IgA Interaction with Carboxy-Terminal 43-kD Fragment of Fibronectin in IgA Nephropathy

SHINOBU WAGA, KAZUHIKO SUGIMOTO, HIROSHI TANAKA, TATSUO ITO, TOHRU NAKAHATA, TAKASHI TATEYAMA, YOSHIKI KAKIZAKI, and MASARU YOKOYAMA

Department of Pediatrics, Hirosaki University School of Medicine, Hirosaki, Aomori, Japan.

Abstract. IgA deposition in the glomerular mesangial matrix is a prerequisite for the diagnosis of IgA nephropathy, and circulating IgA-containing complex has been implicated in this process. Since fibronectin is known to be involved in the assembly of extracellular matrix, this study was conducted to investigate whether fibronectin and its fragments are present in sera of patients and are capable of binding IgA1. Sera from patients with IgA nephropathy were purified by heparin-affinity chromatography, and column eluate were analyzed for the presence of fibronectin using Western blot and a set of anti-fibronectin monoclonal antibodies. Native fibronectin was digested with cathepsin D to obtain fragments similar to those of serum fibronectin. The capacity of fibronectin to bind IgA was examined with a mixture of purified IgA1 and cathepsin D-digested fibronectin fragments. A 43-kD carboxy-terminal fragment of fibronectin was detected in samples derived from sera of patients with IgA nephropathy but not in healthy control subjects. A similar-sized fragment was generated by cathepsin D digestion of the native molecule and was shown to bind to IgA1 *in vitro*. Since the carboxy-terminal domain is known to be critical in assembling exogenous fibronectin into the extracellular matrix, the affinity to IgA1 to a fragment found in patients may have pathogenic potential to mediate extracellular IgA deposition in IgA nephropathy.

IgA nephropathy is a common form of renal disease characterized by deposition of IgA in the mesangial matrix of glomerulus, and the disease progresses slowly to renal failure in a substantial percentage of patients (1). Recurrent deposition of IgA frequently occurs in transplanted kidneys (2). The mesangial deposits contain IgA1, which is the major subclass of serum IgA (3). These findings indicate that circulating IgA-containing complexes capable of depositing in the mesangium may play a key factor in the pathogenesis of IgA nephropathy.

The composition of IgA1-containing complexes has been extensively studied. A limited number of endogenous components including C3, IgG, IgM, fibronectin, and polymeric IgA have been demonstrated (4–6). Among them, polymeric IgA was demonstrated to bind mesangial cells in culture through Fc-α receptor and lead to proliferation and IL-6 secretion (6,7). The possible self-aggregation of IgA1 via its glycosylation properties has been proposed recently (8). In addition, an IgA–fibronectin complex was detected by enzyme-linked immunosorbent assay (ELISA), in which a fibronectin-binding molecule, such as denatured collagen I (5) or anti-fibronectin antibody (9), was immobilized on plastic. Contrary to the initial report that this ELISA could detect IgA complexes in a

statistically significant number of IgA nephropathy patients (9), subsequent studies suggested that the specificity of the assay was ambiguous. Detection of IgA–fibronectin complex was not suppressed by the addition of excess amount of fibronectin or anti-fibronectin antibody, and the reactivity was dependent on the concentration of IgA in the sample (10). Other investigators argued that only plasma polymeric IgA, not monomeric IgA, was able to bind fibronectin (11), although Cederholm *et al.* (12) reported comparable reactivity in serum samples.

The finding common to these studies was that IgA was the unique Ig detected by this type of assay system. However, Rostagno *et al.* (13) reported that IgA did not bind fibronectin under physiologic conditions and that a preferential binding order of fibronectin to the major Ig classes was IgG > IgM > IgA. Thus, it is crucial to elucidate how IgA is able to interact with fibronectin in IgA nephropathy.

Fibronectin fragments have been shown to have comparable biologic activities to the native protein. In addition to chemotactic and opsonic activities (14,15), recent studies demonstrated that both the amino-terminal 70-kD fragment and carboxy-terminal region containing a disulfide bond are essential to be assembled into extracellular matrix (16). Although fibronectin is one of the major components of mesangial cell matrix (17) and matrix expansion is characteristic of IgA nephropathy (4), fibronectin fragments have not been considered in the pathogenesis of the disease. Therefore, a study was undertaken to search for the presence of fibronectin fragment(s) in the serum of patients with IgA nephropathy and to determine its capacity for binding IgA.

Materials and Methods

Sera

Sera were obtained from patients with primary IgA nephropathy. Informed consent was obtained at the time of routine examination. The diagnosis of IgA nephropathy was assessed by the observation of predominant mesangial IgA deposits in renal biopsies using immunofluorescence microscopy. None of the patients had systemic lupus erythematosus, Henoch-Schönlein purpura, or cirrhotic liver disease. Sera from healthy volunteers were used as controls.

Heparin Affinity Chromatography of Serum Fibronectin

One milliliter of serum was passed though a 1-ml column of Sepharose 4B beads (Pharmacia, Uppsala, Sweden) before chromatography on a 1-ml column of heparin-Sepharose CL-6B (Pharmacia) equilibrated with 0.15 M NaCl/10 mM Tris-HCl, pH 7.4. Fibronectin and IgA bound to heparin affinity column was eluted with 0.5 M NaCl/10 mM Tris-HCl, pH 7.4. The effluent was immediately desalted with Bio-Gel-6DG desalting gel (Bio-Rad, Richmond, CA). The protein concentration was determined by Bio-Rad protein assay (Bio-Rad), and a range of 144 to 588 μg/ml was detected from the analysis of 10 IgA nephropathy patients and 10 healthy control subjects.

ELISA for IgA

The IgA concentration of the eluate of heparin affinity chromatography was determined in six patients and nine control subjects by an ELISA technique, as described in previous studies (18,19). In brief, Immulon II microtiter plates (Dynatec Laboratories, Alexandria, VA) were coated overnight at 4°C with 100 μl/well of affinity-purified goat antihuman IgA (Tago, Camarillo, CA) at a concentration of 20 μg/ml in PBS (0.01 M phosphate, 0.15 M NaCl, pH 7.4). The plates were blocked with 150 μl of 0.5% casein in PBS overnight at 4°C. Before use, the plate was washed twice with PBS containing 0.05% Tween 20 (PBS-T). Each assay was standardized with human IgA preparation (Sigma) of 0.25×10^{-3} to 5×10^{-3} μg/ml in PBS. For the detection of IgA, 100 μl of the effluent obtained from heparin affinity chromatography was added in duplicate. The plates were incubated at room temperature for 2 h and washed four times with PBS-T. To all wells except the blanks was added 100 μl alkaline phosphatase-conjugated affinity-purified goat antihuman IgA (Tago) diluted 1:10,000 in 0.5% casein in PBS-T. After agitation at room temperature for 2 h, the wells were rinsed three times with PBS-T and twice with diethanolamine buffer, pH 9.8. Two hundred microliters of substrate solution containing 1 mg/ml p-nitrophenyl phosphate in diethanolamine buffer was added, and optical density was determined after 1 h by using an automated spectrophotometer at 405 nm (model 2550; Bio-Rad). The mean of duplicate readings was calibrated, and the experimental values were determined by computerized linear regression analysis for comparison to the IgA standard curve. The ratio of IgA to the total protein in the eluate was calculated to standardize assay results.

Electrophoresis and Western Blot Analysis

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis on a 15% acrylamide slab gel or 5 to 20% (wt/vol) linear gradient acrylamide slab gel with a 5 or 3.5% acrylamide stacking gel, respectively, and subsequent transfer of samples to nitrocellulose (Bio-Rad) were performed as described previously (19). The nitrocellulose was then cut into strips, which were subsequently soaked in 2% casein (Sigma Chemical Co., St. Louis, MO) in PBS (0.05 M, pH 7.2) for 2 h at room temperature to block nonspecific binding sites. A set of monoclonal antibodies (0.2 mg/ml) with antihuman fibronectin activity (described below), diluted at 1:80 with 2% casein PBS-T, were allowed to react with the nitrocellulose strips for 1.5 h at room temperature. The strips were washed by agitation in two changes of PBS-T for 1 h. Bound antibodies were detected by reaction for 1.5 h at room temperature with peroxidase-conjugated F(ab')$_2$ sheep antimouse Ig (Amersham, Buckinghamshire, United Kingdom) diluted in 2% casein in PBS-T at 1:300. After extensive washing with PBS-T, the strips were subjected to substrate solution composed of 3,3'-diaminobenzidine tetrahydrochloride (Sigma) and hydrogen peroxide in 0.05 M Tris-HCl buffer, pH 7.4. The efficiency of proteins transferred to nitrocellulose was monitored by staining with 0.1% amido-black 10B in 7% acetic acid.

Purification of Fibronectin

Five hundred milliliters of outdated human plasma, a kind gift of Aomori Branch of The Japan Blood Center, was processed to obtain fibronectin, according to Hayashi and Yamada (20). Briefly, a flow-through of plasma from Sepharose CL-4B (Pharmacia) was treated serially with gelatin-Sepharose (Pharmacia) and heparin-Sepharose (Pharmacia). The yield of product was 43%, and purity on 15% SDS-polyacrylamide and Coomassie Blue R250 staining was 95%. Aliquots at 4.3 mg/ml in 10 mM Tris/0.15 M NaCl, pH 7.0, were stored at -80°C until use.

Purification of IgA1

According to the method of Loomes et al. (21), 30 ml of serum derived from the recalcification of 80 ml of plasma was processed serially with ammonium sulfate, gel filtration column (Sepharose 6B; Pharmacia), anion exchange column (DEAE Sephacel; Pharmacia), and jacalin-affinity column (jacalin-agarose; Vector Laboratories, Burlingame, CA). Material bound to jacalin-agarose was eluted with PBS/0.8 M D-galactose. The eluate was dialyzed against PBS. The yield of product was 15%, and purity on 15% SDS-polyacrylamide slab gel was 95%. Aliquots at 0.38 mg/ml in PBS were stored at -30°C until use.

Cathepsin D Treatment of Fibronectin

Fibronectin was treated with cathepsin D (Sigma) with 2 mM phenylmethylsulfonyl fluoride (Sigma), pH 3.0, for 15 min at 37°C at an enzyme substrate ratio of 1:100. The reaction was terminated by addition of a 10-fold pepstatin A (Sigma), followed by neutralization to pH 7.4.

Jacalin-Agarose Affinity Isolation of Fibronectin Fragment Bound to IgA1

Purified IgA1 and cathepsin D-digested fibronectin were mixed at the ratio of 2:1 (wt/wt, 380:190 μg), and incubated for 2 h at 36°C with gentle stirring. The protein mixture was then incubated with 200 μl of hydrated jacalin-agarose for 2 h at room temperature with shaking. The agarose was sedimented in a tube ($15,770 \times g$, 30 s), and the supernatant containing nonbinding protein was removed. The pellet was washed 10 times with PBS/CaCl$_2$ by vortexing and resedimenting. The final pellet was resuspended in 200 μl of 2× concentrated sample buffer, heated, applied to SDS-polyacrylamide gel electrophoresis, and subjected to Western blot analysis. As controls, jacalin-agarose to which intact fibronectin, digested fibronectin, and
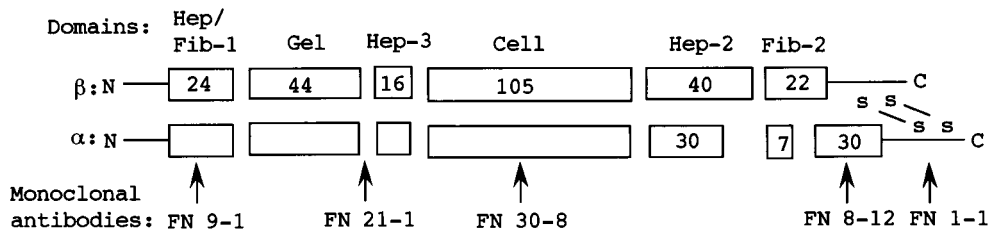
Figure 1. Schematic diagram of plasma fibronectin and specificities of monoclonal antihuman fibronectin antibodies to distinct functional domains. The majority of plasma fibronectin is a two-chain (α and β), disulfide-bridged dimer of approximately 450 kD. Each chain consists of six functional domains with binding specificities to heparin (Hep), fibrin (Fib), gelatin (Gel), and cell surface (Cell). Binding sites (arrows) of monoclonal antihuman fibronectin antibodies (FN9-1, FN21-1, FN30-8, FN8-12, FN1-1) used in this study are indicated.
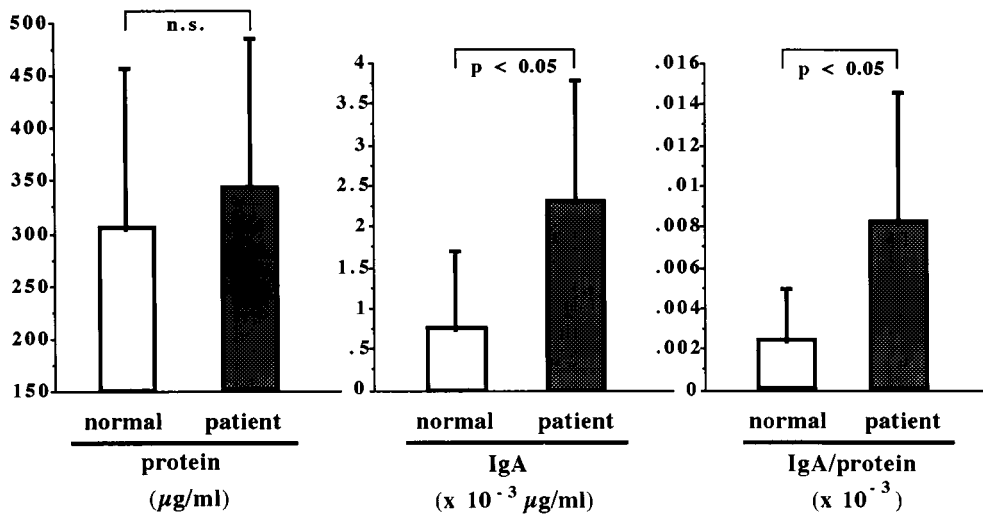

Figure 2. Concentration of IgA in serum heparin-binding proteins. Protein, IgA, and the ratio of IgA to protein (IgA/protein) in the eluate of heparin affinity chromatography from serum of healthy control subjects ($n = 9$) compared with patients ($n = 6$) with IgA nephropathy showed that patients have significantly higher IgA and the ratio of IgA to protein. Values are means + SD.
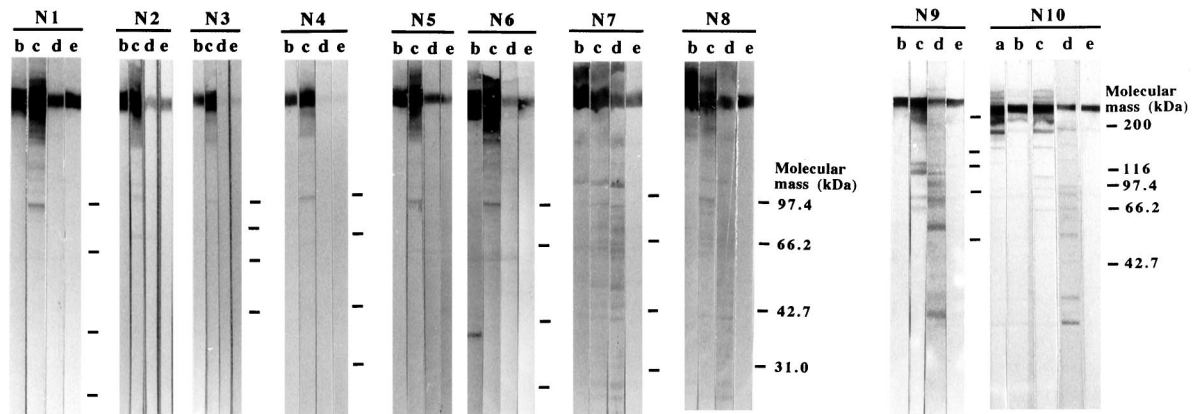

Figure 3. Western blot analysis of fibronectin fragments in normal human serum. Heparin-binding proteins from serum of healthy control subjects (N1-10) were separated by 5 to 20% acrylamide linear gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), electrotransferred to nitrocellulose, and probed with the following monoclonal antibodies: a, FN30-8; b, FN1-1; c, FN21-1; d, FN9-1; e, FN8-12. Molecular masses from standards are shown on the right.
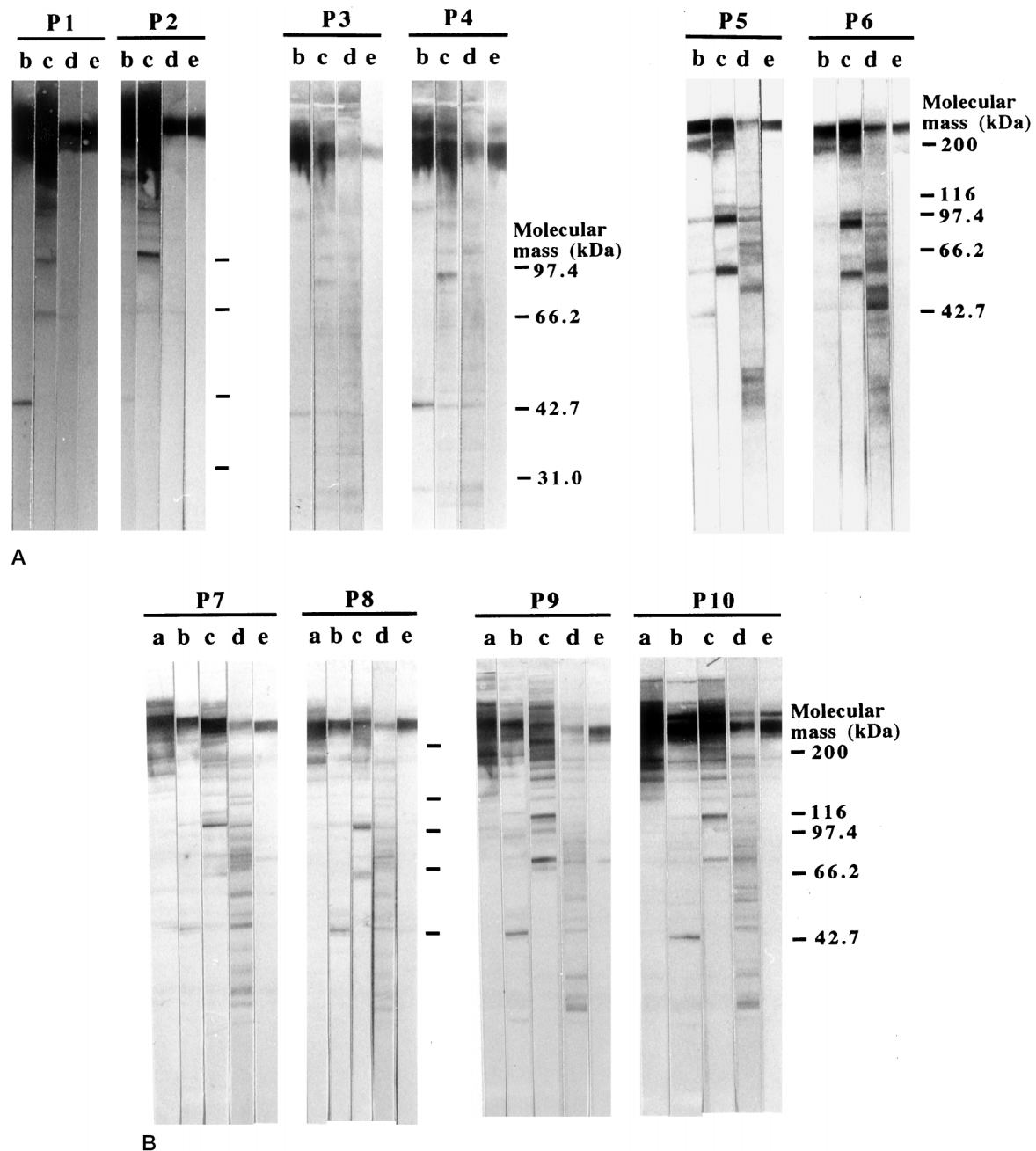
Figure 4. (A) Western blot analysis of fibronectin fragments in patient's serum. Heparin-binding proteins from serum of patients (P1-6) were separated by 5 to 20% acrylamide linear gradient SDS-PAGE, electrotransferred to nitrocellulose, and probed with the following monoclonal antibodies: b, FN1-1; c, FN21-1; d, FN9-1; e, FN8-12. Molecular masses from standards for P5 and P6 were shifted upward because of shorter running time for electrophoresis. (B) Western blot analysis of fibronectin fragments in patient's serum. Heparin-binding proteins from serum of patients (P7-10) were separated by 5 to 20% acrylamide linear gradient SDS-PAGE, electrotransferred to nitrocellulose, and probed with the following monoclonal antibodies: a, FN30-8; b, FN1-1; c, FN21-1; d, FN9-1; e, FN8-12.

IgA1 were added, were subjected to the same procedure. In addition, a protein mixture of purified IgG (Sigma) and fibronectin fragments at the ratio of 1:1 (wt/wt, 380:380 μg) was incubated with 25 μl of hydrated protein G-agarose (protein G-Sepharose 4B Fast Flow; Sigma), an IgG affinity matrix, and was processed as an IgG control.

Monoclonal Antihuman Fibronectin Antibodies

Fibronectin and its fragments were detected using a set of monoclonal antihuman fibronectin antibodies (FN8-12, FN9-1, FN21-1,

FN1-1, FN30-8) obtained from Takara Shuzo, Ltd. (Kyoto, Japan) (22). The binding sites of the monoclonal antibodies on fibronectin molecule are depicted in Figure 1.

Statistical Analyses

Data of both the protein and IgA concentration of the eluate of heparin affinity chromatography are given as mean $\pm$ SD. Statistically significant results, determined by an unpaired t test, were taken as $P < 0.05$.

Results

IgA in the Serum Eluate from Heparin Affinity Chromatography

Six patients and nine healthy control subjects were subjected to the measurement of IgA in the eluate (Figure 2). Protein concentration of the eluate did not show significant differences between patients and healthy control subjects (342.5 ± 141.4 *versus* 306.7 ± 149.2 μg/ml, $P = 0.65$). A small amount of IgA was detected in the eluate, and its concentration in the patients was significantly higher than in control subjects ($2.33 \pm 1.46 \times 10^{-3}$ *versus* $0.77 \pm 0.95 \times 10^{-3}$ μg/ml, $P = 0.0255$). Accordingly, the ratio of IgA to protein in the eluate was also higher in patients than control subjects ($0.0083 \pm 0.0063 \times 10^{-3}$ *versus* $0.0026 \pm 0.0024 \times 10^{-3}$, $P = 0.0258$).

Detection of Fibronectin Fragments in Patient's Serum

Antibodies FN30-8 (Figure 3 and Figure 4, A and B; antibody "a") and FN8-12 (antibody "e") reacted in Western blot with serum fibronectin preparations at the positions around 220 kD in the patients (P7-10 for antibody "a," P1-10 for antibody "e"), but not with the bands under molecular marker of 116 kD. The reactivity at 220 kD corresponds to native fibronectin monomer consistent with that demonstrated with other anti-fibronectin monoclonal antibodies. FN1-1 (antibody "b") reacted with native fibronectin and a band at approximately 43 kD in patients with variable intensity (antibody "b," P1-10), but not in healthy control subjects (antibody "b," N1-10). FN21-1 (antibody "c") reacted with bands at approximately 90 to 100 and 60 to 70 kD in patients (antibody "c," P1-10). Although the patterns of reactivity were similar in control subjects, the intensity of the 90- to-100-kD band in patients was higher than those in control subjects (antibody "c," N1-10). FN9-1 reacted with many bands (antibody "d"), and there were no apparent differences between patients and healthy control subjects. Thus, the 43-kD polypeptide detected with FN1-1 appeared to be specific for serum fibronectin preparations from patients with IgA nephropathy.

Jacalin-Agarose Affinity Chromatography of IgA1–Fibronectin Complex

The possibility that fragments of fibronectin observed only in patients, such as the 43-kD polypeptide, were part of an IgA–fibronectin complex was further analyzed using IgA1-specific jacalin-agarose chromatography (23) with a mixture of IgA1 and *in vitro*-derived fibronectin fragments. In previous studies, several proteases were used to determine the topological assignment of soluble fibronectin. Among these, cathepsin D produced well characterized carboxy-terminal fragments, including the one at approximately 40 kD (24). In our current study, when cathepsin D was used to digest native fibronectin as described in Materials and Methods, intense bands at approximately 45 kD were determined with monoclonal FN1-1 (data not shown). As shown in "1" of Figure 5, amido-black staining of proteins eluted from jacalin-agarose and transferred onto nitrocellulose strips demonstrated heavy and light chain of IgA. Fibronectin fragments copurified with IgA1 were detected
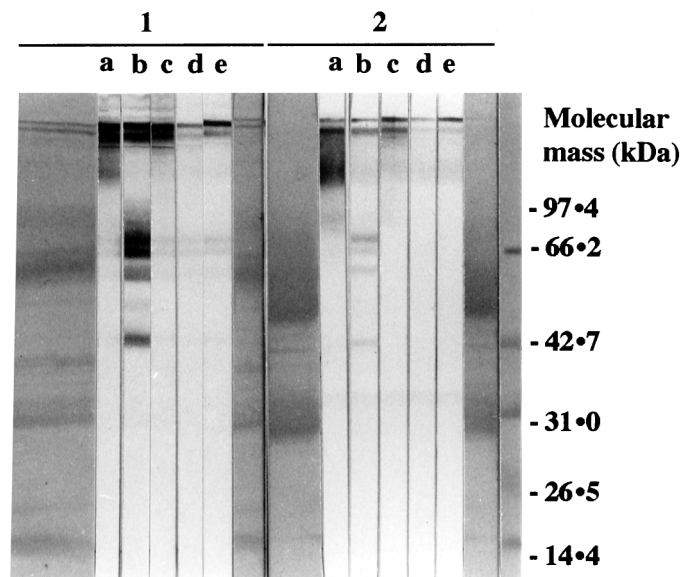

Figure 5. In vitro interaction of cathepsin D-digested fibronectin fragment and IgA1. A mixture of IgA1 and cathepsin D-digested fibronectin was incubated with jacalin-agarose ("1") or protein G-Sepharose ("2"). Binding proteins were eluted and analyzed by Western blot using a set of monoclonal antihuman fibronectin antibodies (a through e) as depicted in Figure 3. Incubation with jacalin-agarose produced bands at the positions of 60 to 97.5 kD, and 43 kD detected only by antibody "b," and bands at the top of nitrocellulose by other antibodies. Parallel analysis for IgG-binding proteins ("2") showed similar but fainter bands. Major bands "1" and "2" correspond to heavy and light chain of IgA and IgG, respectively. Molecular masses of marker proteins stained with amido-black are depicted.

only with FN1-1 (Figure 5, "1b") at approximately 60 to 97 kD and 43 kD. Parallel analysis using protein G-agarose showed similar but fainter bands using FN1-1 (Figure 5, "2b"), suggesting that fibronectin fragments bound more readily to IgA1 than IgG. As controls, only IgA1 or fragmented fibronectin, instead of a mixture of both, was subjected to the affinity purification using jacalin-agarose. As expected, IgA1 did not display any bands (Figure 6, "1"). However, fragmented fibronectin alone produced bands of particularly high molecular weight (Figure 6, "2b,c"), similar to those of a mixture of IgA1 and fibronectin (Figure 6, "3b,c"). If bands produced by fibronectin alone (Figure 6, "2") were considered as background activity for IgA-related reactivity, only the band of about 43 kD remained as significant. Further washing (15×) of jacalin-agarose after incubation of a mixture confirmed this tendency (Figure 7). Thus, the 43-kD fibronectin fragment observed in patients was also detected by FN1-1 in cathepsin D-digested fibronectin and was demonstrated to bind IgA1, not IgG, under physiologic conditions.

Discussion

The carboxy-terminal 43-kD fragment of fibronectin was found in the serum of the patients with IgA nephropathy, as determined by Western blot analysis of the eluate of a heparin-affinity column. A similar-sized fragment derived from car-
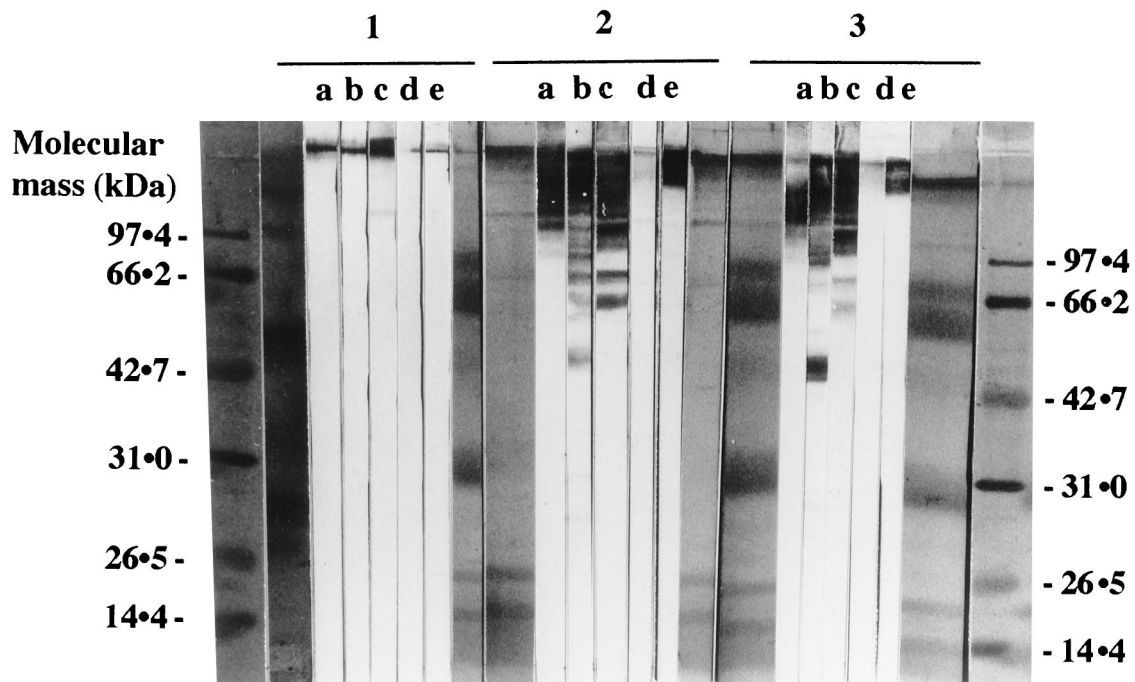
Figure 6. Background reactivity of the interaction of cathepsin D-digested fibronectin fragment and IgA1 on jacalin-agarose. Jacalin-agarose incubated with IgA1 ("1"), cathepsin D-digested fibronectin ("2"), or a mixture of IgA1 and cathepsin D-digested fibronectin ("3") was subjected to Western blot analysis with a set of monoclonal antihuman fibronectin antibodies (a through e) depicted in Figure 3. Major bands in "1" and "3" correspond to heavy and light chain of IgA. Molecular masses of marker proteins stained with amido-black are depicted.

boxy terminus of fibronectin was demonstrated to be able to bind purified IgA1 by jacalin-agarose affinity chromatography of a mixture of cathepsin D-digested fibronectin and purified IgA1. Native fibronectin has been proposed as one of many endogenous components in IgA-containing immune complex in IgA nephropathy, but fragmented fibronectin has not been reported.

It has been demonstrated that fibronectin may associate strongly with IgA from patients with IgA nephropathy, since the fibronectin-rich eluate of heparin affinity chromatography has contained higher amounts of IgA than control subjects (12), as confirmed in this study. We further attempted to measure fibronectin concentration in the eluate with a sandwich ELISA to determine what percentage of protein in the eluate is accounted for by the IgA–fibronectin complex. However, this was not successful because the assay system using several monoclonal antibodies and polyclonal antibodies to human fibronectin failed to generate a reproducible standard curve. Some degree of degradation of the fibronectin might have hampered the assay.

The specificity of assays to detect IgA–fibronectin complex in IgA nephropathy has been controversial (9–11). Since these assays used denatured collagen or anti-fibronectin antibody to bind fibronectin, a macromolecule having many domains with different binding specificities (Figure 1), it is anticipated that if fragmented fibronectin without the gelatin-binding domain or the epitope of anti-fibronectin antibodies is associated with IgA, then such a complex will not be detected by the assays. Indeed, the results in one report (9) and our own observations
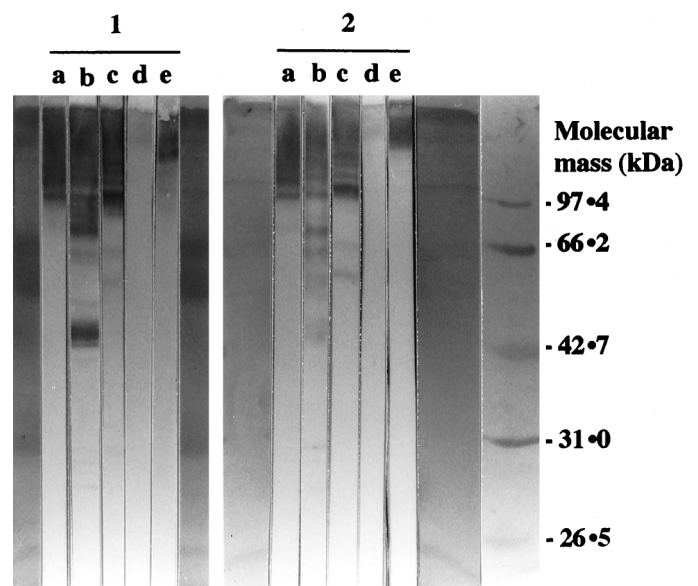

Figure 7. Effect of extensive washing on the binding of fibronectin fragments to jacalin-agarose. Jacalin-agarose incubated with a mixture of IgA1 and cathepsin D-digested fibronectin ("1") or with cathepsin D-digested fibronectin alone ("2") was washed 15× rather than 10×, eluted, and analyzed by Western blot using a set of monoclonal antibodies depicted in Figure 3. Compared with "1," the 43-kD band detected with antibody "b" was almost quenched by this procedure ("2"). The 98-kD band and others detected with antibody "c" were not affected by this procedure, suggesting that bands seen in "1" may not be associated with IgA1. Electrotransferred proteins and marker standard are stained with amido-black and depicted.

(data not shown) indicated that many patients with definite IgA nephropathy were still "negative" by these assays. Moreover, because the majority of fibronectin in the eluate was the intact molecule with many different binding sites for other molecules in addition to IgA, the detection of IgA-associated fibronectin may be misrepresented in these assays. For these reasons, it is important to determine the existence of fibronectin fragments in patient's serum and its binding capacity to IgA.

Fibronectin fragments have been demonstrated in synovial fluid from rheumatoid arthritis (15), but there are few reports concerning serum or plasma fibronectin (25). Some artifactual fragmentation of native fibronectin may not be avoidable during experimental procedures (26) when serum or plasma is applied to affinity chromatography. Indeed, in our study, fibronectin preparations from normal serum also generated fragmented products detectable with certain monoclonal antibodies. However, the 43-kD fragment recognized by FN1-1 was observed only in patients with IgA nephropathy.

Many studies have been performed to explore the function of fibronectin fragments, and structural modulation of the fibronectin molecule has been shown to result in loss or acquisition of function of the native protein (14,16,27–29). In addition, recent reports have demonstrated that the carboxy-terminal fragment in disulfide dimeric form, in cooperation with the amino-terminal 70-kD fragment, is essential for fibronectin to assemble into the extracellular matrix (16). The concept that fibronectin fragment may be able to assemble into the extracellular matrix has not been considered in the expansion of mesangial extracellular matrix, which is an important hallmark in IgA nephropathy. Furthermore, the mechanism for the deposition of IgA on the increased mesangial matrix is not known. Therefore, it is interesting that the 43-kD carboxy-terminal fragment was detected in patients and that similar fragments generated by cathepsin D digestion were capable of binding IgA.

IgG has been shown to have the highest affinity for fibronectin among monomeric Ig classes (13). Its major binding sites on fibronectin were identified in the amino-terminal 22-kD domain under physiologic conditions. The carboxy-terminal 44-kD domain also bound IgG under low ionic conditions. The binding sites for IgA were similar, but this was determined under the condition of low ionic strength. Thus, the observation that only serum or plasma IgA, not IgG or IgM, could bind immobilized fibronectin on plastic plate (10,11) is confusing by itself. There might be some factors that hamper the straightforward explanation of IgA–fibronectin interaction in serum or plasma. Degraded fibronectin may be one of the factors.

Whether IgA–fibronectin fragment complex is in the circulation and is accessible to mesangial matrix in IgA nephropathy should be analyzed further. Preliminary studies have demonstrated that IgA was able to deposit on extracellular matrix of cultured fibroblast only in the presence of fibronectin fragments (30). The existence of carboxy-terminal fibronectin fragment capable of binding IgA1 may have pathogenic potential to participate in IgA deposition in IgA nephropathy.

Acknowledgments

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