Humoral immunity against the proline-rich peptide epitope of the IgA1 hinge region in IgA nephropathy

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Abstract

Background. The human IgA1 hinge region is a unique mucin-like O-linked proline-rich glycopeptide, and its core peptide was found to be exposed aberrantly by the underglycosylation in IgA nephropathy (IgAN). We describe here the presence of humoral immunity against the IgA1 hinge peptide epitope in IgAN and evaluate the relationship between the underglycosylation of the IgA1 hinge region and humoral immunity.

Method. The serum anti-IgA1 hinge peptide antibody (anti-z1HP ab) titre was measured and compared between the IgAN (n=37) and control groups (n=34) by enzyme-linked immunosorbent assay (ELISA) using a synthetic peptide corresponding to the human IgA1 hinge region, PVSTPPTSPSTPPTSPS, as an antigen. Next, to evaluate the relationship between the underglycosylation of the IgA1 hinge region and the humoral immunity, the reactivity of the serum IgG from the patients with IgAN against monoclonal IgA1 which had been digested enzymatically to remove the carbohydrates from the IgA1 hinge region was measured by ELISA.

Results. The anti-z1HP ab titre was significantly higher in the IgAN group than in the control group (OD value: IgG class, 0.564±0.344 vs 0.331±0.154, P=0.0014; IgM class, 0.272±0.148 vs 0.141±0.072, P<0.0001) and it was positive in ~40% of the patients with IgAN. In addition, the reactivity of the serum IgG from the IgAN patients against the monoclonal IgA1 which had been digested enzymatically to remove the carbohydrates from the IgA1 hinge region was measured by ELISA.

Conclusion. These results suggested that the peptide epitope of the IgA1 hinge region which was aberrantly exposed by underglycosylation could induce the humoral immune response in IgAN.

Key words: IgA nephropathy; humoral immunity; IgA1 hinge region; proline-rich peptide; O-glycosylation

Introduction

IgA nephropathy (IgAN) is the most common glomerular disease which is characterized immunohistologically by the predominant deposition of the IgA1 subclass in the mesangial area [1,2]. The structural characteristics of the IgA1 molecule recently were associated with the pathogenesis of IgAN, because the IgA1 molecule is obviously different from the other immunoglobulins in its hinge region which is a unique mucin-like glycopeptide. The IgA1 hinge region has a proline-, serine- and threonine-rich amino acid sequence in which the serine and threonine residues are able to carry O-linked oligosaccharides consisting of sialic acid (NeuNAc), galactose (Gal) and N-acetylgalactosamine (GalNAc) with microheterogeneity [3,4]. Some investigators, including us, revealed the presence of defective O-glycans in the hinge region of the IgA1 molecules derived from sera of patients with IgAN [5–9]. Additionally, we showed that the core peptide of the IgA1 hinge region was exposed by its underglycosylation in IgAN using a rabbit polyclonal antibody against a synthetic peptide corresponding to the human IgA1 hinge region [10].

Regarding the biological significance of O-glycans, very interesting findings have been reported in studies of mucin. In patients with several different kinds of cancer, it was shown that the core peptide of mucin expressed on the malignant cells was exposed by the underglycosylation, resulting in humoral and cellular immune responses to its peptide epitope [11–13]. Since the IgA1 hinge region is very similar to mucin in proline residue abundance and the presence of O-glycans [14], the IgA1 hinge peptide core exposed by underglycosylation may induce some immune response in patients with IgAN.

In this study, we examined the antibody production against the peptide epitope of the IgA1 hinge region
in patients with IgAN by enzyme-linked immunosorbent assay (ELISA) using a synthetic peptide corresponding to the human IgA1 hinge region as an antigen, and evaluated the relationship between the underglycosylation of the IgA1 hinge region and the humoral immune response.

Materials and methods

Sera were collected from 37 patients (17 males, 20 females, mean age 37 years, range 16–56 years) with biopsy-proven IgAN and 34 control patients (22 males, 12 females, mean age 43 years, range 17–74 years) with other biopsy-proven renal diseases without glomerular IgA deposition (membranous nephropathy, n=6; non-IgA mesangial proliferative glomerulonephritis (GN), n=4; membranoproliferative GN, n=4; tubulointerstitial nephritis, n=4; focal glomerulosclerosis n=3; lupus nephritis, n=3; lipoid nephrosis, n=2; nephropersisclerosis, n=2; rapidly progressive GN, n=2; amiloidosis, n=2; acute GN, n=1; diabetic nephropathy, n=1).

A 21mer peptide corresponding to the amino acid sequence of the human IgA1 hinge region, PVPSPPPSTPSPPTPPSPSC, was synthesized by and purchased from Bio-Synthesis, Inc. (Lewisville, TX). The purity and molecular weight were confirmed by HPLC and MALDI-TOFMS. The synthetic IgA1 hinge peptide (sz1HP) was conjugated with bovine serum albumin (BSA, Sigma Chemical Co., St Louis, MO) for plate coating. The conjugation of BSA with the peptide was performed by the following procedure. A 1.5 ml aliquot of BSA (13.4 mg/ml) was mixed with 100 μl of N-ε-maleimidocaproyloxy succinimide (100 mg/ml, Wako Junyaku Co., Tokyo) and incubated for 1 h at room temperature. After desalting, 250 μl of the peptide (20 mg/ml) was added and incubated for 3 h at 4°C. After desalting, the BSA-conjugated peptide was used as the antigen for ELISA.

The 96-well microtitration plates (Limbro/Titertek, Flow General Company, McLean, IL) were coated with 100 μl of the BSA-conjugated sz1HP diluted to a concentration of 10 μg/ml with 0.015 M carbonate buffer, pH 9.6. After blocking of the unreacted sites with 0.01 M phosphate buffer, pH 7.5, 0.15 M NaCl (PBS) containing 1% BSA, 100 μl of sera (1/100 dilution in PBS) from patients with IgAN (n=37) and controls (n=34) were added to the sz1HP-coated and non-coated wells. After incubation for 3 h at room temperature and washing with PBS containing 0.1% BSA and 0.05% Tween-80 (PBS/BSTA/Tween), 100 μl of peroxidase-conjugated goat anti-human IgG (1/500, Fab portion-specific, Organon Teknika Corp., West Chester, PA), anti-human IgA (1/500, chain-specific, Organon Teknika Corp.) and anti-human IgM (1/500, μ-chain-specific, Organon Teknika Corp.) antibodies were added to the wells. After incubation for 1 h at room temperature and washing with PBS/TBA/Tween, the plates were exposed to an enzyme substrate consisting of 0.4 mg/ml of o-phenylenediamine dihydrochloride (Sigma Chemical Co.) and 0.03% hydrogen peroxide in a solution containing 0.1 M disodium hydrogen phosphate and 0.05 M citric acid monohydrate. The developed colour was read at 490 nm with a microplate reader (Bio-Rad Laboratories, model 450, Richmond, CA). Each absorbance level was reduced by that of the non-sz1HP-coated well. All of the assays were performed in triplicate. Each median was expressed. The statistical difference between the two groups was analysed using the Mann–Whitney U-test. A P-value of <0.05 was considered to be a significant difference.

Preliminarily, we examined the specific reactivity of the IgG from patients with IgAN against the sz1HP. The dose-dependent reactivity of the IgG (serum dilution, 1/1600–1/100) against the plate-coated sz1HP was tested using the sera from patients with IgAN (n=6) and controls (n=6) with the above-mentioned method. Each mean ±SD of the six cases was expressed, and the statistical difference between IgAN and the control was analysed by ANOVA. In addition, the inhibition assays were performed using a free sz1HP and an unrelated 21mer synthetic peptide, human β-endorphin (1–5) + (16–31), YGGFMFLFKNAIKNAYKKGE (American Peptide Company, Sunnyvale, CA). Fifty μl of the sera (1/50 dilution) from the patients with IgAN (n=6) were added with 50 μl of the free synthetic peptides (0–20 μg/ml) to the sz1HP-coated wells. After incubation for 3 h and washing, the IgG binding to the plate-fixed sz1HP was detected with peroxidase-conjugated anti-human IgG antibody in the same way. The reactivity without inhibitors was regarded as 100%, and each mean ±SD of the percentage reactivity was expressed.

In order to evaluate the relationship between the underglycosylation of the IgA1 hinge region and the humoral immune response, the reactivity of the IgG from patients with IgAN against a monoclonal IgA1 which was digested enzymatically to remove the carbohydrates from the IgA1 hinge region was measured by ELISA. Neuraminidase (NA) from Streptococcus 6646K (1 U/100 μl; Seikagaku Co. Tokyo, Japan), β-galactosidase (GA) from bovine testis (1.0 U/ml; Sigma Chemical Co.) and α-N-acetylgalactosaminidase (GNA) from Acremonium sp. (13 U/ml; Seikagaku Co.) were used in this examination according to the following procedure. A 1.5 ml aliquot of BSA (13.4 mg/ml) was mixed with 100 μl of N-ε-maleimidocaproyloxy succinimide (100 mg/ml, Wako Junyaku Co., Tokyo) and incubated for 1 h at room temperature. After desalting, 250 μl of the peptide (20 mg/ml) was added and incubated for 3 h at 4°C. After desalting, the BSA-conjugated peptide was used as the antigen for ELISA.

To examine the exposure of the IgA1 hinge peptide core in IgAN, the reactivity of a rabbit polyclonal anti-synthetic IgA1 hinge peptide antibody (anti-sz1HP ab), which was
reported in our previous study [10], against the serum IgA was measured by ELISA and compared in the IgAN and control groups. The microtitration plates were coated with 100 μl of goat anti-human IgA antibody (10 μg/ml, Fab fraction, Organon Teknika Corp). After blocking with BSA, 100 μl of the sera (1/100 dilution) from the IgAN group (n = 24) and control group (n = 24) were added to the wells. After incubation for 3 h at room temperature and washing, 100 μl of the rabbit anti-sz1HP ab (1/100 dilution) was added to the wells. After incubation for 3 h at room temperature and washing, the absorbance levels were measured using the above-mentioned method. These assays were performed in triplicate. Each median was expressed. The statistical difference between the two groups was analysed using the Mann–Whitney U test.

Results

Specific reactivity of serum IgG from IgAN patients against sz1HP

As shown in Figure 1, the serum IgG from the patients with IgAN reacted against the plate-fixed sz1HP in a dose-dependent manner. The reactivity was significantly higher in the patients with IgAN (n = 6) than in the controls (n = 6) (ANOVA, P = 0.017). In addition, the binding of the IgG from the patients with IgAN (n = 6) to the plate-fixed sz1HP was clearly inhibited by the free sz1HP but not by a control peptide (Figure 2).

Serum anti-sz1HP ab titres in IgAN

As shown in Figure 3, the serum anti-sz1HP ab titres of the IgG and IgM classes were significantly higher in the IgAN group (n = 37) than in the control group (n = 34) (IgG class, 0.564 ± 0.344 vs 0.331 ± 0.154, P = 0.0014; IgM class, 0.272 ± 0.148 vs 0.141 ± 0.072, P < 0.0001). However, the antibody titre of the IgA class was not detectable in all cases of both groups. When more than the mean +2 SDs of the control group was regarded as a positive level of the anti-sz1HP ab titres (IgG class, >0.639; IgM class, >0.285), the positive rate was 40.5% (15/37) in the IgG class and 43.2% (16/37) in the IgM class in the IgAN group, and 0% (0/34) in both antibody classes in the control group. There is a significant correlation between the anti-sz1HP ab titres of the IgG and IgM classes (R² = 0.366, P < 0.0001). However, no significant correlation...
was found between the anti-ζ1HP ab titres and the serum concentration of IgG or IgM.

**Reactivity of serum IgG from IgAN patients against deglycosylated IgA1 (monoclonal)**

The serum IgG from patients with IgAN (n = 6) reacted strongly against the deglycosylated IgA1 compared with native IgA1. When the reactivity against native IgA1 was regarded as 100%, the values were 122 ± 9.5% (mean ± SD) against the asialo IgA1, 167 ± 11.5% against the agalacto IgA1 and 188 ± 3.9% against the naked IgA1. The reactivity was increased as the carbohydrates were removed from the IgA1 hinge region, and the reactivity against naked IgA1 was ~2-fold higher than that against native IgA1 (Figure 4). In addition, there was no significant difference in the reactivity of the patient’s IgG against a monoclonal IgA2 between native and deglycosylated IgA2.

**Reactivity of rabbit polyclonal anti-ζ1HP ab against serum IgA in IgAN**

As shown in Figure 5, the reactivity of the rabbit polyclonal anti-ζ1HP antibody against serum IgA was significantly higher in the IgAN group (n = 24) than in the control group (n = 24) (0.321 ± 0.167 vs 0.189 ± 0.137; P = 0.0042). There was no significant correlation between this reactivity and the serum IgA level.

**Discussion**

The results of the ELISA using a synthetic peptide corresponding to the human IgA1 hinge region, PVPSTPPTPSPTPPTPSPS, as an antigen showed that the serum anti-ζ1HP ab titre was significantly higher in the IgAN group than in the control group, and it was regarded as positive in ~40% of the patients with IgAN. It was also observed that this titre was increased in both the IgG and IgM classes but not detectable in the IgA class, indicating that antibody production against the peptide epitope of the IgA1 hinge region occurred in the IgG and IgM classes. These observations suggested that these humoral immune responses could result in the formation of IgA–IgG and IgA1–IgM immune complexes in the circulation of patients with IgAN. Czerkinsky et al. previously reported that IgA-containing circulating immune complexes were found in patients with IgAN [17]. The IgA1–IgG and IgA1–IgM interactions mediated by the IgA1 hinge peptide observed in the present study may be one of the mechanisms involved in the formation of IgA-containing immune complexes in IgAN.

Next, we examined the reactivity of serum IgG from the patients with IgAN against native and deglycosylated monoclonal IgA1. This reactivity was found to be greatest in the naked, followed by the agalacto, asialo and native IgA1, in that order; i.e. the more carbohydrates that were removed from the IgA1 hinge region, the greater the reactivity. This result suggests that the accessibility of the anti-ζ1HP ab to the peptide epitope of the IgA1 hinge region was increased by the enzymatic removal of the carbohydrates from the IgA1 hinge region, and that the carbohydrate moieties including NeuNAc, Gal and GalNAc attached to the hinge region function to protect the peptide epitope. Therefore, if there is some defect in the O-glycans of the IgA1 hinge region, the probability of the peptide epitope being recognized by the anti-ζ1HP ab expressed on B lymphocytes is thought to increase and, consequently, the B cells will be activated and produce the anti-ζ1HP ab.

Furthermore, we also showed that the reactivity of a rabbit polyclonal anti-ζ1HP ab against serum IgA
was significantly higher in the IgAN group than in the control group, suggesting that the peptide core of the IgA1 hinge region is aberrantly exposed by the defective O-glycosylation in IgAN. This result also supports the hypothesis that the underglycosylation of the IgA1 hinge region causes the humoral immune response to its peptide epitope in IgAN. Tomana et al. recently reported that the Gal deficiency in IgA1 molecules was related to the formation of IgA1–IgG complexes in IgAN [8]. Our data are consistent with theirs in terms of the relationship between the formation of the IgA1–IgG immune complex and the aberrant glycosylation of the IgA1 hinge region.

Concerning the structure and function of the O-linked glycopeptides, the study of mucin, a typical O-linked glycoprotein, has been well developed in the field of cancer. Kotera et al. showed the presence of humoral immunity against a tandem repeat peptide epitope of the human mucin MUC-1 in sera from breast, pancreatic and colon cancer patients [11]. In that study, it was shown that the anti-MUC-1 antibody titre was positive in 8–17% of these cancer patients by ELISA using a synthetic peptide corresponding to the MUC-1, PDTDPAPEGSTAPPAGHVTSV. Also, anti-MUC-1 antibody production was suggested to result from the exposure of the MUC-1 core peptide by underglycosylation of the mucin expressed on the malignant cells [18,19]. Since the IgA1 hinge region is very similar to mucin in terms of the abundance of proline residues and the presence of O-glycans, it is likely that the IgA1 hinge region has a structure and function similar to mucin [14,20–22].

In addition, Rughetti et al. showed that a synthetic peptide corresponding to MUC-1 could induce a B-cell immune response in an ovarian cancer patient [12]. Jerome et al. reported that cytotoxic T lymphocytes derived from patients with breast adenocarcinoma recognized a peptide core epitope of mucin preferentially expressed by the malignant cells [13]. These findings indicated that the mucin peptide core epitope could induce cellular immunity as well as humoral immunity in humans. Therefore, the production of antibody against the peptide core epitope of the IgA1 hinge region may provide a possible explanation not only for the formation of the IgA1-containing immune complexes, but also for the abnormalities in the activity of the cellular immune system observed in IgAN [23].

Recent studies in this field provided further interesting information. Agrawal et al. described that T cells from multiparous women specifically proliferated in response to the MUC-1 peptide epitope, indicating that there is a natural immunization against it during pregnancy [24,25]. These observations provided an explanation for a negative correlation with the occurrence of breast cancer in multiparous women [26] and led to the potential use of synthetic peptides corresponding to MUC-1 as ‘vaccines’ for therapy of breast cancer [27,28]. Thus, it is clear that the immune responses to the MUC-1 peptide epitope serve as a preventive factor against breast cancer by eliminating the cancer cells expressing the underglycosylated mucin. Therefore, if the immune response to the peptide epitope of the IgA1 hinge region observed in the present study also functions to eliminate the underglycosylated IgA1 molecules from the circulation of patients with IgAN, then synthetic peptides corresponding to the IgA1 hinge region may be useful for an ‘immunotherapy’ against IgAN. It was suggested recently that IgA1 molecules which have defective O-glycans in their hinge region could cause direct glomerular deposition by non-immunological mechanisms [5–9,16,29–34]. However, there is also the possibility that the immune response to the IgA1 hinge peptide plays a pathogenetic role in IgAN as a result of the IgA1–IgG immune complex formation or other immune mechanisms. Therefore, it is necessary to evaluate carefully the relationship between the anti-MUC-1 antibody titre and the clinical course of many patients with IgAN and to reveal the pathophysiological implication of this antibody production in IgAN.

We previously indicated that the underglycosylation of the IgA1 hinge region caused the non-immunological IgA1 self-aggregation and adhesion to extracellular matrix (ECM) proteins such as type IV collagen, fibronectin and laminin, and proposed that these phenomena might be one of the mechanisms of the glomerular deposition of IgA1 in IgAN [16]. We also inferred that the IgA1 self-aggregation and adhesion to ECM proteins could result from the exposure of the proline-rich peptide core of the IgA1 hinge region due to the defect of O-linked sugars in IgAN [10,32], because, as Williamson described, the proline-rich peptide can mediate various molecular interactions by non-specific physicochemical reactions such as hydrogen bonding and/or hydrophobic interaction [21]. However, in this study, we showed that the exposure of the proline-rich IgA1 hinge peptide by underglycosylation could cause not only non-immunological, but also immunological IgA1-containing protein–protein interactions in IgAN.

In conclusion, it was shown that the serum anti-MUC-1 antibody titre was significantly higher in the IgAN group than in the control group and it was positive in ~40% of the patients with IgAN. The reactivity of the serum IgG from the IgAN patients against a monoclonal IgA1 was increased as the carbohydrates were enzymatically removed from the IgA1 hinge region. These results suggested that the peptide epitope of the IgA1 hinge region which was aberrantly exposed by the underglycosylation could cause the humoral immune response in IgAN.

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