CLINICAL STUDY

Glycosylation of sera thyroglobulin antibody in patients with thyroid diseases

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Abstract

Objective: Thyroglobulin antibody (TgAb) is an important autoantibody in thyroid diseases, which is a glycoprotein, predominantly of IgG class. Glycosylation of the IgG-Fc contributes to many effector functions exhibited by antibodies. The aim of our study was to investigate the glycosylation of sera TgAb in patients with different thyroid diseases.

Design and methods: Sera from 146 patients were collected and divided into four groups: Hashimoto's thyroiditis (HT, $n=90$), Graves' disease (GD, $n=20$), papillary thyroid carcinoma (PTC, $n=17$), and PTC with histological lymphocytic thyroiditis (PTC-T, $n=19$). HT patients were further divided into euthyroidism and subclinical and overt hypothyroidism groups. Lectin-ELISAs were performed to detect the relative amount of core fucose, terminal galactose, and sialic acid on each TgAb respectively. **Results:** Among HT, GD, and PTC groups, HT patients had significantly lower core fucose content on TgAb than the other two groups; an increasing trend of sialylation was found in PTC sera ($P=0.076$) compared with HT groups. PTC-T patients had significantly higher sialylated TgAb than HT and GD patients, and no significant difference was found between PTC and PTC-T. There was no significant difference in the three carbohydrate residue contents on sera TgAb among HT subgroups. In all the patients, negative correlation was found between sialic acid content and TgAb IgG levels ($r=-0.736$, $P<0.001$).

Conclusions: Our study showed that glycosylation of sera TgAb varied in different thyroid diseases and it might be involved in pathogenesis of thyroid disorders.

European Journal of Endocrinology 168 585–592

Introduction

Circulating thyroglobulin antibody (TgAb) is a hallmark of autoimmune thyroid disease (AITD). It could be detected in 80–90% patients with Hashimoto's thyroiditis (HT) and 50–70% patients with Graves' disease (GD) (1). Compared with the general population, the prevalence of TgAb is increased nearly threefold in patients with differentiated thyroid carcinoma (2). TgAb can mediate antibody-dependent cell-mediated cytotoxicity (ADCC) in artificial systems (3) and it may be involved in thyroid destruction in AITD. In papillary thyroid carcinoma (PTC), as the presence of TgAb usually invalidates the serum Tg result, investigators have found that TgAb might be used as a surrogate tumor marker (4).

TgAb is predominantly of IgG class (5), and IgG is a glycoprotein with a sugar moiety attached to each of the asparagine 297 residues in the CH2-domains of the two Fc-fragments (6). Variable attachment of outer arm sugars (sialic acid, galactose, and fucose) to a heptasaccharide GlcNAc2Man3GlcNAc2 core structure results in the generation of heterogeneous array of IgG

glycoforms. These glycoforms can differ in their efficacy of effector function activation as it influences binding of IgG molecules to Fc receptors and C1q (7).

As is known to all, glycosylation of IgG plays an important role in disease pathogenesis, not only in autoimmune disorders but also in tumors. It has been known that the absence of core fucose residues in the Fc glycans substantially increases the ADCC activity of IgG (8, 9). Increased sialylation of Fc glycans results in decreased ADCC activity (10, 11). Reduced IgG-Fc galactosylation has been documented to be limited mainly to patients with certain inflammatory and autoimmune diseases, such as rheumatoid arthritis (RA) and Crohn's disease (12, 13). Interestingly, higher levels of agalactosylated IgG oligosaccharides have recently been reported in patients with prostate cancer (14), ovarian cancer (15), and gastric cancer (16).

As far as we know, most studies on antibody glycosylation focused on total IgG; by contrast, there was little information on the glycosylation of a specific antibody. As AITD is an organ-specific disease, the study on TgAb glycosylation might provide more information on humoral factors in the pathogenesis of thyroid

diseases. The aim of our study was to investigate the glycosylation of sera TgAb in patients with different thyroid diseases including HT, GD, PTC, and PTC with histological lymphocytic thyroiditis (PTC-T).

Materials and methods

Study groups

A total of 146 subjects (137 women and nine men) with thyroid disorders were consecutively referred to Peking University First Hospital in the period December 2010 to August 2012 and enrolled in the current study before receiving treatment. The study groups included 110 AITD (including 90 HT and 20 GD), 17 PTC (without HT or GD), and 19 PTC-T patients confirmed by histopathology. All of them had TgAb, which were detected by electrochemiluminescence immunoassays. The PTC-T group included 57.9% of patients with a clinical diagnosis of nodular thyroid disease (NTD) without any clinical features of HT. None of the PTC or PTC-T patients had been previously treated with ^{131}I . None of the patients had evidence of hereditary or acquired variations in the concentration of thyroxine-binding globulin. There was no evidence of other autoimmune diseases, including systemic lupus erythematosus, RA, type 1 diabetes mellitus, or pernicious anemia. The HT patients were further divided into three subgroups according to thyroid function: patients with euthyroidism (Eu, $n=30$), subclinical hypothyroidism (sH, $n=26$), and overt hypothyroidism (H, $n=34$). This study complied with the Helsinki declaration and was approved by the Ethics Committee of Peking University First Hospital. All the patients gave written informed consent.

Detection of thyroid function and TgAb

Sera samples of all the patients were collected at diagnosis and kept frozen at -80°C until use. Chemiluminescence immunoassays were used to detect total triiodothyronine (TT_3), total tetraiodothyronine (TT_4), and TSH (ADVIA Centaur (Siemens Healthcare Diagnostics, Tarrytown, NY, USA)). TgAb was detected by electrochemiluminescence immunoassays (Cobas e 601 Analyzer (Roche Diagnostics)).

Detection of total TgAb IgG by antigen-specific ELISAs

Half of the 96-well microtiter plates (Costar, Data Packaging Corporation, Spencer, MA, USA) were coated with $4\text{ }\mu\text{g/ml}$ human native Tg (Calbiochem Merck KGaA) in 0.05 mol/l bicarbonate buffer (pH 9.6) for 90 min and the other half were coated with bicarbonate buffer alone to act as antigen-free wells. The volume in each well was $50\text{ }\mu\text{l}$ in all the steps. All incubations were

carried out at 37°C for 1 h unless otherwise specified, and the plates were washed three times with PBS containing 0.1% Tween 20 (PBST) between stages. After washing, all the plates were blocked with 3% BSA (Sigma) and were then oxidized with $200\text{ }\mu\text{l}$ 0.1 mol/l sodium periodate (17) in citrate buffer (pH 4.0) for 10 min at room temperature in the dark. The serum samples diluted 1:100 were added in duplicate to both antigen-coated and antigen-free wells. Every plate contained a positive control, a negative control, and a blank. Then, an HRP conjugate goat anti-human immunoglobulin (Jackson ImmunoResearch Laboratories, Inc., Baltimore Pike, West Grove, PA, USA), 1:2500 dilution, was subsequently employed for antibody detection. Following washing, *ortho*-phenylenediamine diluted in citrate buffer containing 0.1% hydrogen peroxide was used as substrate/chromogen mixture. The reaction was stopped by the addition of 1 mol/l hydrochloric acid. Finally, the results were recorded at 490 nm by a Varioskan Flash Multimode Reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and expressed as percentages of the positive control.

Detection of carbohydrate residues on sera TgAb by lectin-ELISAs

Lectin-ELISAs (L-ELISA) were applied to detect carbohydrate on sera TgAb. Three biotinylated lectins (Vector Laboratories, Burlingame, CA, USA) were used in the current study: Aleuria Aurantia Lectin (AAL) (18) for fucose detection, Ricinus Communis Agglutinin I (RCA I) (19) for galactose detection, and Elderberry lectin (EBL) (20) for sialic acid detection. Similar assay procedures as mentioned earlier were conducted to detect sera TgAb glycosylation. Briefly, after the sera diluted 1:100 were added in duplicate to both antigen-coated and antigen-free wells, diluted biotinylated AAL (1:1000), RCA I (1:500), and EBL (1:2000) were employed respectively and then peroxidase-labeled avidin D (Vector Laboratories) (1:10 000 for AAL and 1:12 000 for RCA I and EBL) was added. The subsequent procedures were the same as described earlier. The results were expressed as percentages of the positive control as well. The relative amount of fucose in each TgAb IgG was calculated as the percentage of fucose-positive control/the percentage of TgAb IgG-positive control $\times 100\%$. The calculation of the relative amount of galactose and sialic acid was performed similarly.

Statistical analysis

Statistical analysis was performed using the SPSS 13.0 (SPSS) statistics package. Comparisons were carried out by the Mann–Whitney U test, one-way ANOVA, χ^2 test, Kruskal–Wallis H test, Pearson correlation, and Spearman's test. A P value under 0.05 was considered statistically significant.

Table 1 Demographic data, thyroid functional status, and TgAb IgG levels of the patients in different groups. Numbers expressed as mean $\pm$ s.d., median (interquartile range). Reference value range: TT₃: 0.92–2.79 nmol/l; TT₄: 58.1–140.6 nmol/l; TSH: 0.35–5.5 mIU/l.

Groups	HT (n=90)	GD (n=20)	PTC (n=17)	PTC-T (n=19)
Age (years)	50.5 (33.5–57.0)*	34.0 (23.0–41.7)	54.0 (22.4–64.0)*	48.0 (41.8–52.0)*
Gender (M/F)	6/84	0/20	2/15	1/18
TT ₃ (nmol/l)	1.5 (1.3–1.8)*	4.6 (3.6–8.5)	1.6 (1.4–1.8)*	1.6 (1.4–1.9)*
TT ₄ (nmol/l)	88.6 (64.7–104.1)*,†,‡	201.9 (133.1–249.1)	112.0 (89.6–122.6)*	113.4 (100.1–127.9)*
TSH (mIU/l)	6.4 (4.3–18.0)*,†,‡	0.01 (0.00–0.04)	2.3 (1.3–4.1)*	2.5 (1.8–3.4)*
Positive percentage of TgAb IgG (%)	41.2 (33.2–62.4)	48.6 (39.6–61.8)	46.3 (38.6–52.8)	39.2 (32.7–55.1)

HT, Hashimoto's thyroiditis; GD, Graves' disease; PTC, papillary thyroid carcinoma; PTC-T, papillary thyroid carcinoma with histological lymphocytic thyroiditis. * $P < 0.05$ vs GD; † $P < 0.05$ vs PTC; ‡ $P < 0.05$ vs PTC-T.

Results

Demographic data of all the patients

As shown in Table 1, there was no significant difference in gender distribution in HT, GD, PTC, and PTC-T groups. GD patients were younger than the other three groups ($P < 0.05$ respectively). TT₃ and TT₄ levels in GD group were significantly higher than those in the other three groups ($P < 0.05$ respectively). TT₄ in the HT group was lower than that in PTC and PTC-T groups ($P < 0.05$). TSH levels in HT were significantly higher than those in the other three groups ($P < 0.05$ respectively), and no significant difference was found in TSH levels between PTC and PTC-T groups.

In HT patients, there were no significant differences in age and gender distribution in patients of H, sH, and Eu subgroups. TT₃ and TT₄ levels in H were significantly lower than those in the other two groups ($P < 0.05$ respectively) (Table 2), and TSH levels in the H group was significantly higher than those in the sH and Eu groups ($P < 0.05$ respectively).

Oxidation of thyroglobulin antigen

As Tg is a glycoprotein, sodium periodate was used to cleave Tg carbohydrate in order to minimize the interference in the detection of glycosylation on sera TgAb. As shown in Fig. 1, all three carbohydrate residues on Tg could be cleft by sodium periodate, and terminal sialic acid was the most susceptible for the oxidation cleavage. As shown in Fig. 2, with

prolongation of oxidation reaction, the OD values of TgAb binding oxidized Tg fell only slightly, which indicated that Tg oxidation might have little influence on its antigenicity. Ten minutes was finally chosen as the reaction conditions with sodium periodate, giving consideration to the detection of TgAb IgG and all the three carbohydrate residues.

Results of total TgAb IgG levels

As shown in Table 1, there was no significant difference in sera TgAb IgG levels among the four groups. In HT patients (Table 2), TgAb IgG levels in the Eu group were significantly lower than those in the H group ($P < 0.05$), and there was no significant difference between the sH and H groups ($P > 0.05$).

Results of carbohydrate residues on each TgAb

The relative amount of three carbohydrate residues on each TgAb in HT, GD, PTC, and PTC-T groups is shown in Fig. 3 respectively.

The relative amount of fucose on sera TgAb in different thyroid diseases Among HT, GD, and PTC groups, sera TgAb in HT patients had significantly lower core fucose content than that in the other two groups ($P < 0.05$ respectively). No significant difference was found between GD and PTC groups and PTC and PTC-T groups. There was significantly less fucosylated TgAb in HT sera than in PTC-T groups ($P = 0.001$).

Table 2 Demographic data, thyroid functional status, and TgAb IgG levels of the patients in the HT subgroups. Numbers expressed as mean $\pm$ s.d., median (interquartile range). Reference value range: TT₃: 0.92–2.79 nmol/l; TT₄: 58.1–140.6 nmol/l; TSH: 0.35–5.5 mIU/l.

Groups	Eu (n=30)	sH (n=26)	H (n=34)
Age (years)	44.7 $\pm$ 16.0	50.0 $\pm$ 12.5	47.0 $\pm$ 14.0
Gender (M/F)	1/29	1/25	4/30
TT ₃ (nmol/l)	1.6 (1.4–1.7) [†]	1.8 (1.6–2.0) [†]	1.2 (1.0–1.5)
TT ₄ (nmol/l)	98.1 (85.0–107.0) [†]	94.5 (85.5–108.0) [†]	53.0 (37.2–71.0)
TSH (mIU/l)	3.4 (2.0–4.5)*,†	6.5 (6.1–8.4) [†]	39.6 (10.2–100.3)
Positive percentage of TgAb IgG (%)	35.7 (31.3–47.8) [†]	46.8 (34.4–64.1)	52.7 (33.8–67.0)

Eu, euthyroidism; sH, subclinical hypothyroidism; H, hypothyroidism. * $P < 0.05$ vs sH; † $P < 0.05$ vs H.

Figure 1 The biotinylated lectins binding to thyroglobulin (Tg) in different oxidation time with sodium periodate. (A) Aleuria Aurantia lectin (AAL) for fucose detection, (B) Ricinus Communis Agglutinin I (RCA I) for galactose detection, and (C) Elderberry lectin (EBL) for sialic acid detection.

The galactose on sera TgAb in different thyroid diseases No significant differences in TgAb galactosylation were found among the four groups.

The relative amount of sialic acid on sera TgAb in different thyroid diseases Although there was no significant difference in the relative amount of sialic acid on each TgAb in the HT, GD, and PTC groups, an increasing trend of sialylation was found in PTC sera compared with the HT group ($P=0.076$). PTC-T patients had significantly more sialylated TgAb than HT and GD patients ($P=0.005$ and $P=0.021$ respectively).

The three carbohydrate residues on TgAb in HT subgroups There were no significant differences in the relative amount of all the three carbohydrate residues on TgAb among the three HT subgroups (data not shown).

The relationship between carbohydrate residues on TgAb and TgAb IgG levels For all the patients with different thyroid diseases, a negative relationship was found between the relative amount of sialic acid and TgAb IgG levels ($r = -0.736$, $P < 0.001$) (Fig. 4). There were no relationships between the levels of glycosylation for all three carbohydrate residues and the levels of TSH, TT_3 , and TT_4 respectively.

Discussion

During the last decade, it became apparent that glycosylation of the IgG-Fc, the most important step of posttranslational modification, contributes to many effector functions exhibited by antibodies such as complement binding and activation, induction of ADCC, and binding to macrophage Fc receptors (21). Notable alterations in glycosylation of the IgG-Fc region have been described not only in autoimmune disorders but also in some malignancy diseases (16). For example, Chen *et al.* (22) had reported that the changes in total IgG-Fc N-linked glycosylation was associated with thyroid cancer.

Structural features of glycan moieties in the Fc portion of IgG were regulated in an antigen-specific fashion (23). As TgAb is produced by thyroid-derived lymphocytes, and B cells at the site of chronic inflammation might be the major source of differentially glycosylated (auto)antibodies (24), we studied the glycosylation in thyroid-specific antibody rather than total IgG in different thyroid diseases.

In our study, the levels of fucosylation and sialylation on TgAb varied in different thyroid disorders. The IgG

Figure 2 TgAb IgG binding to thyroglobulin with a serial dilution (diluted 1:12.5–1:800) in different oxidation times with sodium periodate.

Figure 3 Comparisons of the relative amount of carbohydrate residues on each sera TgAb from patients with different thyroid diseases. (A) Sera TgAb in HT patients had significantly lower core fucose content than that in the GD, PTC, and PTC-T groups. (B) There was no significant difference in the relative galactosylation on TgAb in different thyroid groups. (C) The relative amount of sialic acid on each sera TgAb from HT and GD patients was lower than that from patients with PTC-T. HT, Hashimoto's thyroiditis ($n=90$); GD, Graves' disease ($n=20$); PTC, papillary thyroid carcinoma ($n=17$); PTC-T, papillary thyroid carcinoma with histological lymphocytic thyroiditis ($n=19$).

molecule comes in close contact if fucose residues are present (25); therefore, non-fucosylated antibodies bind to the Fc γ RIIIa receptor with significantly increased affinity. By this way, the commercial therapeutic monoclonal antibody such as trastuzumab (26) and rituximab (27) is constantly improved to work more efficiently. It has also been reported that the sialic acid-containing IgG displays an anti-inflammatory effect (11) and sialic acid negatively affects antibody binding to the Fc γ RIIIa. In the current study, among the HT, GD, and PTC groups, the lowest fucosylation levels of TgAb were found in HT sera, and an increasing trend in sialylated TgAb was found in the PTC group compared with the HT group. As non-fucosylated and non-sialylated antibodies may have stronger ability to participate in ADCC, we speculated that low levels of fucosylation and sialylation might be the property of TgAb in HT, which might have more capacity to induce thyroid destruction.

Current evidence does not support an important role of terminal galactose residues in either enhancing or attenuating the activity of IgG *in vivo* (6). And in some reports, terminal galactose content of IgG does not affect ADCC but complement-dependent cytotoxicity activity (28). In our study, no significant difference was found in the galactosylation on TgAb in the AITD, PTC, and PTC-T groups. As TgAb does not fix the complement (29), the importance of galactose residues on TgAb need to be further studied.

In thyroid cancer, the TgAb concentration can serve as a surrogate tumor marker for recurrence (4). Whether different pathogenesis in AITD and PTC is involved in the production of TgAb is still not known. Latrofa *et al.* (30) found different epitope specificities for TgAb in PTC compared with AITD. Our study found that the levels of TgAb glycosylation were also different between AITD and PTC groups. As different B-cell subsets produce differentially glycosylated IgG (31), we assumed that TgAb arose in PTC from different pathogenetic mechanisms of AITD. In PTC, TgAb production might be the result of an immune response to the inflammation associated with tumorigenesis that may have the potential to release posttranslational modified Tg antigens with enhanced immunogenicity (4).

In the current study, the PTC-T group, confirmed by histopathology, included 57.9% of patients with a clinical diagnosis of NTD. Latrofa *et al.* (30) had found that TgAb epitope pattern in PTC-T resembled that of AITD. As only a few PTC-T patients had classical clinical features of HT in our research, no significant difference in the levels of TgAb glycosylation was found between PTC and PTC-T groups.

It was interesting that the levels of sialylation on each TgAb had a negative relationship with TgAb IgG levels, independent of thyroid function. In an epidemiological study, the individuals with thyroid antibodies might be at high risk of developing thyroid failure (32). We speculated that the changing of carbohydrate residues

on each TgAb IgG might be a consequence of elevated Ig synthesis by B cells (33). Chen *et al.* (34) had reported that breaking B-cell self-tolerance occurred first for Tg and subsequently for TPO, and TgAb arose first followed later by TPOAb in HT. We assumed that with TgAb levels increasing, more TgAbs with lower terminal sialic acid were produced, which might have higher capacity in mediating ADCC and contributing to thyrocyte damage, and this process triggered thyroid autoimmunity further.

Figure 4 The correlation between the relative amount of carbohydrate residues on each TgAb and TgAb IgG levels in all the patients ($n=146$). There was a negative correlation between the relative amount of sialic acid and TgAb IgG levels. (A) Core fucose, (B) galactose residue, and (C) sialic acid residue.

Overall, as sugar residues such as fucose and sialic acid can dramatically alter IgG activity, the change in IgG glycosylation on thyroid antibodies might provide a new view to investigate the role of autoantibodies in the pathogenesis of thyroid diseases.

In clinical practice, HT patients with TgAb may have different thyroid functional status. It is an evolutionary process from Eu to sH, and even to H (35). When thyroid cell hyperplasia cannot compensate for follicular destruction and $\sim 90\%$ of the thyroid gland is destroyed, thyroid failure will develop (36). As there were no significant differences in the levels of glycosylation on each TgAb among the three HT subgroups with different thyroid functional status, the levels of glycosylation on TgAb might not represent thyrocyte hyperplasia but merely reflect the capacity of inducing thyroid destruction by ADCC.

In the current study, the total glycosylation of TgAb was measured by antigen-specific ELISAs. TgAb is predominantly of IgG class, and IgA and IgM isotype could be detected only in sera from a few patients and at much lower levels (5). Therefore, compared with the N-linked glycosylation of TgAb IgG, glycosylation of IgA and IgM (37) might be neglected. And it is well known that human serum IgG has on average 2.8 N-glycoside-type sugar chains per protein molecule. Two of them are invariably located in the conserved N-glycosylation site of asparagine 297, and additional ones are found in the variable regions of the light and heavy chains (38). In our study, we did not distinguish the glycosylation on TgAb between IgG-Fab and Fc fragments. Further study focused on Fc regions might provide more convincing evidence on the role of the glycosylation on TgAb in the pathogenesis of thyroid diseases.

In consideration of the sample size and the cost for detection, the simple L-ELISAs were used to detect relative glycosylation on TgAb in our study, instead of more complex but more accurate assays such as liquid chromatography mass spectrometry and high performance liquid chromatography. Furthermore, removal of the terminal sugar residues from Tg by oxidation is a necessary step to avoid the antigen's reactivity with the lectins used in this study. Sodium periodate is a strong oxidant and can cleave the carbohydrate nonspecifically. After the oxidation step, the ability of TgAb IgG binding to oxidized Tg changed little. This result was in accord with the notion that the TgAb epitope does not involve carbohydrate (39). In addition, an oxidation time of 10 min was used in ELISAs for TgAb IgG detection and L-ELISAs in order to guarantee the same amount of TgAb IgG was captured in these assays.

Taken together, our study showed for the first time that glycosylation of sera TgAb varied in AITD and PTC, and that the levels of sialylation of TgAb might decrease with increasing TgAb levels. Further studies on the glycosylation of thyroid autoantibodies may be helpful to elucidate the potential role of autoantibodies in the pathogenesis of thyroid diseases.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by Beijing Natural Science Foundation (grant numbers 7122185), Beijing Nova Program (grant numbers 2011011), and Program for New Century Excellent Talents in University (grant numbers BMU20110266) and Sector Funds of Ministry of Health (grant numbers 201002002).

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Received 1 November 2012

Revised version received 17 December 2012

Accepted 29 January 2013