Selective Binding Affinity of Human Plasma Fibronectin for the Collagens I–IV

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The binding of plasma fibronectin to the collagens I, II, and III was greater in a cohort of glaucoma patients. In contrast, binding of plasma fibronectin to collagen type IV was less in 39 glaucoma patients than in any of 3 other diagnostic categories, including 92 patients that were normal, cataract, and glaucoma suspect patients. This observation may have significance in further understanding the control of aqueous outflow resistance in glaucoma and nonglaucoma patients. Invest Ophthalmol Vis Sci 26: 1740–1744, 1985

Fibronectin is one of the glycoproteins and extracellular substances found in greater amounts in the trabecular meshwork of human eyes with glaucoma as contrasted to age-matched controls.[1] Based on the hypothesis that such extracellular substances can increase the resistance to outflow of aqueous humor, we have investigated the plasma levels of fibronectin as measured by enzyme immunoassay in patients with glaucoma, glaucoma suspects, and normal patients. The mean concentration in 26 patients with glaucoma was 552 ± 373 μg/ml as compared to 472 ± 352 μg/ml in 12 glaucoma suspects and 254 ± 103 μg/ml in 15 normal patients. There was no statistically significant difference in the concentration between groups, largely because of the great variation among patients in each group.

In an effort to detect any difference in the binding affinity of fibronectin to various collagens, we measured the binding affinity to Collagen I and III using enzyme immunoassay and found no difference between normal patients and patients with glaucoma.[2]

In 1984 we presented an analysis of human plasma fibronectin binding to Collagens I–IV which showed a significant difference in the binding of fibronectin to Collagen II between normal patients, glaucoma patients, and glaucoma suspects.[3]

The data reported in that abstract was based on analysis of a supersaturated concentration of collagen bound to the immunoassay plate. A further refinement of the method depends on determining the optimum concentration of each collagen which results in maximum binding of plasma fibronectin. We discovered that the efficiency of absorbing collagens to the plastic wells varies from time to time even with the same batch of collagens. Therefore, the entire experiment for all plasma samples was repeated using wells coated on the same day with the four collagens at the same time. In addition, each plasma sample was run at several different dilutions. A different set of dilutions was used for each collagen because each collagen type has an optimum plasma concentration for maximum binding. This two-way optimization of concentration results in the maximum binding capacity of each collagen and the greatest constancy between collagens. This refinement in technique enhances the ability for defining differential binding affinity. The purpose of this report is at present the result of an analysis of this new refinement in enzyme immunoassay.

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Supported by the Veterans Administration Research Service.

Submitted for publication: March 8, 1985.

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Materials and Methods

Patient Population

Patients were drawn from a private practice setting where glaucoma was defined as the presence of optic nerve head cupping with corresponding visual field loss as measured by kinetic and static perimetry. Glaucoma suspects were those patients with elevated intraocular pressure, but no detectable visual field loss. That group4.5.4 varied in the degree of optic nerve head cupping and number of glaucoma risk factors. Normal patients were those with no elevated intraocular pressure, changes in the optic nerve head, or risk factors of glaucoma. Informed consent was obtained from all patients prior to drawing blood.

Purification of Rabbit IgG

Five ml of rabbit antifibronectin (Cappel Labs; Westchester, PA) was added to a 5 ml column of aga-

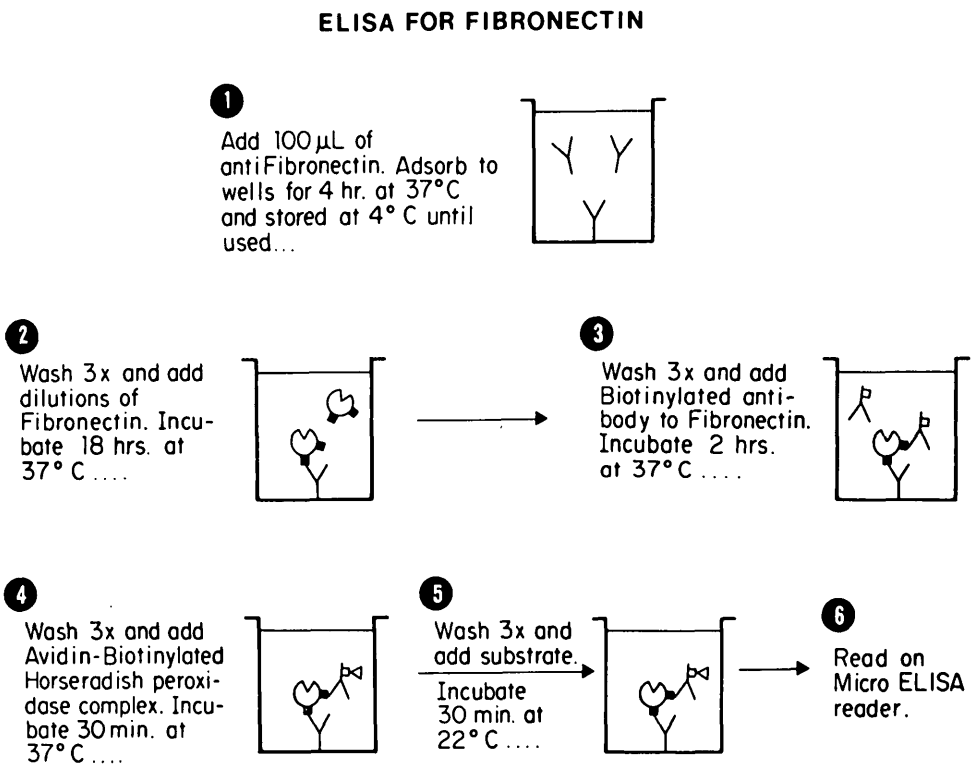

Fig. 1. Flow diagram of the immunoassay to quantitate the amount of fibronectin in plasma.

rose-staphylococcal protein A (SPA) (Zymed, Inc.; Burlingame, CA) at a flow rate of 15 to 30 ml/hr, maintained with phosphate buffered saline (PBS, pH 7.4). The column effluent was monitored for absorbance at 280 nm, and 1 ml fractions was collected. After non-IgG fractions had passed through the column, it was rinsed with 10 ml PBS. The IgG was eluted by adding 10 ml of 0.1 M glycine HCl, pH 3.0. Each fraction was neutralized with 0.1 N NaOH. The IgG fractions were pooled and dialyzed overnight at 4°C against 2 l of 0.1 M $NaHC_3$, pH 8.4.

Biotinylation

The protein concentration of the purified IgG solution was adjusted to 1 mg/ml with the bicarbonate buffer, and to each ml of antibody, was added 100 µl of dimethyl sulfoxide containing 1.1 mg/ml of the N-hydroxy succinimide ester of biotin (Calbiochem; La Jolla, CA). The reaction was allowed to proceed at room temperature for 2 hr with occasional shaking. Unreacted ester was blocked by adding 100 µl of 1.0 M NH_4Cl to each ml of antibody and incubating for an additional 10 min. To each ml of starting material, we added 1 ml of PBS containing 1% bovine serum albumin, then the sample was dialyzed overnight at 4°C against 2 liters of PBS containing 0.01% thimerosol.

Plasma samples (EDTA) were obtained from patients and stored frozen at −20°C. Samples were then thawed in a 37°C water bath, vortexed and diluted with 0.1 M phosphate buffered saline plus 0.25% bovine serum albumin pH 7.4 (BSA buffer) and assayed for fibronectin levels and binding to collagens I–IV.

Immulon I plates (Dynatech; Alexandria, VA) were used in the immunoassay. Immunoassay to quantitate the amount of fibronectin concentration present in the plasma, was performed as illustrated in Figure 1. Immunoassay to determine the amount of plasma fibronectin binding to the various collagens is depicted in Figure 2. Briefly, in these methods polyclonal antibody to fibronectin (Cappel Laboratories; Westchester, PA) and collagens I, II, and III (Seragen Inc.; Boston, MA) were diluted in 0.1 M sodium carbonate buffer at pH 9.6 and 50 µl was absorbed to wells 50 µl at the following concentrations: Anti-fibronectin–1.07 µg/ml; collagen I + III–6.25 µg/ml; collagen II–3.125 µg/ml. Plates were sealed, incubated at 37°C for 4 hr and then refrigerated at 4°C until used. Collagen IV (Bethesda Research Laboratories; Gaithersburg, MD) was diluted in 0.1 M acetic acid, to 40 µg/ml, and 50 µl added to each well, and air dried overnight before sealing and refrigeration. All the plates were aspirated and washed three times with BSA buffer prior to assay. The plasma dilutions (50 µl/well) were then added to triplicate wells at two concentrations for each collagen or antifibronectin as follows: Anti-fibronectin 1/40,000 and 1/160,000; Collagen I, II, and III 1/10,000 and 1/40,000; Collagen IV 1/1,250 and 1/5,000. By using two dilutions, one can select the dilution closest to the mid-

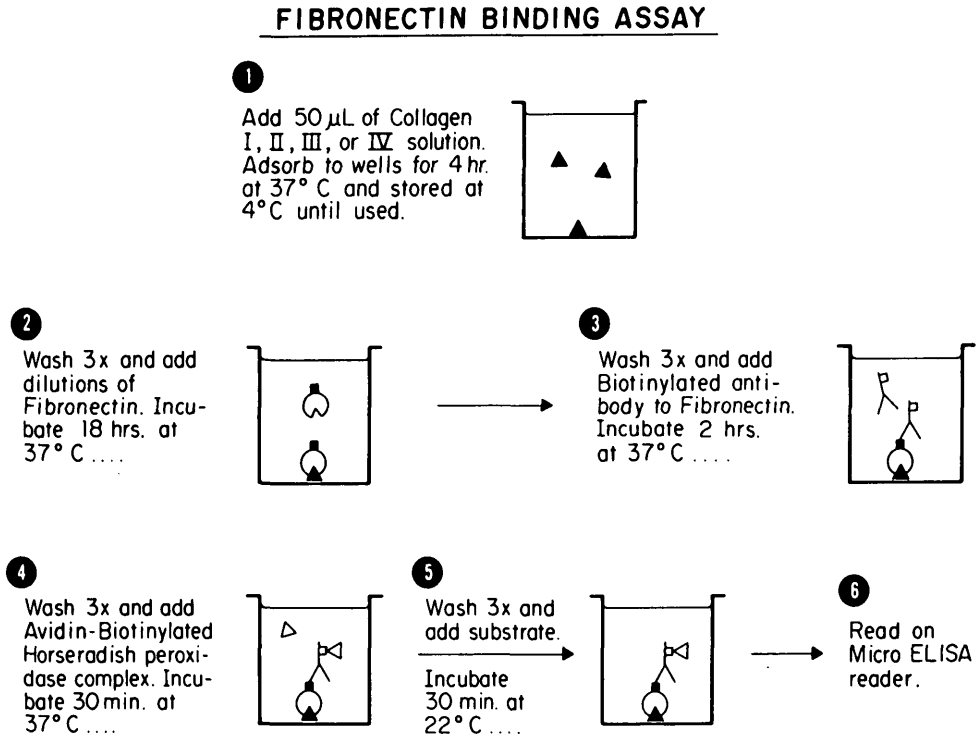

Fig. 2. Flow diagram of the immunoassay to quantitate the amount of fibronectin binding to collagens I–IV.

range of the standard curve to insure the accuracy of the data.

Plates were covered and incubated for 18 hr at 37°C to allow the fibronectin to bind. The unbound fibronectin was aspirated, and the plates washed three times, then a 1/150 dilution of Biotin labeled polyclonal rabbit antifibronectin (50 µl/well) was added and incubated for 2 hr at 37°C. The plates were aspirated and washed three times, and a 1/480 dilution avidin biotinylated horseradish peroxidase complex (ABC) (Vector Laboratories; Burlingame, CA) (50 µl/well) was added and plates incubated at 37°C for 30 min. Aspiration and washing were repeated and 200 µl/well of substrate solution (0.05 M citrate–0.1 M phosphate buffer [pH5] containing 0.04% O-phenylenediamine and 0.006% hydrogen peroxide) was added, and the plates incubated for 30 min at room temperature in the dark. The reaction was stopped by addition of 100 µl of 2 M H_2SO_4. The intensity of color (amount of reaction product) in each well was determined in a Dynatech MR580 automatic MicroELISA reader (Dynatech; Alexandria, VA) at 490 nm using BSA buffer as the blank. The data from the MicroELISA reader was transferred to a floppy disk of an Apple II microcomputer (Compton, CA) and the concentration of plasma fibronectin present (antifibronectin plate) and that bound to the various collagens was determined by using a standard curve and a Logit Transformation program which mathematically determines the concentration of fibronectin present. The standard curves for each of the five immunoassays are relatively linear when plotted on a log × log chart (Fig. 3) and values taken for the purpose of calculation were always selected from those in the mid-range of the curve.

Results

Complete data including exact age was available on 125 patients. Among those 125 were patients with chronic open angle glaucoma, 28 glaucoma suspects (ocular hypertensives), 34 cataract patients, and 30 control patients.

Patient Population

There was no correlation with fibronectin and vacation activity. The patients were a stable, resident population. There was no seasonal variation in binding activity.

Age

Tabulation of the 125 patients by decades showed no age trend for plasma fibronectin level or binding of plasma fibronectin to any of the Collagens I through IV. The values for each interval from under age 60, 60–69, 70–79, and age 80 and above are listed in Table 1. Statistical analysis by ANOVA (analysis of variance) failed to show a statistical significant difference between values at the $P < 0.005$ level except for ages 60 versus 70. Analysis of each diagnostic category failed to show

any statistical difference as a function of age when analyzed by regression analysis.

Because there was no specific age correlation with plasma fibronectin levels and no correlation with age to collagen binding, the results were compared among four diagnostic categories: cataract, normal, glaucoma suspect and glaucoma patients.

Table 2 lists the values for each of these groups. The total number of patients, 131, includes an additional 6 patients in whom there was a question of their exact age.

One way analysis of variance (ANOVA) showed a statistical significant difference in the binding capacity of plasma fibronectin to the Collagens I through IV is shown in Table 3. The values which show $P < 0.005$ are indicated by a letter indicating the higher of the two values. Comparison showed statistically significant difference in plasma fibronectin levels among the four diagnostic categories.

A comparison of the values for glaucoma patients versus normal patients shows the binding capacity value among patients with glaucoma to be higher for Collagens I (192 vs 158), II (163 vs 110), and III (167 vs 102), but lower for Collagen IV (33 vs 41).

A comparison of the values for glaucoma patients versus cataract patients showed the binding capacity to Collagen IV to be higher for cataract patients (33 vs 40).

A comparison of the values for normal patients versus cataract patients showed the binding capacity to Collagen III to be higher for cataract patients (102 vs 142).

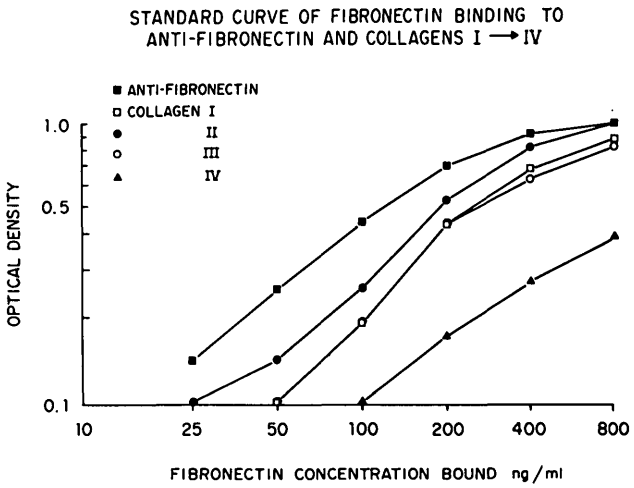

Fig. 3. Standard curves for each of the immunoassays. Log × log plot.

Discussion

Collagen types are specific to different eye tissues. The aqueous outflow pathways contain predominantly Collagens I, III and IV. Collagen I is the most common collagen in eye tissues. Collagen I is found in cornea, sclera, and interstitial connective tissue. Collagen II is most often found in cartilage but is also found in the vitreous. Collagen III is found in loose connective tissue, blood vessel walls, and sclera. Collagen IV is found in the basement lamina on cell surfaces such as the lens capsule and Descemet's membrane.[4]

The binding of fibronectin to Collagens I, II and III was greater in the glaucoma population. In contrast,

Table 1. Differential binding of plasma fibronectin to the four collagen types as a function of age and total amount in plasma*

Age	*Number*	*Total plasma*	*Collagen 1*	*Collagen 2*	*Collagen 3*	*Collagen 4*
<60	30	522 ± 140	160 ± 58	123 ± 61	130 ± 63	36 ± 12
60–69	39	584 ± 143	195 ± 53	171 ± 78	159 ± 67	37 ± 9
70–79	39	530 ± 168	160 ± 50	118 ± 53	110 ± 44	39 ± 12
80+	17	545 ± 127	176 ± 46	153 ± 73	151 ± 52	37 ± 9
Total	125					

* There was no statistically significant difference as a function of age for binding to any of the collagen types or the plasma values when tested by ANOVA.

† Values in μg/ml.

Table 2. Plasma fibronectin values in μg/ml in diagnostic categories

Group	*N*	*Total fibronectin in plasma*	*Fibronectin bound to collagen*			
			I	*II*	*III*	*IV*
Normal	30	514 ± 158	158 ± 51	110 ± 51	102 ± 41	41 ± 10
Cataract	34	550 ± 131	183 ± 52	149 ± 70	142 ± 65	40 ± 10
Glaucoma Suspect	28	570 ± 178	166 ± 50	145 ± 67	129 ± 53	39 ± 14
Glaucoma	39	576 ± 147	196 ± 56	163 ± 78	167 ± 61	33 ± 8.6
Total	131					

Table 3. Statistical significant differential binding capacity of plasma fibronectin to collagens I through IV

Groups	*I*	*II*	*III*	*IV*
Glaucoma (G) vs Normal (N)	G*	G	G	N
Glaucoma vs Cataract (C)	—	—	—	C
Glaucoma vs Glaucoma Suspect	—	—	—	—
Glaucoma Suspect vs Normal	—	—	—	—
Glaucoma Suspect vs Cataract	—	—	—	—
Normal vs Cataract (C)	—	—	C	—

* Letters indicate binding value differences determined by ANOVA where $P < 0.005$. The letter is for the group with the higher value.

binding to Collagen IV was less in the glaucoma population than any of the other three diagnostic categories. The increased binding capacity to collagens found in the interstitial spaces is an interesting finding when combined with the finding of a reduced binding to cell surfaces (basement lamina). It is assumed that plasma fibronectin reflects tissue fibronectin binding. It might be speculated that an increased amount of fibronectin in the interstitial space versus on cell surfaces could be correlated with a reduced rate of aqueous flow. The demonstration of a differential binding capacity in and of itself might prove helpful in differentiating normal patients from glaucoma patients using a blood test. However, in its present form the analysis lacks sufficient selectivity and specificity to serve as a reliable screening blood test. The finding also lends itself to a variety of speculations regarding cause and effect. For example, there may be fibronectins with differential binding capacity to each of the collagens; an assay of plasma values measures those with lower tissue affinity which are therefore still circulating free in the plasma. Other substances could be the causative agent by binding at a site shared by the collagens and would therefore depress binding. A single binding site could also be regulated by competitive inhibition. If all three of the Collagens I, II, and III are present, they could occupy all the binding site space, thereby lowering the collagen binding of Collagen IV. In contrast to the normals, where collagen binding values for Collagen I, II and III were respectively 158, 110 and 102 μg/ml, are closer to the Collagen IV binding of 41 μg/ml; the glaucoma patients had Collagen I, II and III values of 192, 163 and 167 μg/ml, all a considerable gap from the value of 33 μg/ml for Collagen IV.

The variation in binding capacity between patients does not make the measure accurate for predicting a normal versus a glaucoma patient. However, the difference, particularly for Collagen IV alone or in comparison to the other collagens, is worthy of further refinement and analysis in hopes of developing a test of prospective and predictive value.

Key words: fibronectin, glaucoma, collagen I, collagen II, collagen III, collagen IV

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