may influence their biologic properties in the diseased joint. (J Rheumatol 2002;29:896–902)

Key Indexing Terms:
SYNOVIAL FLUID   FIBRONECTIN
PEANUT AGGLUTININ   OLIGOSACCHARIDE

Fibronectins (Fn) isolated from the synovial fluid (SF) of patients with rheumatic disease differ from the major Fn isoforms previously identified in terms of charge, molecular weight, and glycosylation[1]. Synovial fluid Fn has been shown to contain a reduced amount of sialic acid, a greater molar amount of N-acetyl galactosamine, and a greater degree of reactivity to the lectin peanut agglutinin (PNA)[2], the latter indicating the presence of an altered O linked oligosaccharide on SF Fn when compared to plasma fibronectin (PFn). In addition, we have demonstrated that an O linked oligosaccharide previously found only on embryo derived Fn is present on the carboxyterminal domain of the SF form but not the plasma form of Fn isolated from patients with rheumatic disease[3]. In this investigation we found that the reactivity of SF Fn to PNA lectin is due to the presence of a desialyated O linked glycan. The domain localization, effect on ligand binding, and quantitation of this carbohy-drate-specific Fn isoform in various forms of arthritis are described.

MATERIALS AND METHODS

Normal human plasma was purchased from the New York Blood Center. SF was aspirated from patients with inflammatory arthritis, with informed consent. Patients with rheumatoid arthritis (RA) met the revised American Rheumatology Association criteria[4]. Patients with osteoarthritis (OA) had typical clinical and radiographic findings. Fluid was collected aseptically into polyvinylpropylene tubes in the absence of anticoagulant. Fluid was centrifuged at $1000 \times g$ to remove cellular elements and the supernatant stored frozen at –30°C.

Purified Fn. Fn was purified from normal human plasma, SF, and the condi-tioned media of HFL-1 fibroblasts and fibroblast-like synoviocytes (FLS)[5] by chromatography on Sepharose 4B and gelatin-Sepharose 4B[6]. Purified protein was dialyzed against 0.05 M Tris-HCl, 1 mM PMSF, pH 7.2.

Enzymatic digestion. Limited proteolytic digestion of purified plasma and SF Fn was performed with the following enzymes: TPCK-Trypsin (Sigma, St. Louis, MO, USA), 1/100 w/w in 0.1 M Tris, 0.01 M $CaCl_2$, pH 7.4; chymotrypsin (Calbiochem, La Jolla, CA, USA), 1/50 w/w in 0.5 M Tris, 1 mM PMSF, pH 7.5; thermolysin (Calbiochem) 2.5 mg/ml in 0.05 M Tris, 0.15 M NaCl, pH 7.2. Limited glycosidase digestion was performed with the following enzymes: neuraminidase (Sigma) 1:50 w/w in 0.1 M NaAcetate, 0.01 M $CaCl_2$, pH 6, for 3 h at 37°C; and N-acetylgalac-

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tosaminidase 0.1 mu/µg in 0.02 N phosphate, 0.1% NP-40, 0.1% sodium dodecyl sulfate (SDS), 1% 2-mercaptoethanol, 0.01 M EDTA, pH 6, for 16 h at 37°C.

Gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) electrophoresis was performed according to the method of Laemmli[7]. Proteins were transferred to nitrocellulose according to Towbin[8]. Lectin binding was detected using peroxidase labeled PNA (Sigma). Low molecular weight markers were insulin (3000), bovine trypsin inhibitor (6000), lysozyme (14,300), ß-lactoglobulin (18,400), α-chymotrypsinogen (25,700), and ovalbumin (43,000). High molecular weight markers were bovine serum albumin (68,000), phosphorylase B (97,400), and myosin H chain (206,000). Fn peptides were detected using the following antibodies: polyclonal anti-Fn[6], a monoclonal antibody reactive with the C-terminal extension of the collagen-binding domain (Mab CE-9, provided by Dr. J. Czop, Harvard Medical School)[9], and a Mab reactive with a 61 kDa C-terminal fragment (Mab 61K, provided by Dr. G. Balian, University of Virginia)[10]. Binding of polyclonal antibodies was detected using peroxidase labeled goat anti-rabbit IgG (BioRad, La Jolla, CA, USA). Mab were detected using peroxidase labeled goat anti-mouse immunoglobulins. Thin layer isoelectric focusing was performed using a 0.5 mm acrylamide gel containing 4% ampholines (pH 3.5–10).

Antibody-lectin sandwich immunosorbent assay (ALSIA). To quantitate levels of Fn isoforms containing specific glycans an ALSIA was utilized: Anti-fibronectin IgG was isolated from rabbit anti-human fibronectin serum by chromatography on DEAE Sepharose (Pharmacia, Uppsala, Sweden). The low ionic strength breakthrough fraction was then chromatographed on protein-A agarose (Sigma). Purified IgG was eluted with 0.2 M glycine, pH 2, neutralized with 1 M NaOH, and dialyzed against 0.02 M Na_2CO_3, 0.02 M NaN_3, pH 9.6. Ninety-six well microtiter plates were coated with anti-Fn IgG in 0.02 M Na_2CO_3, 0.02M NaN_3, pH 9.6, at 1 µg/well by incubation for 48 h at 4°C. Plates were then washed 3 times with PBS-0.05% Triton X-100 (PBS-Triton). For the assay, anti-fibronectin coated plates were incubated with increasing concentrations of Fn isoforms either as purified protein or in complex solution as plasma or SF. Dilutions were made in PBS-Triton. Dilutions of purified protein solution ranged from 1:20 to 1:320 corresponding to Fn concentrations of 1.3–20 µg. Fn were incubated in the antibody coated wells for 1 h at 37°C. After washing the plate 3 times with PBS-Triton, wells were incubated with the lectin-peroxidase conjugate (PNA-peroxidase, 0.06 µg lectin/ml; Sigma) at a dilution of 1:100 in PBS containing 60 mg/ml bovine serum albumin (BSA) for 1 h at 37°C. After 3 wash cycles with PBS-Triton, ABTS substrate was added for 20 min at 37°C. Optical density at 414 nm was measured using an automated scanner (Titertek). Binding of lectin conjugate to antibody coated plates in the absence of Fn was < 12% of maximal values.

To assess the specificity of the lectin–carbohydrate interaction, the lectin conjugate was preincubated with the ligand sugar (200 mM D-galactose) for 45 min at room temperature. Incubation of PNA-peroxidase with 200 mM D-galactose reduced reactivity to baseline values.

Gelatin-binding assay. Ninety-six well microtiter plates were coated with gelatin, 5 µg/well in 0.05 M Tris, pH 7.4, for 16 h at 23°C and then were washed 3 times with PBS, 0.05% Triton X-100, 0.02% NaN_3 (PBS-Triton). Unbound sites were blocked with 1% BSA in Tris buffered saline, pH 7.4, for 20 min followed by washing 3 times with PBS-Triton and aspiration of residual buffer. Plates were stored at 4°C. Samples of Fn isoforms or their fragments (200 µl) diluted in PBS-Triton were incubated in wells for 1 h at room temperature. After washing 3 times with PBS-Triton, wells were incubated with anti-fibronectin followed by goat anti-rabbit IgG conjugated to alkaline phosphatase (1:1000; Sigma). After washing 3 times, a 1:1000 dilution of alkaline phosphatase substrate was added. The reaction was terminated with 50 µl of 4 N NaOH. Color was read with an automated ELISA reader (Titertek) at 405 nm.

Statistics. Statistical differences between means were determined using Mann-Whitney rank-sum tests (Crisp program, Crunch Software).

RESULTS

Oligosaccharide heterogeneity of plasma and synovial Fn. Plasma and SF Fn display significant carbohydrate heterogeneity. SF Fn reacts strongly with the lectins PNA and wheat germ agglutinin while plasma Fn do not. Results of a typical experiment utilizing PNA are shown in Figure 1. Since PNA reactivity suggests the presence of O linked oligosaccharides, additional experiments were performed to further investigate its specificity for O-glycan moieties present on synovial Fn. As shown in Figure 1, treatment with α-N acetylgalactosaminidase eliminated the PNA reactivity of synovial Fn (lane 3), whereas treatment with enzyme buffer alone did not affect PNA reactivity (lane 2).

α-N acetylgalactosaminidase treatment did not result in nonspecific proteolytic degradation of the Fn band (lane 1).

Distribution of the PNA reactive O linked oligosaccharide on SF Fn. Limited digestion of SF Fn with thermolysin (Figure 2A, lane 3) results in characteristic 155 and 145 kDa carboxyterminal fragments and 60 and 40 kDa amino terminal fragments. The 60 and 40 kDa fragments contain the gelatin-binding domain and can be isolated on gelatin-Sepharose (Figure 2A, lane 5). Only the gelatin-binding amino terminal fragments react with PNA (Figure 2A, lane 7). In contrast, the larger 145–155 kDa domains, which do not react with PNA (Figure 2A, lanes 4 and 8), are derived from the carboxyterminal, as shown by their reactivity with a monoclonal anti-fibronectin specific for a cathepsin D fragment derived from the carboxyterminal region (Figure 2A, lane 8). Limited digestion with trypsin (5 min) cleaves the major Fn interchain disulfide bridge so that A and B chains can be clearly distinguished. As can be seen in Figure 2B, lanes 6 and 7, PNA reactivity is confined to the Fn B
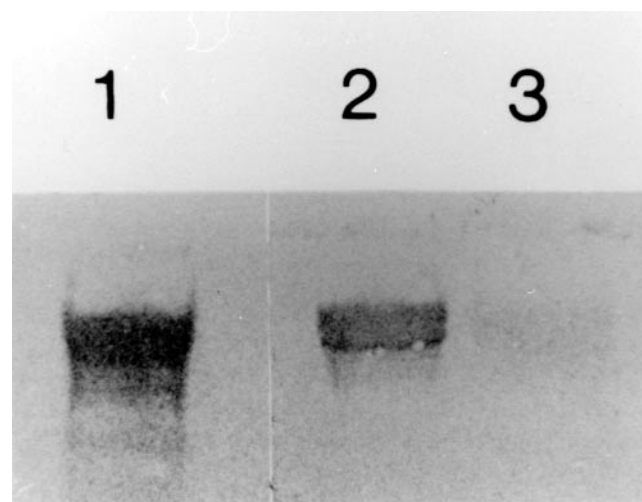

Figure 1. Western blot of purified Fn. Lane 1: RA SF Fn stained with fast green after incubation in glycosidase digestion buffer. Lane 2: RA SF Fn stained with PNA-peroxidase after incubation in glycosidase digestion buffer. Lane 3: RA SF Fn stained with PNA-peroxidase after digestion with α-N acetylgalactosaminidase in glycosidase digestion buffer.
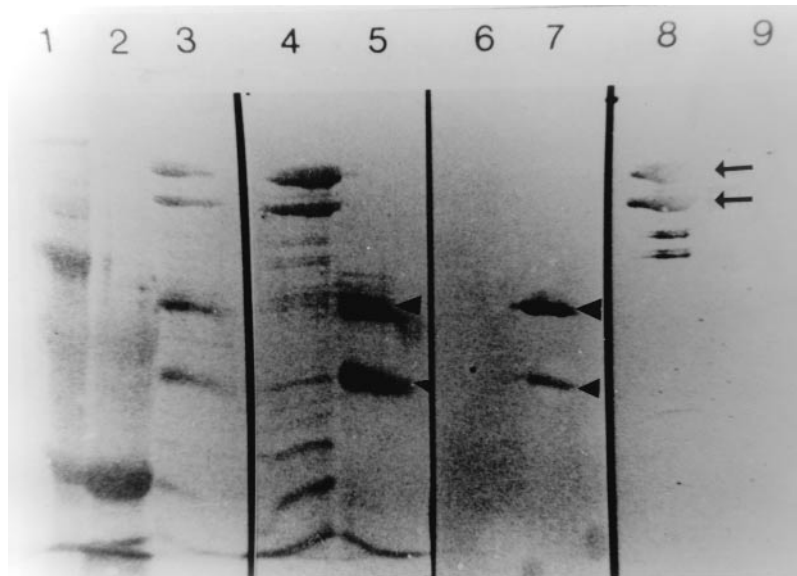
Figure 2A. Digestion of SF Fn with thermolysin resulting in typical 155 and 145 kDa carboxyterminal fragments and 60 and 40 kDa aminoterminal gelatin-binding fragments. Lanes 1 and 2: high and low MW markers, respectively; lane 3: total digest; lanes 4, 6, and 8: flow-through from gelatin-Sepharose column; lanes 5, 7, and 9: fragments bound to gelatin-Sepharose and eluted with 8 M urea. Lanes 1–5 stained with fast green. Lanes 6 and 7 stained with PNA-peroxidase; lanes 8 and 9 stained with Mab 61K, followed by peroxidase labeled antimouse IgG. PNA reactivity localizes to fragments bound to and eluted from gelatin (arrowheads, lanes 5 and 7). Arrows indicate carboxyterminal fragments reactive with Mab 61K (lane 8), but nonreactive with PNA (lane 6).
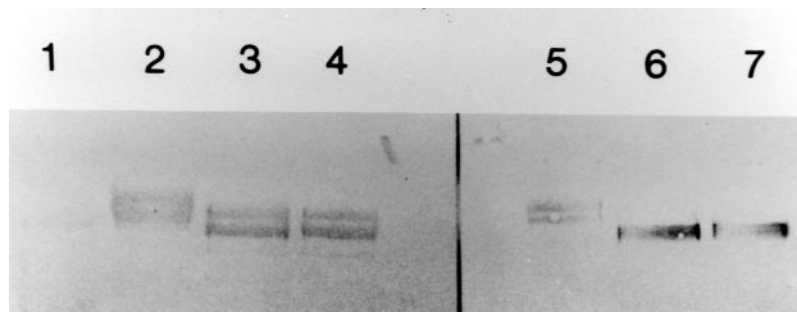

Figure 2B. Digestion of SF Fn with TPCK-trypsin. Lane 1: high MW markers. Lanes 2 and 5: SF Fn undigested. Lanes 3, 4, 6, 7: five minute tryptic digest of SF Fn. Lanes 1–4 stained with fast green. Lanes 5–7 stained with PNA-peroxidase. PNA reactivity is seen only on the lower MW B chain.

chain. Further localization of the PNA binding site was accomplished by limited chymotryptic digestion followed by chromatography of fragments on gelatin-Sepharose and heparin-Sepharose with examination of separated fragments by SDS-PAGE (Figures 3A, 3B). The major PNA reactive species were observed to be 60, 40, and 30 kDa. The 60 and 40 kDa peptides bound firmly to gelatin but not to heparin. The 60 kDa peptide reacted with Mab CE9 (Figure 3B), which identifies the 18 kDa carboxyterminal extension of the gelatin binding domain.

Effect of terminal sialyation of the gelatin-binding domain oligosaccharide on charge and binding affinity. PNA generally binds to oligosaccharides of the form O-GalNac-β_{1-3} Gal. That SF Fn reacts with PNA without prior neura-

minidase treatment suggests that these aminoterminal oligosaccharides are nonsialyated, in agreement with studies describing a lower quantity of sialic acid in SF Fn[2] and charge differences between plasma Fn and SF Fn[11]. Isoelectric focusing of plasma and SF Fn chymotryptic fragments (Figure 4A) reveals significant charge heterogeneity. Gelatin-binding domains derived from plasma and SF Fn yield sets of peptides with significant microheterogeneity. While one set of fragments appears to have a common PI, PFn displays an additional set of peptides with an acidic PI, whereas SF Fn yields a set of peptides with a basic PI. Treatment of Fn chymotryptic digests with neuraminidase does not alter the migration of the synovial derived fragments (lanes 1 and 2), but converts the acidic subset of PFn
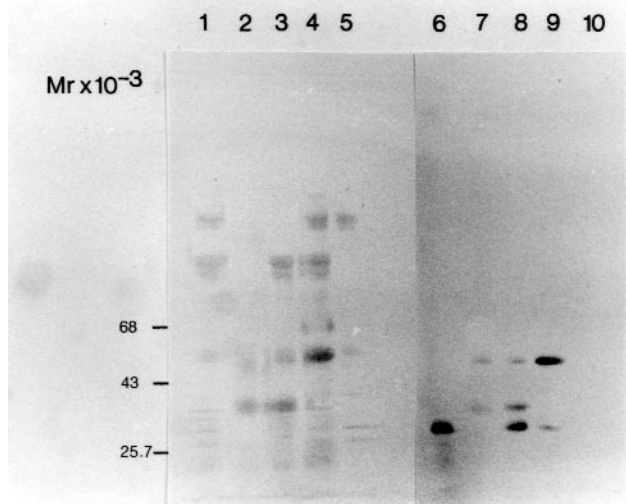
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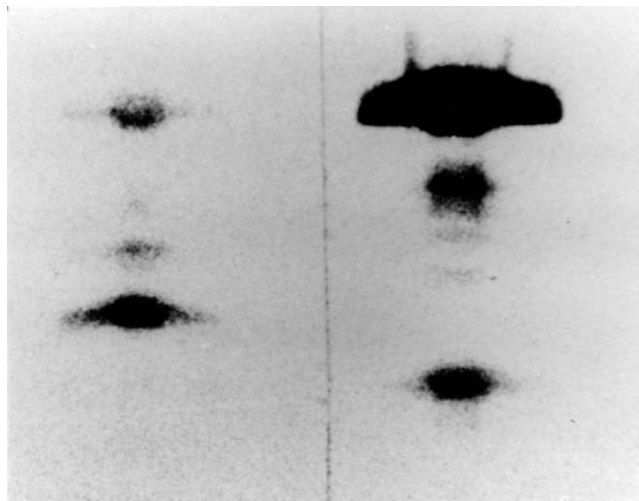
B

Figure 3. Analysis of SF Fn chymotryptic fragments by SDS-PAGE. A. Lanes 1 and 6 gelatin-Sepharose unbound fraction; lanes 2 and 7 gelatin-Sepharose bound fraction; lanes 3 and 8 heparin-Sepharose unbound fraction; lanes 4 and 9 heparin-Sepharose weakly bound fraction; lanes 5 and 10 heparin-Sepharose strongly bound fraction. Lanes 1–5 stained with fast green; lanes 6–10 stained with PNA-peroxidase: PNA reactivity localizes to 60 and 40 kDa aminoterminal fragments, which bind gelatin and bind heparin weakly. No reactivity is seen on carboxyterminal fragments, which bind heparin strongly (lanes 4 and 5 vs lanes 9 and 10). B. Reactivity of 60 kDa aminoterminal chymotryptic fragment with PNA (left lane) and Mab CE9 (right lane). CE9 is specific for the 18 kDa carboxyterminal extension of the gelatin-binding fragment.

peptides to a more basic charge, resulting in comigration with the SF basic subset (lanes 3 and 4).

The ability of chymotryptic Fn gelatin-binding fragments to bind denatured collagen is illustrated in Figure 4B. Fragments derived from SF Fn display a higher affinity for collagen than do fragments derived from PFn (panel a). When PFn fragments are treated with neuraminidase (panel b), an enhanced affinity for collagen can be seen.
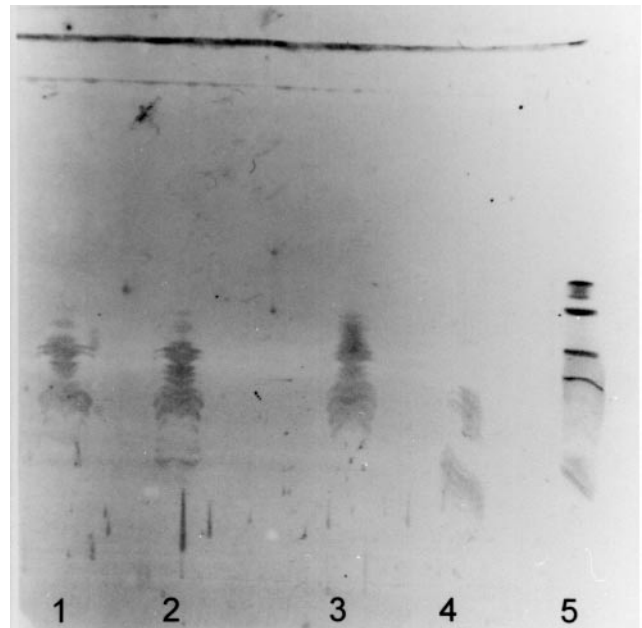

Figure 4A. Isoelectric focusing of chymotryptic gelatin-binding fragments derived from plasma (lane 4) and RA SF (lanes 1 and 2). Plasma Fn reveals an acidic set of peptides (lane 4; bottom), whereas SF Fn has a more basic set of peptides (lanes 1 and 2; top). Treatment of PFn with neuraminidase prior to chymotrypsin digestion (lane 3) alters the migration of the PFn peptides to resemble the SF Fn peptides (lanes 1 and 2). Neuraminidase treatment of SF Fn does not alter migration (lane 1). PI markers, lane 5; anode at bottom.

Source of PNA reactive Fn. To determine if the fibroblast-like synoviocyte is the source of PNA reactive SF Fn, Fn isolated from the conditioned medium of HFL-1 fibroblasts and fibroblast-like synoviocytes were examined (Figure 5). These Fn were very weakly reactive with PNA.

Quantitative estimation of PNA expression on SF Fn. PNA reactivity of Fn in whole SF was quantitated by ALSIA (Figure 6). The PNA reactivity of 21 SF Fn samples (28.1 ± 2.9 U/μg) significantly exceeded that of 25 normal PFn samples (7.5 ± 1.2 U/μg; p < 0.001). The PNA reactivity of OA samples (34.9 ± 2.8 U/μg) exceeded that of RA samples (19.8 ± 3.4 U/μg; p < 0.02).

DISCUSSION

It has been shown that the glycosylation of Fn isolated from SF differs from Fn isolated from plasma[2]. SF Fn contains a higher carbohydrate content, a reduced content of sialic acid, a greater amount of N-acetylgalactosamine, and enhanced reactivity to the lectins WGA and PNA, the former indicating the presence of an additional N linked oligosaccharide at the carboxyterminal end of the molecule. In addition it has been shown that SF Fn reacts with Mab FDC-6, indicating the presence of an O linked glycan adjacent to the carboxyterminal heparin-binding site[3]. This glycan was shown to be restricted to Fn synthesized by
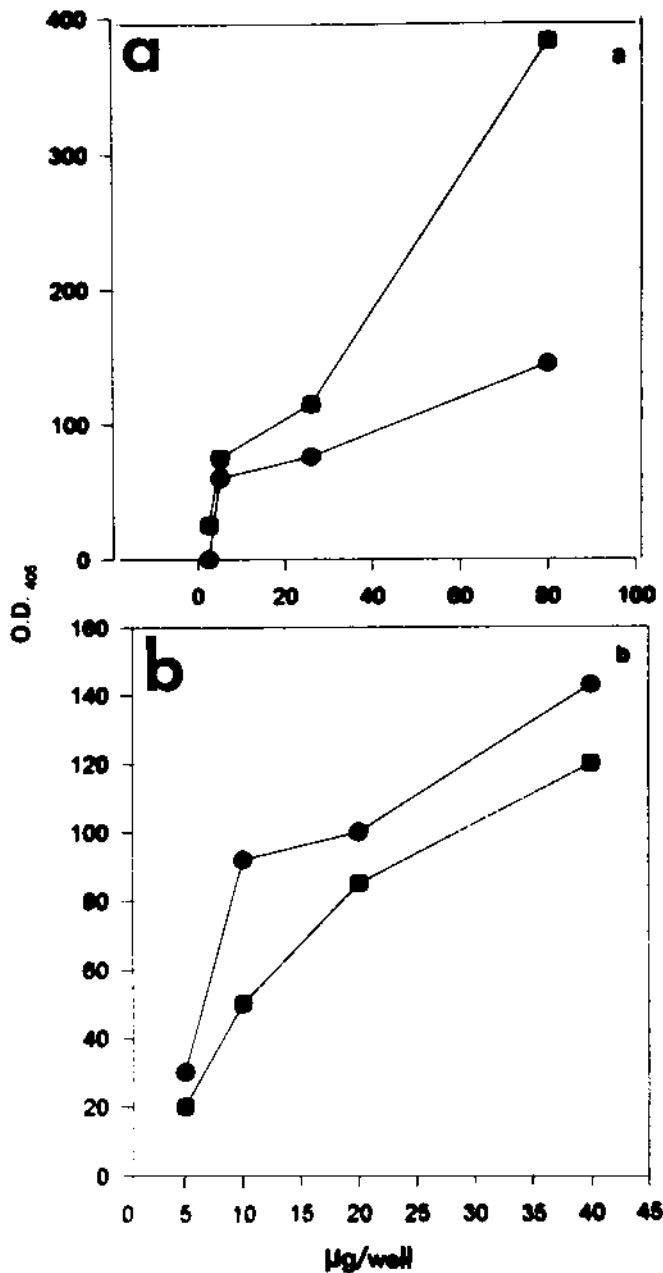
Figure 5. Analysis of Fn secreted by cultured fibroblasts. Lanes 1 and 6: plasma Fn; lanes 2, 5, 7, 10: RA SF Fn; lanes 3 and 8: Fn secreted by HFL-1 fibroblasts; lanes 4 and 9: Fn secreted by fibroblast-like synoviocytes. Lanes 1–5 stained with fast green; lanes 6–10 stained with PNA-peroxidase.

Figure 6. Antibody-lectin sandwich immunosorbent assay (ALSIA) for quantitation of glycosylated Fn isoforms. Bars indicate means ± SE. White bar: plasma Fn; black bar: RA SF Fn; shaded bar: OA SF Fn.

Figure 4B. Enzyme linked collagen-binding assay of chymotryptic Fn fragments. (a) ■: binding of serial dilutions of gelatin-binding SF Fn fragments; ●: serial dilutions of gelatin-binding PFn fragments. (b) Neuraminidase treatment of PFn fragments (●) enhances their binding to gelatin compared to fully sialyated PFn fragments (■).

embryonal or malignant tissues[12], suggesting that the enhanced O glycosylation observed on RA SF Fn indicates transformation of fibroblast-like synoviocytes (FLS). The enhanced PNA reactivity shown by SF Fn potentially represents an additional site of enhanced O glycosylation on SF Fn. To confirm this, SF Fn were digested with N-acetyl-galactosaminidase, which resulted in abrogation of PNA reactivity. Additionally, SF Fn reacted with VVA lectin (data not shown), which also possesses known specificity for O
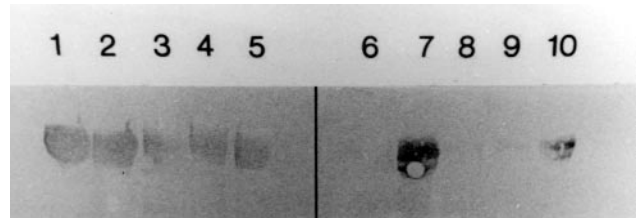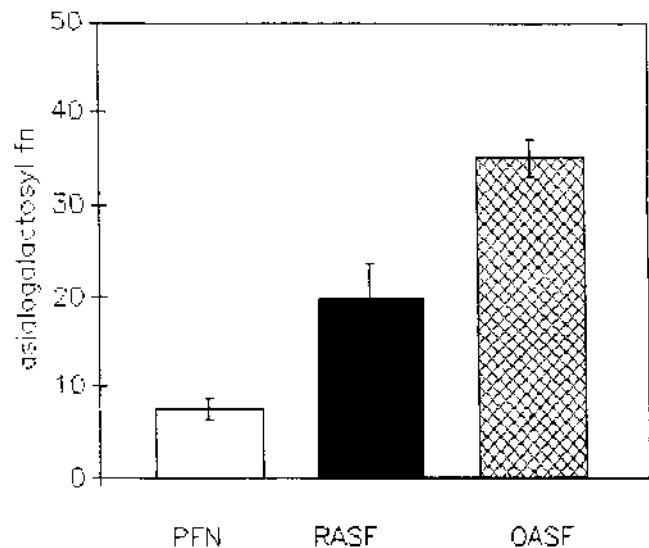
linked glycans. To confirm that PNA binding represents expression of an O linked glycan on SF Fn, unique from the FDC-6 epitope, limited enzymatic digestion was performed with a series of proteolytic enzymes. These experiments revealed that PNA reactivity localized to the carboxyterminal portion of the gelatin-binding fragment, which is located toward the amino terminal of the molecule. The presumed structure of the PNA reactive glycan is O-GalNac-ßGal, suggesting that PNA reactivity is unmasked by the *in vivo* loss of the terminal sialic acid[13]. It is interesting that there appears to be selective PNA expression of the Fn B chain (Figure 2B), since the sequence of the gelatin binding regions of both chains of the Fn dimer should be nearly identical. A desialyated, lower molecular weight form of osteopontin has recently been shown to arise by desialyation at or immediately after secretion[14]. It is possible that postsecretory desialyation may have occurred and may have preferentially affected the Fn B chain due to stereochemical considerations secondary to overall differences in intrachain structure (i.e., the absence of the IIICS domain on the B chain).
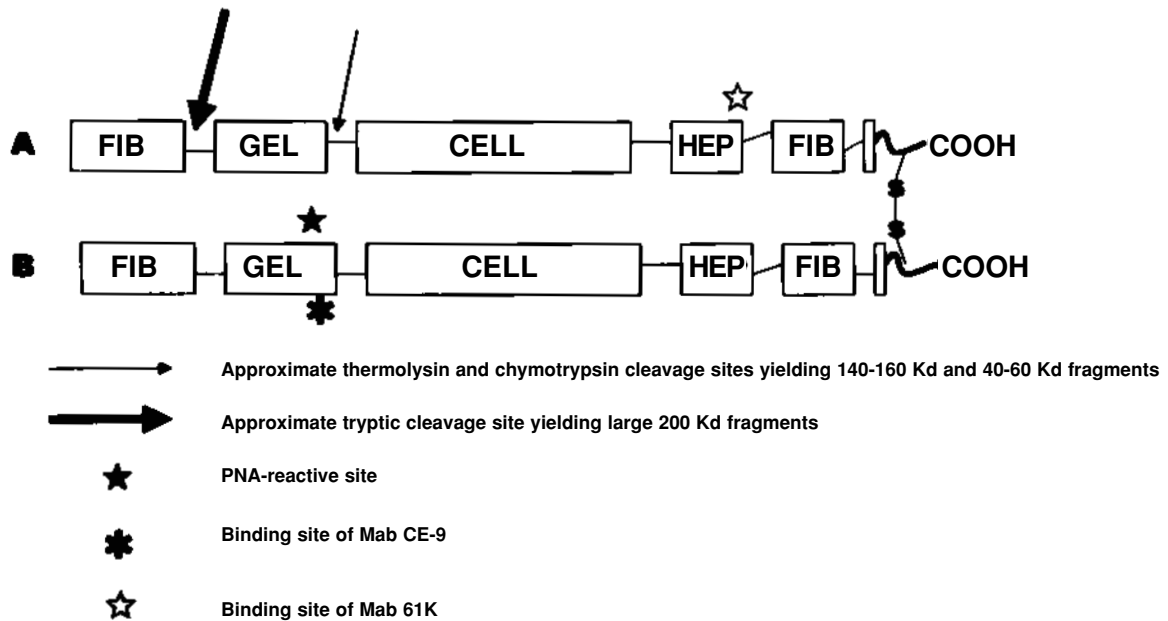
Figure 7. The domain structure of the fibronectin dimer showing major proteolytic cleavage sites and binding sites for PNA lectin and Mab CE-9 and Mab 61K.

Carbohydrate moieties of glycoproteins serve several functions, including protection of the protein from proteolytic digestion in an inflammatory environment and steric modulation of ligand-binding properties[15]. The glycosylation heterogeneity described for SF Fn differs significantly from that described for plasma IgG in RA[16]. IgG have been shown to be hypogalactosylated, whereas galactose is required for PNA binding. Hypogalactosylation of IgG in RA is thought to reduce its binding to monocytes and C1q[16]. Further, altered glycosylation of RA IgG occurs on N linked side chains, whereas alteration of glycosylation of RA Fn involves O linked side chains as well. Hyposialyation of SF Fn may serve to reduce steric hindrance and enhance its potential binding to specific ligands. As shown here, the charge displayed by a set of SF Fn fragments is significantly more basic than PFn fragments; these appear to represent hyposialyated collagen-binding SF Fn fragments that have enhanced affinity for denatured collagen compared to corresponding PFn fragments. The glycosylation modification of the gelatin-binding domain described here differs from that seen in human amniotic fluid Fn, where lactosaminoglycanation reduces binding to gelatin[17]. These results suggest that basic, hyposialyated Fn collagen-binding domains in the synovial microenvironment may be more avidly bound to extracellular matrix collagen in inflamed synovium and diseased cartilage, where they may modulate cell attachment and invasion. Since PNA reactive Fn is not found in plasma, it is assumed that it is either locally produced by FLS or formed by local modification of Fn in the synovial environment. Fn isolated from HFL-1 fibroblasts barely reacted with PNA, whereas Fn isolated from RA FLS was weakly reactive. This suggests that PNA reactive SF Fn may be derived in part from FLS.

Phenotypic characteristics of FLS including the expression of the FDC-6 glycan epitope suggest that they possess transformation-like properties. It is likely that this phenotype is induced by the inflammatory microenvironment of the diseased joint. Cytokines do have the potential of altering protein glycosylation as reported by Levesque and Haynes[18], who described interleukin 4 (IL-4) mediated inhibition of tumor necrosis factor-α dependent CD44-hyaluronate binding associated with alterations in CD44 N-glycosylation. In addition, Chintalacharuvu, *et al* found that IL-4 and IL-5 treatment of CH12LX cells decreased terminal glycosylation of secreted IgM and MHC class I[19].

When an antibody-lectin solid phase immunoassay was utilized to measure expression of the PNA reactive oligosaccharide, elevated levels were found in all SF Fn specimens compared to plasma Fn sample levels. Expression of this oligosaccharide, however, was significantly higher on OA SF Fn in contrast to RA SF Fn. This is in contrast to the FDC-6 reactive glycan, whose expression in RA is roughly 2-fold higher than in OA[3]. Thus, while there appears to be enhanced expression of 2 O-glycosylation sites on SF Fn, expression appears to be independently regulated in OA and RA. The finding of high levels of PNA reactive Fn in OA SF suggests that it may be a product of OA cartilage, since OA cartilage has been shown to have an elevated Fn content[20], and Fn isolated from cartilage of osteoarthritic dogs have been shown to possess altered glycosylation[21]. Extracts of human OA cartilage, however, have not revealed PNA reactive glycans when examined by lectin Western blotting

(unpublished data). Nonetheless, Fn fragments of molecular size comparable to the amino-terminal chymotryptic fragments studied here have been identified in OA SF[22].

This is the third modification in glycosylation described for fibronectins isolated from synovial fluid. Each appears to be structurally distinct and is localized to a specific domain of the molecule. Fibronectin glycosylation isoforms have the potential to be markers of rheumatic disease activity and progression, as well as indicating potential mechanisms for structural extracellular matrix alteration in arthritis.

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