Original Article

Tonsillar IgA1 as a possible source of hypoglycosylated IgA1 in the serum of IgA nephropathy patients

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Abstract

Background. There are many reports of incompletely glycosylated O-linked oligosaccharides on the IgA1 hinge region in certain IgA nephropathy patients. In addition, other reports have noted a relationship between tonsillectomy and IgA nephropathy.

Methods. Immunoglobulins from extracts of tonsillectomized tissue and other sources were analysed by isoelectric focusing (IEF) and by enzyme-linked immunosorbent assay (ELISA).

Results. The IEF profile of tonsillar IgA differed from that of serum IgA and it was enriched in cationic IgA. However, extracts from tonsillitis controls and IgA nephropathy patients exhibited profiles that were very similar. Enzymatic removal of sialic acid induced a shift of the peaks to the cathode side. The profiles of IgA from treated tonsillar extract and treated serum were closely overlapped. In addition, asialo Gal\(^{b,3}\)GalNAc was clearly present in cationic IgA from tonsillar extract and in aberrant IgA1 from serum following enzymatic transfer of sialic acid to IgA1. Serum IgA also contained partly sialylated IgA1. Quantitative analysis of IgA and IgG in the extracts indicated that IgA was significantly higher, whereas IgG was significantly lower in IgA nephropathy patients.

Conclusions. We found that the IgA1 produced in tonsillar tissue differed from serum IgA1. Furthermore, an overproduction of asialo IgA1 resulted from the disordered balance between IgA- and IgG-producing cells in the tonsils from the IgA nephropathy patient. Although it is unclear how such asialo IgA1 molecules are transferred from tonsil tissue to serum, a tonsillar source may produce a few micrograms of aberrant IgA1 that then appears in serum.

Keywords: hypoglycosylated IgA1; IgA nephropathy; isoelectric focusing; \(\alpha2,3\)-(O) sialyltransferase; secretory IgA; tonsillectomy

Introduction

Among serum glycoproteins, human serum IgA1 is one of the most exceptional because it has O-linked oligosaccharides in its hinge portion in addition to the N-linked carbohydrate chains in its structure [1,2]. In our previous report, we analysed the glycoform of the O-linked oligosaccharide of the IgA1 subclass from a healthy control and from IgA1 myeloma patients. In myeloma patients, three glycoforms for IgA1 were found and only one glycoform was identified in healthy individuals. However, the IgA1 from a healthy individual could be further fractionated depending on its differential affinity for jacalin and its differential heat stability. These subfractions had mutually different glycoforms of the O-linked oligosaccharide. The aggregated IgA1 was especially abundant in Gal\(^{b,3}\)GalNAc [Thomsen–Friedenreich (TF) antigen] and the heat-stable IgA1 subfraction was abundant in a sialylated TF antigen [3]. We also reported that enzymatic removal of sialic acid from normal human serum IgA1 induced self-aggregation of a portion of IgA1. Aggregated human serum IgA1, induced by neuraminidase treatment, had a lower number of O-linked sugar chains on the hinge portion [4]. The removal of the N-glycan sugar chains from IgA1 by peptide N-glycanase treatment did not induce self-aggregation or binding to the asialo IgA1–Sepharose column. Thus, the N-glycan sugar chain was shown to have no involvement in the IgA1–IgA1 interaction. The artificially produced IgA1 with incomplete sugar chains exhibited a strong binding affinity towards the representative matrix proteins, type IV collagen, fibronectin and laminin. In addition to the removal
of sialic acid, the removal of the galactose residue especially strengthens binding ability. Based on these results, a sialic acid-containing sugar chain on IgA1 was found to play an important role in inhibiting the aggregation of IgA1 [5].

IgA nephropathy is a common disease characterized by predominant IgA deposits in the renal mesangium. Of the two glycoprotein subclasses, IgA1 and IgA2, it is well known that IgA1 is predominantly deposited in the glomeruli during IgA nephropathy [6]. The most prominent structural difference between the IgA1 and IgA2 subclasses is the duplicated proline-rich hinge portion and the characteristic O-linked oligosaccharide chains on the IgA1 hinge portion. Several groups have reported the presence of an incompletely glycosylated O-linked oligosaccharide(s) on the IgA1 hinge region in some IgA nephropathy patients [7–13]. Although these are animal data, Hiki et al. [12] and Sano et al. [14] reported sugar-chain incompleteness in IgA1 deposits found in glomeruli.

The mechanism responsible for hypoglycosylated IgA1 production is still unclear. There are reports of a relationship between tonsillectomy and IgA nephropathy. When comparing tonsillectomized and non-tonsillectomized normal subjects, Amelio et al. [15] found significantly lower levels of serum IgA in tonsillectomized cases. Furthermore, immunohistomorphometric analysis of a tonsillar plasma cell revealed 65% IgG-secreting cells and 29% IgA plasma cells in the control, contrasting with 37% IgG-producing cells and 56% IgA-producing cells in the IgA nephropathy patient [16]. Thus, these findings indicate that tonsillotomy affected IgA content in serum.

In the present experiment, IgA in the extract of tonsilllectomized tissue was qualitatively and quantitatively examined by isoelectric focusing (IEF), as reported by Shuib et al. [17]. Our findings indicate that asialo IgA1 may be produced in excess in the tonsils of IgA nephropathy patients.

Subjects and methods

Subjects

Normal human serum was purchased from China Newtech Development and Trade Corp. and was stored at −20°C. Neuraminidase from Streptococcus 6646K was obtained from Seikagaku Co. (Tokyo, Japan). 2,3-O-Sialyltransferase (rat, recombinant) was purchased from Calbiochem (Los Angeles, CA) and Immobiline DryStrip® was from Amersham Bioscience Co. (Uppsala, Sweden).

Right-side tonsils were obtained from seven patients with IgA nephropathy (five males and two females; age range: 18–38 years; mean age: 30.4 ± 6.9 years) who had been identified with IgA nephropathy (five males and two females; age range: 18–38 years; mean age: 30.4 ± 6.9 years) who had been identified with IgA nephropathy (five males and two females; age range: 18–38 years; mean age: 30.4 ± 6.9 years) who had been identified with IgA nephropathy [5].

Tonsillar IgA1 in IgA nephropathy

Preparation of tonsillar extract

The tonsillar extract was prepared from fresh tonsilllectomized tissue as summarized in Figure 1. The tonsilllectomized tissue was first removed from other connective tissues and cut into small species on ice. The tissue was homogenized from 4 vol of wet tissue weight in a solution containing 10 mM phosphate buffer, pH 7.0, with 0.25 M sucrose, 25 mM KCl and 1 mM EDTA. The homogenate was first centrifuged at 3000 r.p.m. for 10 min and the supernatant was then ultracentrifuged at 40 000 r.p.m. for 70 min. Clear supernatant was then used as tonsillar extract. The tonsillar extract from the control was designated CTS. The protein concentration of tonsillar extracts ranged from 6.0 to 15.8 mg/ml. The precipitate containing the microsomal fraction was stored for additional analysis.

Preparation of IgA1-binding protein (IgA1-BP)

IgA1-BP from human serum was prepared as previously reported [18]. Ten ml of pooled serum was applied to asialo-, agalacto-IgA1–Sepharose (5 ml) and was equilibrated with the 0.1 M Tris–HCl buffer, pH 7.5, containing 0.02% sodium azide. The column was thoroughly washed with buffer and the IgA1-BP was then eluted from the column using the same buffer containing 1.0 M NaCl. The IgA1-BP fraction was collected, dialysed, concentrated and half of the concentrate was subjected to IEF.

IEF using IPGphor instrumentation

IEF was carried out according to IPGphor instrument instructions (Amersham Pharmacia Biotech Co). Ten µl serum and 30 µl tonsillar extract were analysed as follows. Each sample was mixed with 350 µl of the solution containing 8 M urea, 0.5% Triton X-100, 0.6% dithiothreitol, 0.1% IPGphor, 1.0 µl of carrier protein (IGG10) in 0.3 M mesylate, 0.1% ampholines, pH 3.9–4.9. The IPG strips were rehydrated for 10 min, then kept at 20°C for 12 h. The IPG strips were then subjected to IEF in the IEF unit IPGphor. The IPG strips were stained with Coomassie Brilliant Blue R-250 and destained.
0.5% Ampholine and 0.02% bromophenol blue. The sample solution and the Immobiline DryStrip pH 3–10 NL (18 cm) were carefully placed on the strip holder and covered with the DryStrip Cover Fluid and the strip holder was placed in the IPGphor instrument. The gel was swollen with the above solution after standing at 20°C for 12 h. IEF was then begun according to standard condition steps 1 through 3. Step 1 was at a gradient ranging from 0 to 500 V for 1 h, step 2 at a gradient from 500 to 1000 V for 1 h and step 3 was at a gradient from 1000 to 8000 V for 1 h.

After the focusing, the DryStrip was cut from the anode side into 5-mm long pieces (nos 1–35) and each piece was placed in a tube filled with 300 μl 0.1 M Tris–HCl buffer, pH 7.5, containing 0.02% azide. The tube was stirred vigorously and centrifuged at 10 000 r.p.m. for 3 min. The above treatment was then repeated. The protein in the gel in each tube was detected by the enzyme-linked immunosorbent assay (ELISA) method.

Detection of proteins by ELISA

For preparation of IEF profiles of IgA or IgG, 96-well microtitration plates (Costar; Corning Incorp., Corning, NY) were coated with 100 μl goat affinity purified antibody to human IgA (α chain specific; ICN Cappel) or sheep affinity purified antibody to human IgG (γ chain specific; The Binding Site) diluted to a concentration of 10 μg/ml with 0.015 M sodium carbonate solution. After blocking the unreacted sites with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), 100 μl from each IEF tube was added to the anti-IgA-coated wells. After incubation for 1.5 h at room temperature, the plate was washed six times with PBS washing buffer containing 0.05% Tween-20 and 0.1% BSA. One-hundred μl of peroxidase-conjugated sheep affinity purified antibody to human IgA (1:500 dilution in PBS, α chain-specific; ICN Cappel) or peroxidase-conjugated goat affinity purified antibody to human IgG (1:500 dilution in PBS; Biosource) was added to the wells. After incubation for 1 h at room temperature, the plate was washed 12 times with the PBS washing buffer. The plate was then exposed to a freshly prepared substrate solution (100 μl) containing 40 mg o-phenylenediamine, 30 μl hydrogen peroxide, 3.56 g Na2HPO4 and 1.05 g citric acid in 100 ml water. The reaction was stopped by adding 100 μl of 2 N H2SO4. The developed colour was read at 490 nm with a microplate reader (model 450; Bio-Rad, USA).

For a quantitative comparison of IgA and IgG contents in the tonsillar extract, all of the assays were performed in triplicate at dilutions of 1000–10 000 times for IgA and 10 000–100 000 times for IgG. Each median was expressed as a concentration. After incubation and allowing the sample to stand at room temperature for 5 min, the absorbance at 595 nm was read according to the manufacturer’s instructions.

Results

Typical IEF profile of IgA by Immobiline DryStrip pH 3–10 NL gel

Figure 2 shows a typical IEF profile of serum IgA and a typical pH gradient produced by the Immobiline DryStrip pH 3–10 NL gel. The isoelectric point (pI) of the major portion of serum IgA was distributed from 4.8 to 6.4, corresponding to a linear pH portion on the gel.

Comparative study of the IEF profiles of IgA from various sources

We additionally compared IEF profiles of IgA. As shown in Figure 3, the profiles of IgA in tonsillar extracts from the two controls and two IgA nephropathy patients were similar. In contrast, the IEF profiles of serum IgA from the two controls and two IgA nephropathy patients were also similar. In contrast, the profile of IgA from the tonsillar extract was clearly different from profiles in serum (Figure 4).

Enzymatic treatment of serum and tonsillar extract

Neuraminidase treatment of serum and tonsil extract was carried out by incubating 10 μl serum and 30 μl tonsillar extract with neuraminidase (10 mU) at 37°C for 3 h. Serum (10 μl) and tonsillar extract (30 μl) were treated with 20 μg CMP-N-acetylneuraminic acid and α2,3(O)sialyltransferase (3.4 mU) at 37°C for 2 h. After the treatment, the samples were directly subjected to IEF under standard conditions.

Protein assay

Assay of total protein content in the tonsillar extract was carried out using a Bio-Rad Protein Assay Kit. In brief, 400 μl of dye reagent concentrate was added to 1.6 ml of sample containing 1–5 μl tonsil extract or 2 μl BSA standard (1–10 mg/ml). After stirring and allowing the sample to stand at room temperature for 5 min, the absorbance at 595 nm was read according to the manufacturer’s instructions.
The relative content of cationic IgA was clearly higher in the tonsillar extract than that in serum. In order to determine whether the protein portion was equivalent in both IgAs, the sialic acid residues contributing to its net negative charge were removed from IgA by neuraminidase treatment. As shown in Figures 5A and 5B, removal of sialic acid changed IgA profiles in both tonsillar and serum extracts. As expected, all of the peaks were shifted to the cathode side after decreasing the negative charges. After treatment, we found that the IEF profile of IgA from the tonsillar extract overlapped well with the serum profile (Figure 5C). The separation of the treated IgA from the tonsillar extract into two peaks was due to the different protein portions, IgA1 (cathode side) and IgA2 (anode side), because the peak on the cathode side was bound to jacalin (data not shown).

Fig. 3. Comparison of IEF profiles for IgA in the tonsillar extract and in serum from the control and an IgA nephropathy patient. Open circles and closed circles indicate samples from the control and the IgA nephropathy patient, respectively. Thirty microlitres of each tonsillar extract and 10 μl of each serum sample were analysed by IEF under the standard conditions shown in Figure 2.

Fig. 4. Comparison of IEF profiles for IgA in the tonsillar extract and in serum. Open circles and closed circles indicate the IEF profiles of two control tonsillar IgAs and two control serum IgAs, respectively. IEF and detection of IgA was carried out under standard conditions.

Fig. 5. Change in IEF profile of IgA before and after neuraminidase treatment of control tonsillar extract and control serum. (A), (B) and (C) indicate IEF profiles of IgA before and after the neuraminidase treatment of a control tonsillar extract and of control serum, respectively. Open circles and closed circles in (A) and (B) indicate IgA untreated and treated with neuraminidase, respectively. In (C), open circles and closed circles indicate IgA from tonsillar extract and serum treated by neuraminidase, respectively.
**IEF profile of IgA in IgA1-BP**

IgA1-BP was prepared from pooled human serum as previously described. The IgA in IgA1-BP that was thought to be aberrant IgA exhibited binding characteristics to the hypoglycosylated IgA1. Quantitative analysis of IgA in the IgA1-BP showed that the content (a few mg per ml serum) of the aberrant IgA, assumed to be hypoglycosylated IgA1, was significantly higher in the IgA nephropathy patient [18]. This IgA in IgA1-BP was analysed by IEF. The IEF profile of IgA in the IgA1-BP prepared from serum did not coincide with the profile of serum IgA. This indicated that the IgA in IgA1-BP corresponded to the minor component of serum IgA and that it also coincided with cationic IgA in the tonsillar extract (Figure 6).

**Transfer of sialic acid to IgAs from various sources**

Sialic acid was transferred to IgAs from various sources using α2,3(O)sialyltransferase. This addition of sialic acid shifted all the IEF profiles to the anode side (Figure 7). Because the enzyme is known to transfer sialic acid to the galactose residue of the Galβ1,3GalNAc structure (TF antigen), these findings indicated the presence of the asialo Galβ1,3GalNAc structure, that is, the presence of asialo IgA1. Thus, most of the cationic IgA in tonsillar extract (Figure 7B) and most of the aberrant IgA in IgA1-BP (Figure 7C) were found to be asialo IgA1.

**Quantitative analysis of IgA and IgG in the tonsillar extract**

Quantification of IgA and IgG in tonsillar extract was carried out by ELISA. Each extract was diluted from 1000 to 10 000 times for the detection of IgA and from 10 000 to 100 000 times for IgG. Contents of each immunoglobulin were corrected as amount per total protein. They were expressed as the relative value divided by the amount from the PPP patient. The IgA content (1.577 ± 0.363) was significantly higher than in controls (1.172 ± 0.219; P < 0.05) whereas the IgG content was significantly lower in IgA nephropathy patients (0.630 ± 0.109) than in controls (0.988 ± 0.080; P < 0.01).

**Discussion**

In a previous report, we showed that the degree of completeness of the hinge O-linked oligosaccharide correlated with stability and with the polymerization process of IgA1 [3]. In the IgA nephropathy group, there was a significant shift from the sialyl Galβ1,3GalNAc (sialyl TF antigen) to the asialo-TF antigen compared with the negative control [10]. Recently, this shift towards the asialo-TF antigen in serum IgA1 from an IgA nephropathy patient was reconfirmed in studies using enzyme treatment and amino sugar analysis [19].
In order to remove artefactual effects, we examined sialidase activity in tissue extracts. The sialidase activity in the tonsillar extract was ~10 µU/ml, making it too low to remove sialic acid from IgA under the ice-cold conditions. Instead, the IEF profile of tonsillar IgA was not changed after further storage of the tonsillar extract under ice-cold conditions for 4 h (data not shown). Because removal of sialic acid from both tonsillar IgA and serum IgA produced similar IEF profiles, the difference in their charge was not due to the protein portion, but essentially to sialic acid on the sugar portion.

Secretory IgA1 (sIgA1), obtained from the colostrum, is characterized not only as a dimeric molecule associated with a J chain and secretory component, but also by the presence of asialo O-linked oligosaccharide [20]. Judging from its isoelectric point and the findings presented in Figure 7B, the cationic peaks from tonsillar IgA corresponded to the asialo IgA1. Because the producing cell of dimeric IgA and the J chain in tonsillar tissue were detected, the presence of tonsillar IgA1 having an asialo sugar chain similar to sIgA1 was also expected. Since secretion into the oral cavity of IgA produced in the tonsil was not reported, tonsillar IgA1 having an asialo sugar chain similar to sIgA1 was also expected. Since secretion into the oral cavity of IgA produced in the tonsil was not reported, the produced asialo IgA1 was most likely discharged from tonsillar IgA having an asialo sugar chain similar to sIgA1. Because the producing cell of dimeric IgA and the J chain in tonsillar tissue were detected, the presence of tonsillar IgA1 having an asialo sugar chain similar to sIgA1 was also expected. Since secretion into the oral cavity of IgA produced in the tonsil was not reported, the produced asialo IgA1 was most likely discharged into the serum. Amelio et al. [15] examined the relationship between serum IgA level and tonsillectomy and reported significantly lower levels of serum IgA in normal tonsilllectomized cases than in normal non-tonsillectomized subjects. The change in IgA levels in serum was thought to be an indirect effect of the tonsillectomy [15].

Thus, although there were no qualitative differences in tonsillar IgA between control and IgA nephropathy patients, there were quantitative differences. Significantly higher IgA and significantly lower IgG levels were found in tonsillar extract from IgA nephropathy patients. This pattern of results was reflected in the previously demonstrated distribution of tonsillar plasma cells showing 65% of IgG-secreting cells and 29% of IgA plasma cells in the control but 37% of IgG-producing cells and 56% of IgA-producing cells in the IgA nephropathy patient [16]. The tonsil of the IgA nephropathy patient produced much more asialo IgA1 than the control tonsil.

We previously reported that hypoglycosylated IgA1 was preferentially bound to hypoglycosylated IgA1–Sepharose. In order to estimate aberrant IgA1 in the serum, we quantified IgA in the protein that is bound to the hypoglycosylated IgA1–Sepharose (IgA1-BP). We found a few mg per ml of aberrant IgA in sera from the IgA nephropathy patient, which was significantly higher than in the normal control [18]. Therefore, it remains possible that the tonsil was a major source of this aberrant IgA1, identified as asialo IgA1, in the IgA nephropathy patient. We attempted to analyse the aberrant IgA in IgA1-BP by using IEF. The resulting IEF profile of IgA in IgA1-BP was completely different from the profile of the major portion of serum IgA and corresponded to that of the cationic minor components in serum IgA. The peaks in the profiles also corresponded to the major cationic component in tonsillar IgA. In addition, asialo Galβ1,3GalNAC was clearly present in this aberrant IgA following the enzymatic transfer of sialic acid to IgA1 (Figure 7). Especially prominent was the shift of the IEF profiles of IgA in IgA1-BP and the tonsillar extract to the anode side. These results indicate that the aberrant IgAs were essentially composed of asialo IgA1. As in our previous report [19], serum IgA also contained partly sialylated IgA1.

In summary, we found that IgA1 produced in tonsillar tissue differed from IgA1 in serum. Furthermore, overproduction of asialo IgA1 resulted from the disordered balance between IgA- and IgG-producing cells in the tonsil from the IgA nephropathy patient. Although it is unclear how such sIgA-like molecules are transferred from tonsil tissue to serum, a tonsillar source may produce a few µg of aberrant IgA1 that then appears in serum.

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