ELSEVIER

Journal of Chromatography B, 776 (2002) 101–106

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Quantitative analysis of IgA1 binding protein prepared from human serum by hypoglycosylated IgA1/Sepharose affinity chromatography

Ikuko Nakamura[a], Hitoo Iwase[b,*], Yukichi Ohba[c], Yoshiyuki Hiki[d], Tadashi Katsumata[b], Yutaka Kobayashi[a]

[a]*Department of Internal Medicine, Kitasato University, Sagamihara, Kanagawa 228-8555, Japan*
[b]*Department of Biochemistry, Kitasato University, 1-15-1 Kitasato, Sagamihara, Kanagawa 228-8555, Japan*
[c]*Department of Laboratory Medicine, Kitasato University, Sagamihara, Kanagawa 228-8555, Japan*
[d]*Department of Internal Medicine, Nagoya University, Daiko Medical Center Nagoya, Aichi 461-0047, Japan*

Abstract

The binding protein to a hypoglycosylated IgA1/Sepharose (IgA1-BP) could be prepared from human sera. IgG was a major component in the IgA1-BP. A Protein A column was used to remove the IgG; however, about half of the IgA1-BP was passed from the column [Biochem. Biophys. Res. Commun., 264 (1999) 424]. Quantitative analysis of the passed fraction (PAP) by laser nepherometry indicated that it was composed of a fairly large amount of IgA, IgM and complement C3 besides IgG. The relative content of IgG:IgA:IgM:C3:C4 was 25:10:41:22:2 in the PAP fraction. Meanwhile, the Protein A bound-fraction was essentially composed of IgG (78%) and IgM (19%). The total amount of IgA1-BP was not different between the sera from IgA nephropathy patients and other nephropathy patients. With respect to the IgA content in the IgA1-BP from IgA nephropathy patients, it was significantly higher than that from other nephropathy patients. It was found that the IgA1-BP from some IgA nephropathy patients contained a few micrograms of aberrant IgA per ml of serum. Thus, the obtained results suggested the preferential deposition of the self-aggregated IgA composed of hypoglycosylated IgA1 and co-deposition of IgG, IgM and C3 in the glomeruli in an IgA nephropathy patient.

Keywords: Immunoglobulins; Proteins

1. Introduction

Human serum IgA1 is one of the most exceptional glycoproteins among the serum glycoproteins because it has O-linked oligosaccharides in its hinge portion in addition to the N-linked carbohydrate chains in its structure [1–3]. In our previous report, the glycoform of the O-linked oligosaccharide of the IgA1 subclass from a healthy control and from IgA1 myeloma patients was analyzed. Three glycoforms for IgA1 from myeloma patients and only one glycoform from healthy individuals were found [4]. However, IgA1 from a healthy individual could be further fractionated dependent on its different affinity toward jacalin and its different heat stability. These subfractions had mutually different glycoforms of the O-linked oligosaccharide. Especially, aggregated IgA1 was abundant in asialo-Galβ1,3GalNAc, and

*Corresponding author. Tel.: +81-427-789-267; fax: +81-427-788-441.
E-mail address: iwaseh@med.kitasato-u.ac.jp (H. Iwase).

1570-0232/02/$ – see front matter
PII: S1570-0232(02)00176-9

the heat-stable IgA1 subfraction was abundant in a sialylated disaccharide [5]. Additionally, we found a phenomenon in which the enzymatic removal of sialic acid from normal human serum IgA1 induced self-aggregation of part of the IgA1. Aggregated human serum IgA1 induced by neuraminidase treatment had a lower number of O-linked sugar chains on the hinge portion [6,7]. The removal of N-glycan sugar chains from IgA1 by peptide N-glycanase (PNGase) treatment did not induce self-aggregation. The N-glycan sugar chain is thus shown not to be involved in IgA1–IgA1 interactions [8]. The artificially produced IgA1 having incomplete sugar chains exhibited strong binding ability toward the representative matrix proteins, Type IV collagen, fibronectin and laminin. Especially, in addition to the removal of sialic acid, removal of the galactose residue strengthens the binding ability. Based on these results, a sialic acid-containing sugar chain on the hinge portion of IgA1 was found to play an important role in inhibiting the aggregation of IgA1 [6].

IgA nephropathy is a common disease characterized by predominant IgA deposits in the renal mesangium. It is well-known that IgA1 among two subclasses, IgA1 and IgA2, is the dominant deposit in the glomeruli during IgA nephropathy [9]. The most prominent structural difference between the IgA1 and IgA2 subclasses was the duplicated proline-rich hinge portion and the characteristic O-linked oligosaccharide chains on the IgA1 hinge portion. There are many reports on the presence of an incompletely glycosylated O-linked oligosaccharide(s) on the IgA1 hinge region in some of the IgA nephropathy patients [10–23]. Whether IgA1 having an exposed hinge portion was present in the IgA nephropathy patient was examined using an anti-synthetic hinge peptide antibody. Approximately 46% of the IgA nephropathy patients appeared positive, and the obtained results showed an increment in IgA nephropathy patients of the supposed IgA1 molecule having an exposed hinge portion [24]. The fact that the artificially deglycosylated IgA1 acquired binding ability for fibronectin coincided well with the result that the fibronectin–IgA complex had been seen in the serum from IgA nephropathy patients [25]. All of these results will indicate the abundance of IgA1 molecules having incomplete sugar chains in the patient sera. Recently, direct evidence was also obtained by mass spectrometric analysis for the presence of hypoglycosylated IgA1 in glomerular IgA1 and serum IgA1 from an IgA nephropathy patient [22,23].

We looked for the specific protein binding to incompletely glycosylated IgA1, previously referred to as sticky IgA1 [8], among the human serum proteins. A portion of the serum proteins was found to bind to the asialo-, agalacto-IgA1(IgA1-SG)/Sepharose column. The binding protein (IgA1-BP) was predominantly IgG enriched with the IgG3 subclass [26]. The obtained results coincide well with the previous report of the co-presence of IgG1 and IgG3 with deposited IgA1 in IgA nephropathy patients [27].

In this report, a further detailed analysis of IgA1-BP from normal human serum and patients serum was carried out to examine the presence of serum proteins other than IgG.

2. Experimental

2.1. Reagents and chemicals

The following compounds and materials were commercially obtained: normal human serum was from China Newtech Development and Trade Corp. and was stored at −20 °C. Jacalin-agarose was from Vector Laboratories (Burlingame, CA); CNBr-Sepharose and Sephacryl S-300 were from Pharmacia Biotech (Tokyo, Japan); neuraminidase from *Streptococcus 6646K* and α-*N*-acetylgalactosaminidase from *acremonium* sp. were from Seikagaku Co. (Tokyo, Japan); recombinant β-galactosidase from *Bacillus circulans* was a generous gift from Dr. Ajisaka [28].

Sera were collected from 15 patients (six males, nine females) with biopsy-proven IgA nephropathy and 18 control patients (seven males, 11 females) with biopsy-proven other renal diseases without glomerular IgA deposition.

2.2. Preparation of IgA1 from normal human serum by jacalin-agarose affinity chromatography

The jacalin-agarose affinity chromatography was carried out at room temperature. Ten ml of serum were applied to the jacalin column (6×1.5 cm I.D.)

and washed with 0.1 *M* Tris–HCl buffer, pH 7.5, containing 0.02% sodium azide (abbreviated as J-Buffer) at a flow-rate of 2 ml/5 min. The thoroughly washed column was eluted with 0.8 *M* galactose (50 ml) in the J-Buffer. The IgA1 fraction obtained on stepwise elution with galactose was dialyzed against distilled water and then lyophilized.

2.3. Preparation of asialo-, agalacto-IgA1(IgA1-SG)/Sepharose

Five grams of CNBr-activated Sepharose were washed well with 2 l of 1 m*M* HCl at 4 °C. One hundred mg of IgA1 in 15 ml of the buffer, pH 8.3, containing 0.5 *M* NaCl were added to about 15 ml of wet gel and then stirred at room temperature for 2 h. The remaining active groups on the gel were blocked with excess glycine. To complete the washing of the IgA1/Sepharose, the column was thoroughly washed with J-Buffer containing 1.0 *M* NaCl. Preparation of IgA1-SG/Sepharose was carried out as follows. Five ml of packed gel were suspended in 50 m*M* sodium acetate buffer, pH 5.0, and were treated overnight with 100 mU of neuraminidase and 100 mU of β-galactosidase. The gel was then washed thoroughly with J-Buffer containing 1.0 *M* NaCl.

2.4. Preparation of IgA1-BP from human serum by IgA1-SG/Sepharose column

Normal human serum (5 ml) was applied to an IgA1-SG/Sepharose column (5 ml gel volume). The column was washed with J-Buffer. The bound protein was then eluted stepwise with J-Buffer containing 1.0 *M* NaCl. The eluted materials (IgA1-BP) were dialyzed against distilled water and lyophilized. Patient serum (each 1 ml) was also treated as above (Fig. 1).

2.5. Analysis of IgA1-BP

The IgA1-BP was applied to a Protein A column. Elutions were carried out according to the directions in the ImmunoPure (A) IgG Purification Kit (Pierce Co.). The passed fraction and bound fraction were dialyzed against distilled water and lyophilized. Quantitation of immunoglobulin and complement in these fractions was carried out by the nephelometric method using the Behring Nephelometer Analyzer II (DADE BEHRING, Germany).

In the analysis of the IgA content in IgA1-BP from IgA nephropathy patients and other nephropathy patients, the statistical difference between the IgA nephropathy and other types of nephropathy was analyzed by Mann–Whitney's *U* test. It was regarded as statistically significant if the *P* value was less than 0.05.

2.6. Immunofluorescence studies of deposited proteins

The renal tissue selected for this study consisted of renal biopsy specimens from 100 patients with primary IgA nephropathy. Renal biopsy specimens were embedded in OCT medium (Miles Lab., Elkhart, IN, USA) immediately after sampling, frozen in liquid nitrogen and stored at −80 °C until use. Frozen sections were cut to 2 mm, fixed in absolute acetone for 10 min and then rinsed in phosphate-buffered saline (PBS), pH 7.4. The sections were incubated with properly diluted fluorescein isothiocyanate (FITC)-labeled antibody against human IgA, IgG, IgM and C3. The stained sections were rinsed with PBS and observed under a fluorescent photomicroscope (Nikon, Tokyo, Japan) and were graded as 0–3+ by the authors (Y. Hiki and Y. Kobayashi).

3. Results

As reported previously, IgA1-BP was prepared from normal human serum using the IgA1-SG/Sepharose column (Fig. 1). The pooled IgA1-BP was separated into the passed fraction (PAP, 54%) and the bound fraction (PAB, 46%) to a Protein A column as indicated in a previous report [8]. Analysis of the immunoglobulin class and complements in the PAP fraction and the PAB fraction was carried out. The obtained relative content of IgG:IgA:IgM:C3:C4 was 41:17:67:36:3 in the passed fraction as indicated in Fig. 2. On the other hand, the bound fraction was composed of mainly IgG (78%) with some IgM (19%) (Fig. 2). We have reported already that the human serum IgG1 and IgG3 were preferentially bound to the sticky IgA1 using the same affinity chromatography [26]. The obtained

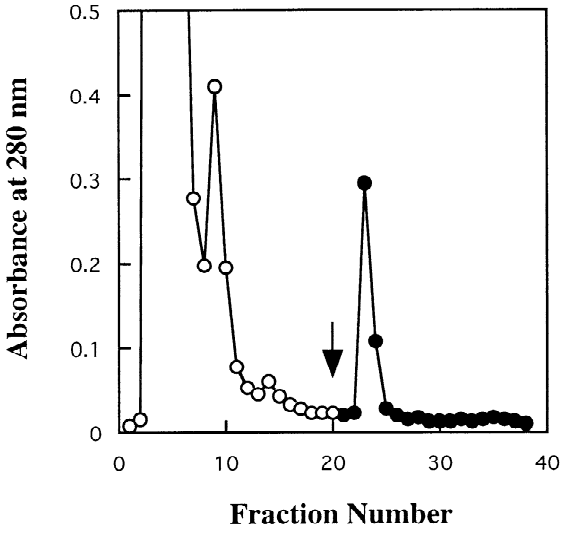

Fig. 1. Typical elution profile of IgA-BP on IgA1-SG/Sepharose. One ml of serum was applied to the column. The elution buffer was changed from the J-Buffer to the J-Buffer containing 1.0 *M* NaCl at the point indicated by the arrow.

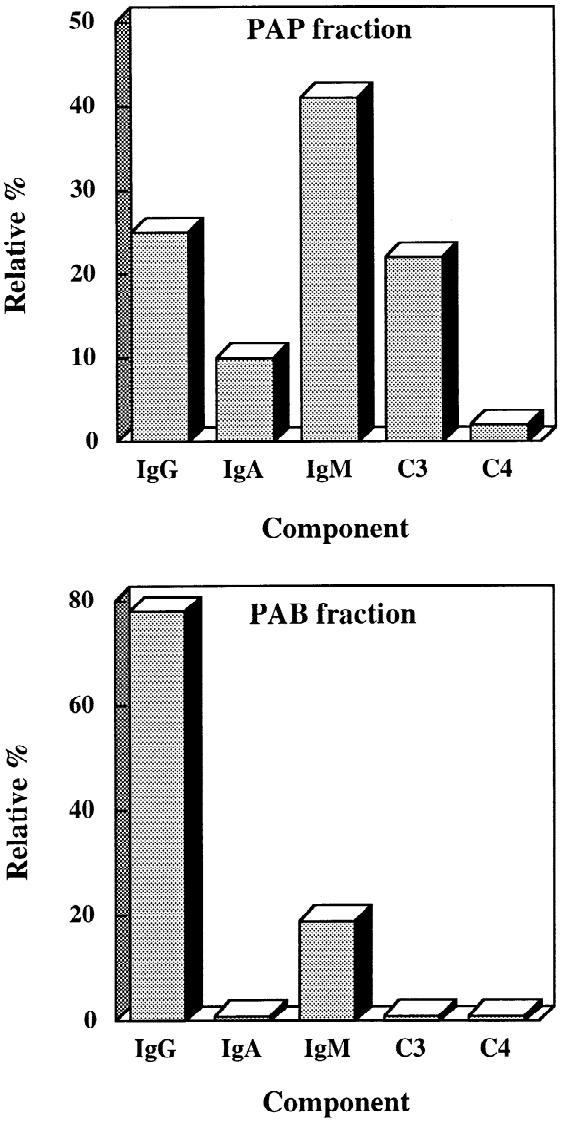

Fig. 2. Relative content of IgG, IgA, IgM, C3 and C4 in the PAP and PBP fractions of IgA1-BP.

results coincide well with the previous report of the co-presence of IgG1 and IgG3 with deposited IgA1 in IgA nephropathy patients [27]. Similarly, the obtained results in this experiment also coincide well with the previous report of the frequent co-presence of IgM and C3 with deposited IgA1 in IgA nephropathy patients. To confirm this result, the frequency of positive cases of IgG, IgM, C3 and C4 with the deposited IgA in 100 IgA nephropathy patients in our institute was examined. The actual frequency of positive cases of IgG, IgM, C3 and C4 was 18%, 70%, 98% and 9%, respectively (Fig. 3). The frequency of positive cases of IgM and C3 was higher than that of IgG. Although the lower frequency of IgG was not clear, the results suggested the reason why IgM and C3 were co-present with deposited IgA1 in IgA nephropathy patients.

In order to examine whether the sticky IgA1 was increased in the IgA nephropathy patient, quantitation of IgA in IgA-BP from IgA nephropathy patients was carried out. As shown in Fig. 4, the total amount of IgA1-BP was not different between the sera from IgA nephropathy patients and other nephropathy patients. On the other hand, it was revealed that the content of IgA in IgA1-BP was significantly higher in the IgAN group (IgA content: 0.486 ± 0.412) than in the other renal disease (control) group (IgA content: 0.206 ± 0.217). The value under the detection limit (0.34) was calculated to be zero. When more than mean+2SD of the control group (>0.630) was regarded as a positive level, the frequency of positive cases was 33% (5/15) in the IgAN group and 0% (0/18) in the control group.

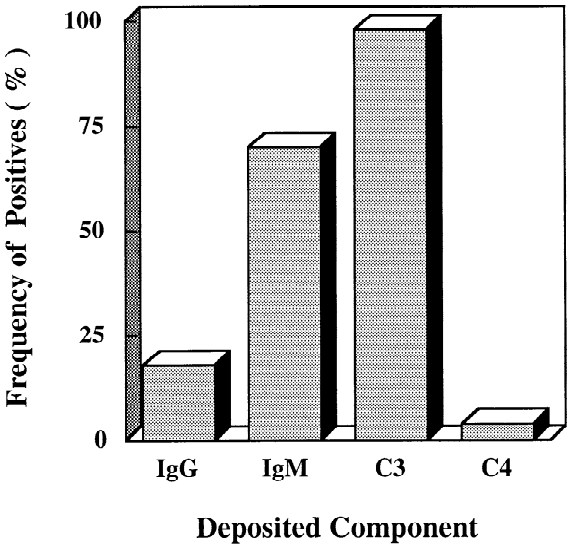

Fig. 3. Frequency of positive cases of IgG, IgM, C3 and C4 with the deposited IgA in IgA nephropathy patients.

4. Discussion

Although it is an animal model experiment, Hiki et al. reported that the involvement of the sugar chain incompletion in the IgA1 deposition in the glomeruli was found. The structural characteristic of the deposited IgA1 subfraction was incomplete mucin-type sugar chains on the hinge region. There are also many other reports about incompletion of the sugar chain of IgA1 in IgA nephropathy patients. Thus, our findings in this report also indicated the possible involvement of the sugar chain incompletion in IgA1 deposition in the patient glomeruli. In a very recent report by Hiki et al., the IgA1 was successfully eluted from the glomeruli isolated from the renal biopsy specimens of IgA nephropathy patients. Comparing the structure of the hinge glycopeptide from serum IgA1 of normal controls and that of IgA nephropathy patients by mass spectrometry, that of the glomerular-deposited IgA1 from IgA nephropathy patients exhibited incompletion of the O-linked oligosaccharide on the hinge portion. The authors suggested based on these results that the altered IgA1 O-glycosylation would promote the mesangial deposition of IgA1 [23]. Kokubo et al. reported that the hypoglycosylated IgA1 strongly bound to matrix

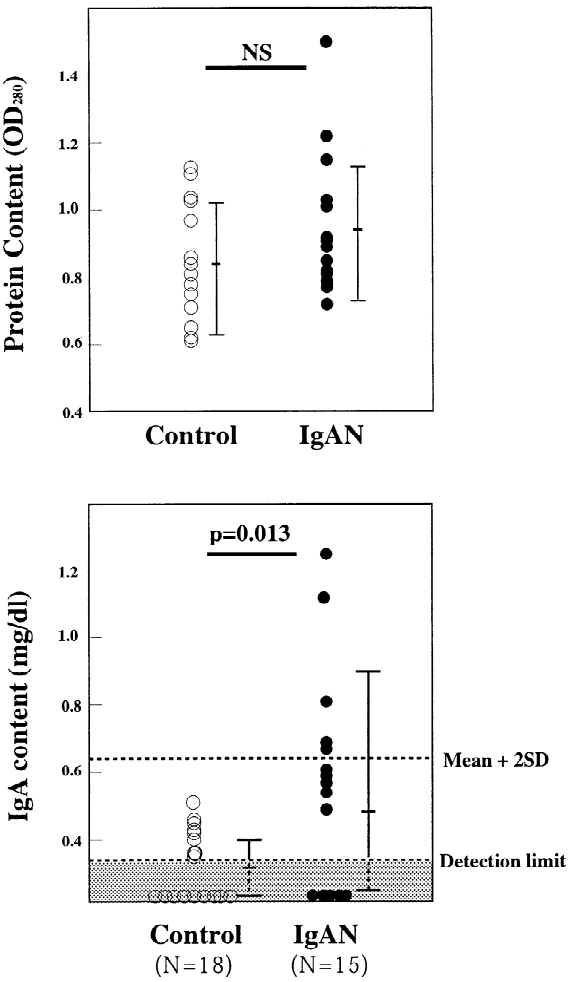

Fig. 4. Total amount of IgA1-BP and content of IgA in IgA1-BP prepared from nephropathy patients. Patient and control in the figure indicate IgA nephropathy patients and other nephropathy patients, respectively. Upper figure: amount of IgA1-BP prepared from 1 ml of patient serum is indicated by the absorbance at 280 nm. Lower figure: IgA in IgA1-BP (1 ml solution) indicated as total mg/dl. Dotted bar represents the value under the detection limit (0.34 mg/dl) and mean+2SD value for the control.

proteins such as fibronectin, laminin and Type IV collagen [6]. Additionally, the preferential binding of IgM, C3 to the hypoglycosylated IgA1 and the elevated amount of IgA in the binding protein from the serum of an IgA nephropathy patient were confirmed by affinity chromatography in this report. Such IgA in the IgA1-BP will be hypoglycosylated IgA1 because the asialo-, agalacto-IgA1 bound pref-

erentially to similar hypoglycosylated IgA1/Sepharose [8]. The biochemical mechanism of the preferential binding of those proteins to the hypoglycosylated IgA1 was difficult to estimate but might be related to the mechanism of the previously reported self-aggregation of hypoglycosylated IgA1 [7,8]. Williamson described that the proline-rich regions (PRRs) can mediate various molecular interactions by non-specific physicochemical reactions such as hydrogen bonding and/or hydrophobic interaction [29]. Therefore, the naked hinge portion having a proline rich-sequence might be implicated in this phenomenon.

Clarification of a common mechanism for the self-aggregation of IgA1 having incomplete hinge mucin-type sugar chains and for the preferential binding of matrix proteins, IgM, C3, IgG1 and IgG3, to the hypoglycosylated IgA1 might provide a basis for better understanding of the biochemical mechanism of the glomerular deposition of IgA1 and the development of inflammation in IgA nephropathy patients.

Acknowledgements

The authors are indebted to Dr. K. Hotta and Dr. H. Ohtani for their encouragement and support. Part of this work was performed as a portion of an R&D Project of the Industrial Science and Technology Frontier Program, supported by NEDO (New Energy and Industrial Technology Development Organization). Additionally, this work was supported in part by a Grant-in-Aid from The Ministry of Education, Science and Culture of Japan (no. 12671046) and Honen Co.

References

[1] J. Baenziger, S. Kornfeld, J. Biol. Chem. 249 (1974) 7260.
[2] J. Baenziger, S. Kornfeld, J. Biol. Chem. 249 (1974) 7270.
[3] T.S. Mattu, R.J. Pleass, A.C. Willis, M. Lilian, M.R. Wormald, A.C. Lellouch, P.N. Rudd, J.M. Woof, R.A. Dwek, J. Biol. Chem. 273 (1998) 2260.
[4] H. Iwase, I. Ishii-Karakasa, E. Fujii, K. Hotta, Y. Hiki, Y. Kobayashi, Anal. Biochem. 206 (1992) 202.
[5] H. Iwase, A. Tanaka, Y. Hiki, T. Kokubo, I. Ishii-Karakasa, Y. Kobayashi, K. Hotta, J. Biochem. 120 (1996) 92.
[6] T. Kokubo, Y. Hiki, H. Iwase, A. Tanaka, J. Nishikido, K. Hotta, Y. Kobayashi, J. Am. Soc. Nephrol. 9 (1998) 2048.
[7] H. Iwase, A. Tanaka, Y. Hiki, T. Kokubo, T. Sano, I. Ishii-Karakasa, K. Toma, Y. Kobayashi, K. Hotta, J. Chromatogr. B 724 (1999) 1.
[8] H. Iwase, S. Ohkawa, I. Ishii-Karakasa, Y. Hiki, T. Kokubo, T. Sano, A. Tanaka, K. Toma, Y. Kobayashi, K. Hotta, Biochem. Biophys. Res. Commun. 261 (1999) 472.
[9] M.E. Conley, M.D. Cooper, A.F. Michael, J. Clin. Invest. 66 (1980) 1432.
[10] P.M. Andre, P. Le Pogamp, D. Chevet, J. Clin. Lab. Anal. 4 (1990) 115.
[11] A.C. Allen, S.S. Harper, Clin. Exp. Immunol. 100 (1995) 470.
[12] A.C. Allen, P.S. Topham, S.J. Harper, J. Feehally, Nephrol. Dial. Transplant. 12 (1997) 701.
[13] J. Mestecky, M. Tomana, P.A. Crowley-Nowick, Z. Moldoveanu, B.A. Julian, S. Jackson, Contrib. Nephrol. 104 (1993) 172.
[14] M. Tomana, K. Matousovic, B.A. Julian, J. Radl, K. Konecny, J. Mestecky, Kidney Int. 52 (1997) 509.
[15] Y. Hiki, H. Iwase, T. Kokubo, A. Horii, A. Tanaka, J. Nishikido, K. Hotta, Y. Kobayashi, J. Am. Soc. Nephrol. 7 (1996) 955.
[16] Y. Hiki, H. Iwase, M. Saitoh, Y. Saitoh, A. Horii, K. Hotta, Y. Kobayashi, Nephron 74 (1996) 429.
[17] T. Kokubo, Y. Hiki, H. Iwase, A. Horii, A. Tanaka, J. Nishikido, K. Hotta, Y. Kobayashi, J. Am. Soc. Nephrol. 8 (1997) 915.
[18] Y. Hiki, A. Tanaka, T. Kokubo, H. Iwase, J. Nishikido, K. Hotta, Y. Kobayashi, J. Am. Soc. Nephrol. 9 (1998) 577.
[19] Y. Hiki, T. Kokubo, H. Iwase, Y. Masaki, T. Sano, A. Tanaka, K. Toma, K. Hotta, Y. Kobayashi, J. Am. Soc. Nephrol. 10 (1999) 760.
[20] A.C. Allen, E.M. Bailey, J. Barratt, K.S. Buck, J. Feehally, J. Am. Soc. Nephrol. 10 (1999) 1763.
[21] A. Amore, G. Conti, P. Crina, L. Peruzzi, M. Alpa, F. Bussolino, R. Coppo, Am. J. Kidney Dis. 36 (2000) 1242.
[22] H. Odani, Y. Hiki, M. Takahashi, A. Nishimoto, Y. Yasuda, H. Iwase, T. Shinzato, K. Maeda, Biochem. Biophys. Res. Commun. 271 (2000) 268.
[23] Y. Hiki, H. Odani, M. Takahashi, Y. Yasuda, A. Nishimoto, H. Iwase, T. Shinzato, Y. Kobayashi, K. Maeda, Kidney Int. 59 (2001) 1077.
[24] T. Kokubo, Y. Hiki, H. Iwase, A. Tanaka, J. Nishikido, K. Hotta, Y. Kobayashi, Nephrol. Dial. Transplant 14 (1999) 81.
[25] S. Waga, K. Sugimoto, H. Tanaka, T. Ito, T. Nakahata, T. Tateyama, Y. Kakizaki, M. Yokoyama, J. Am. Soc. Nephrol. 10 (1999) 256.
[26] H. Iwase, Y. Yokozeki, Y. Hiki, A. Tanaka, T. Kokubo, T. Sano, I. Ishii-Karakasa, K. Hisatani, Y. Kobayashi, K. Hotta, Biochem. Biophys. Res. Commun. 264 (1999) 424.
[27] P. Aucouturier, R.C. Monteiro, L.H. Noel, J.L. Preud'homme, P. Lesavre, Clin. Immunol. Immunopathol. 51 (1989) 338.
[28] H. Fujimoto, M. Miyasato, Y. Ito, T. Sasaki, K. Ajisaka, Glycoconjug. J. 15 (1998) 155.
[29] M.P. Williamson, Biochem. J. 297 (1994) 249.