TGF-β1 mimics the effect of IL-4 on the glycosylation of IgA1 by downregulating core 1 β1, 3-galactosyltransferase and Cosmc

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Abstract. The aberrant glycosylation of IgA1 is pivotal in the pathogenesis of IgA nephropathy (IgAN). The aim of the present study was to investigate the effect of transforming growth factor-β1 (TGF-β1) on the glycosylation of IgA1 and the associated mechanism. The mRNA levels of core1 β1, 3-galactosyltransferase (C1GalT1) and its molecular chaperone, Cosmc, were analyzed, as was the subsequent O-glycosylation of IgA1, in a human B-cell line stimulated with TGF-β1. The IgA1-positive human B-cell line was cultured with different concentrations of recombinant human TGF-β1 (5, 10, 15 and 30 ng/ml). The production and glycosylation of IgA1 were assayed using sandwich ELISA and enzyme-linked lectin binding assays, respectively, and the mRNA levels of C1GalT1 and Cosmc were quantified using reverse transcription-quantitative polymerase chain reaction analysis. The results showed that the production of IgA1 was stimulated by low concentrations of TGF-β1 (5 or 10 ng/ml) and was suppressed by high concentrations (15 or 30 ng/ml). The terminal glycosylation of secreted IgA1 was altered in response to TGF-β1. TGF-β1 stimulation significantly decreased the mRNA levels of C1GalT1 and Cosmc. TGF-β1 may be key in controlling the glycosylation of IgA1, in part via the downregulation of C1GalT1 and Cosmc.

Introduction

IgA nephropathy (IgAN) is the most common primary glomerular disease worldwide and contributes significantly to end-stage renal disease (1). The diagnostic hallmark of IgAN is mesangial deposition of IgA, predominantly polymeric IgA of the IgA1 subclass (pIgA1) (2). IgA1 contains a hinge region in its heavy chain, which serves as the site of attachment for five O-linked glycan chains comprised of N-acetylgalactosamine with a 1, 3-linked galactose, which is sometimes sialylated (3,4). Patients with IgAN exhibit elevated circulating levels of IgA1 linked to O-glycans consisting of galactose-deficient N-acetylgalactosamine (GalNAc) with or without N-acetylneuraminic acid (5). These aberrantly galactosylated IgA1 molecules have an increased tendency to self-aggregate and to form antigen-antibody complexes with IgG antibodies directed against IgA1 hinge epitopes (6,7). In addition, Ig-containing complexes are particularly prone to mesangial trapping. Therefore, the formation of circulating IgA-containing immune complexes may promote mesangial IgA deposition, eventually resulting in glomerular injury (8). There is increasing evidence supporting the involvement of circulating immune complexes containing aberrantly glycosylated IgA1 in the pathogenesis of IgAN. However, the mechanisms underlying the overproduction of abnormal O-glycosylated IgA1 in IgAN remain to be fully elucidated.

The production of abnormal galactosylated IgA1 in patients with IgAN is caused by defective B lymphocytes (9). Core1 β1, 3-galactosyltransferase (C1GalT1) is a key enzyme, which transfers galactose from UDP-galactose to N-acetylgalactosamine residues. The stability of this enzyme is dependent on its interaction with the chaperone, Cosmc (10,11). The decreased expression and activity of C1GalT1, the decreased expression of its dedicated chaperone, Cosmc, and/or the increased expression and activity of a specific sialyltransferase have been demonstrated in the B cells of patients with IgAN (12-14). The production of aberrantly glycosylated IgA1 is likely due to the altered expression of these specific glycosyltransferases in B cells.

The production of IgA in B cells is regulated by T cells (15). Previous investigations have primarily focused on the imbalance of T helper (Th)1/Th2. It has been demonstrated that Th2 cytokines, including IL-4, 5 and 6, promote B cell class switching to IgA, and promote the subsequent proliferation and differentiation of IgA-producing cells (16). In addition, it has been reported that the Th2 cytokines

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IL-4 and IL-5 alter the N-glycosylation of murine IgA (17). As mice have only one subclass of IgA, which generally lacks O-glycans, Yamada *et al* (18) used an IgA1-positive human B-cell line (DAKIKI) to observe the effect of Th2 cytokines on the production of IgA1. The results revealed that IL-4 increases the galactose deficiency of secreted IgA1 by downregulating the expression of C1GalT1 and Cosmc. Furthermore, Suzuki *et al* (19) found that cytokines IL-6 and IL-4 accentuate IgA1 galactose deficiency via modulation of key glycosyltransferases in IgA1-producing cells isolated from patients with IgAN.

TGF-β1 is a key cytokine, which promotes IgA production and is elevated in patients with IgAN. A previous study examining the cytokine expression of peripheral blood mononuclear cells (PBMCs) showed increased levels of TGF-β1 and enhanced Th2 cytokine production in single cells isolated from patients with IgAN (20). In addition, circulating γδ T cells are increased in patients with IgAN and produce higher levels of TGF-β1, which promotes the production of IgA by B cells in culture (21). A previous study of a Chinese patient cohort also suggested that an increase in TGF-β1 in the sera of patients with IgAN was correlated with the levels of IgA, SIgA and galactose-deficient IgA1 (Gd-IgA1), and with pathologic classification (22). Yang *et al* (23) found that the number of type 3, TGF-β-secreting helper T cells was significantly elevated during the acute stage of Henoch-Schönlein purpura, which has a similar pathogenesis to IgAN. Our preliminary investigation demonstrated a significant increase in the percentages of Th2 and Th3 cells in patients with IgAN, compared with healthy controls, and that these percentages were positively correlated with serum levels of IgA (24). Based on these data, it was hypothesized that the Th3 cytokine, TGF-β1, has a similar role to IL-4 in the pathogenesis of IgAN. The present study aimed to determine the effect of TGF-β1 on the production and under-glycosylation of IgA1, and to explain the mechanisms underlying this effect in DAKIKI cells.

Materials and methods

Cell cultures and experimental protocols. The surface IgA1-positive human B lymphoma cell line, DAKIKI, was purchased from America Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% heat-inactivated fetal calf serum (FCS; Gibco; Thermo Fisher Scientific, Inc.), 1 mM sodium pyruvate, 2 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin in a humidified atmosphere at 37˚C with 5% CO_2. To assess the effect of cytokine stimulation on the production of IgA1, and to analyze the glycosylation and sialylation of IgA1, the cells were cultured in different concentrations (0, 5, 10, 15, 30 ng/ml) of recombinant human TGF-β1 (PeproTech, Inc., Rocky Hill, NJ, USA) at a density of $5x10^5$/ml per well in 24-well culture plates at 37˚C for 3 or 7 days. To assess the effect of cytokine stimulation on the mRNA expression of C1GalT1 and Cosmc, the cells were cultured under the same conditions for 12, 24 or 72 h. Previous studies have shown that IL-4 increases the levels of Gd-IgA1 by suppressing the expression of C1GalT1 and its chaperone Cosmc (18,19). Thus, 10 ng/ml of recombinant human IL-4 (PeproTech, Inc.) was used as a positive control in the present study (18). In each experiment, triplicate culture wells were used for each stimulation condition.

Enzyme-linked immunosorbent assay (ELISA) for IgA1. The level of IgA1 in the supernatant of each culture well was determined in duplicate using ELISA, as previously described (25). Briefly, 96-well immunoplates were coated with 5 μg/ml goat anti-human IgA polyclonal antibody (1:200; Southern Biotechnology Associates, Birmingham, AL, USA) and incubated at 4˚C overnight. Following three washes with PBS containing 0.05% Tween-20 (TBST), the plates were blocked in PBS containing 1% bovine serum albumin (BSA) (Sigma-Aldrich Shanghai Trading Co., Ltd., Shanghai, China) for 1 h. Following washing with 0.05% PBST, 50 μl of supernatant samples centrifuged at 100 x g for 5 min at 20˚C or standard human IgA1 (Merck Millipore, Darmstadt, Germany) was added in duplicate and incubated for 2 h at room temperature, followed by incubation with horseradish peroxidase-conjugated mouse anti-human IgA1 antibody (1:1,000 Southern Biotechnology Associates) for 1 h at 37˚C. Color was developed using tetramethylbenzidine solution (BD Biosciences, Franklin Lakes, NJ, USA) and detected using a microplate reader (Spectramax Plus384; Molecular Devices LLC, Sunnyvale, CA, USA) at 450 nm. Standard curves were constructed by serial dilution of an IgA1 standard serum.

Enzyme-linked lectin binding assays for IgA1 glycosylation and sialylation. O-glycosylation of the IgA1 samples was measured by the binding of *Vicia villosa* lectin (VVL; Vector Laboratories, Ltd. Peterborough, UK), which is specific for terminal GalNAc (26). The sialylation of IgA1 was measured by the binding of peanut agglutinin lectin (PNA; Vector Laboratories, Ltd.), which binds to the core1 disaccharide Gal-GalNAc (27). The procedures used to coat, block and wash the plates were the same as for the measurement of IgA1 concentration. Biotinylated VVL and PNA (5 μg/ml) were added to the reaction wells, which were then incubated for 2 h at 37˚C. The plates were washed with 0.05% PBST five times, and lectin binding was detected with avidin-horseradish peroxidase conjugate (Vector Laboratories, Ltd.). The color was developed and measured, as above.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis for C1GalT1 and Cosmc mRNA. Total RNA was extracted from the cells using an E.Z.N.A. Total RNA kit II (Omega Bio-Tek, Inc., Norcross, GA, USA). cDNA was synthesized from the total RNA using EasyScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech Co., Ltd., Beijing, China). The resulting cDNAs (1 μg) were amplified by real time qPCR analysis using a StepOne Plus™ Real-Time PCR system (Thermo Fisher Scientific, Inc.). The 20 μl reaction mixture contained: 10 μl SYBR Green PCR Master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) 0.4 μl forward primer, 0.4 μl reverse primer, 2 μl cDNA template, 0.4 μl passive reference, 6.8 μl ddH_2O. The samples were incubated at 94˚C for 30 sec, followed by 40 cycles of denaturation for a 5 sec at 94˚C, and annealing and extension for 31 sec at 60˚C. All samples were examined in triplicate against the human GAPDH reference gene, which was used to normalize the expression data. The primer pairs for C1GalT1,

Table I. Primers used for reverse transcription-quantitative polymerase chain reaction analysis.

Gene	Primer sequence	Fragment size (bp)
C1GalT1	Forward 5'-AAGGTTGACACCCAGCCTAA-3' Reverse 5'-CTTTGACGTGTTTGGCCTTT-3'	226
Cosmc	Forward 5'-AATGGTTCTGACAATGACTG-3' Reverse 5'-GCTGTATTGGATATGTAGTTACT-3'	272
Human GAPDH	Forward 5'-CAGGGCTGCTTTTAACTCTGGT-3' Reverse 5'-GATTTTTGGAGGGATCTCGCT-3'	203

C1GalT1, core1 β1, 3-galactosyltransferase.

Cosmc and human GAPDH are listed in Table I. The results were analyzed using the $2^{-\Delta\Delta Cq}$ method (28).

Statistical analysis. All data are presented as the mean ± standard deviation. Comparisons among groups were evaluated by using one way analysis of variance and least significant difference t-tests. $P<0.05$ was considered to indicate a statistically significant difference. SPSS software (version, 18.0; SPSS, Inc., Chicago, IL, USA) was used for all calculations.

Results

IgA1 production in DAKIKI cells treated with TGF-1. The concentrations of IgA1 in the supernatants from cells cultured with 5 or 10 ng/ml TGF-β1 were significantly higher, compared with those in the control cells. Culture with 10 ng/ml TGF-β1 had the most marked effect on the production of IgA1, similar to the effect observed with IL-4. By contrast, IgA1 concentrations decreased following treatment with 15 or 30 ng/ml of TGF-β1 (Fig. 1A). These results indicated that low doses of TGF-β1 stimulated the production of IgA1, whereas high doses suppressed the production of IgA1. It was also observed that the concentration of IgA1 increased following prolonged stimulation for 7 days (Fig. 1B).

Effects of TGF-β1 stimulation on the glycosylation and sialylation of IgA1. The VVL reactivity of IgA1 in each sample was calculated as the optical density (OD) U/μg of IgA1, which indicated the degree of glycosylation. The PNA reactivity of IgA1 was also calculated as OD U/μg of IgA1, which indicated the degree of sialylation. Unlike the dose-dependent effect observed on the secretion of IgA1, TGF-β1 had a more direct effect on the glycosylation of IgA1, wherein each dose stimulated the under-glycosylation of IgA1 (Fig. 2A). Regarding sialylation, the binding of PNA to IgA1 derived from cells stimulated by 30 ng/ml of TGF-β1 was significantly higher, compared with that for IgA1 from cells stimulated by other doses of TGF-β1 or IL-4, or from unstimulated cells (Fig. 2B). No significant differences were found between samples treated for 3 or 7 days.

Effects of TGF-β1 on the gene expression of C1GalT1. The mRNA expression levels of C1GalT1 in cells incubated with TGF-β1 or IL-4 were significantly lower, compared with those in the negative controls at 12-72 h, indicating that TGF-β1 downregulated the mRNA expression of C1GalT1 at 12, 24 and 72 h in a time-dependent manner (Fig. 3). No significant difference was observed between the stimulation doses.

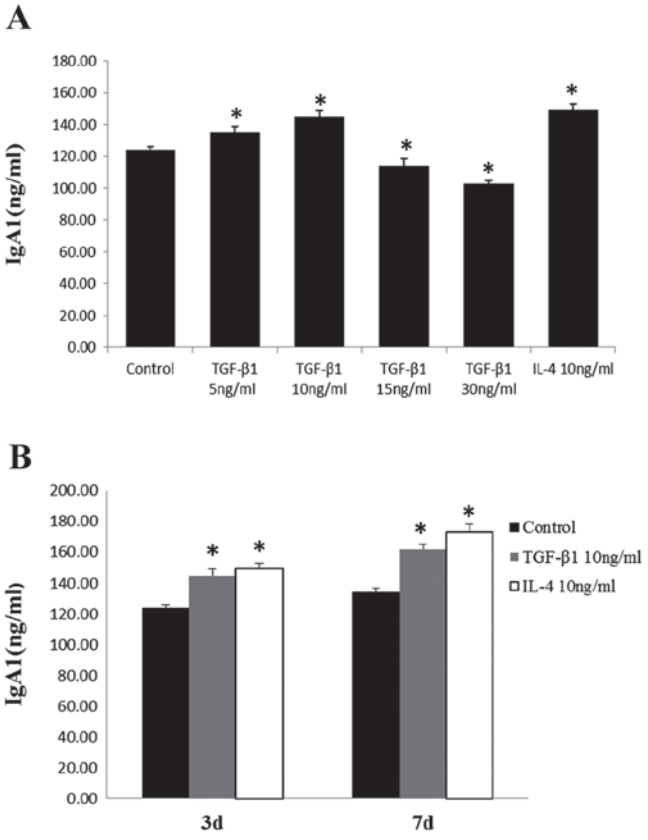

Figure 1. Effect of TGF-β1 on the production of IgA1 in DAKIKI cells. (A) Treatment with 5 and 10 ng/ml of TGF-β1 stimulated the production of IgA1 in DAKIKI cells, with 10 ng/ml TGF-β1 having the most marked effect on the production of IgA1, similar to IL-4. Treatment with 15 and 30 ng/ml TGF-β1 inhibited the production of IgA1. (B) Concentration of IgA1 following stimulation with 10 ng/ml TGF-β1 for 7 days was increased, compared with that following stimulation for 3 days. *P<0.05, compared with the control. TGF-β1, transforming growth factor-β1; IL-4, interleukin-4.

Effects of TGF-β1 on gene expression of Cosmc. The mRNA expression levels of Cosmc, which is the C1GalT1 chaperone, were also examined. Compared with the negative control, the mRNA level of Cosmc was markedly reduced by stimulation with TGF-β1 or IL-4 at each incubation time point (Fig. 4). No

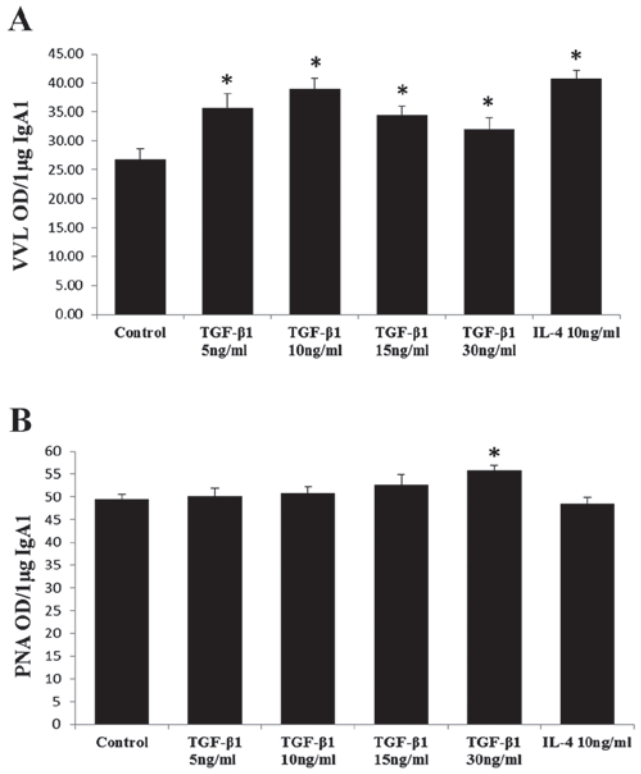

Figure 2. Effects of TGF-β1 stimulation on the glycosylation and sialylation of IgA1. (A) Glycosylation of IgA1 was assessed by the binding of VVL lectin per 1 μg IgA1. Lectin binding to GalNAc of IgA1 was significantly higher following TGF-β1 and IL-4 stimulation, compared with in the control. (B) Sialylation of IgA1 was assessed by the binding of PNA lectin per 1 μg IgA1. The binding of PNA to IgA1 in cells stimulated by 30 ng/ml of TGF-β1 was significantly higher, compared with that from control cells. *P<0.05, compared with the control. TGF-β1, transforming growth factor-β1; IL-4, interleukin-4; VVL, *Vicia villosa* lectin; PNA, peanut agglutinin lectin; GalNAc, galactose-deficient N-acetylgalactosamine; OD, optical density.

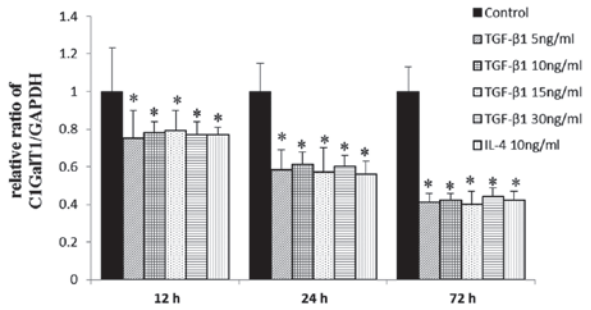

Figure 3. Effects of TGF-β1 on the mRNA expression of C1GalT1. Cells were stimulated with 5-30 ng/ml TGF-β1, or with 10 ng/ml IL-4 as a positive control. TGF-β1 downregulated the mRNA expression of C1GalT1 in DAKIKI cells in a time-dependent manner. *P<0.05, compared with the control. TGF-β1, transforming growth factor-β1; IL-4, interleukin-4; C1GalT1, core1 β1, 3-galactosyltransferase.

significant difference was found between incubation durations or incubation concentrations.

Discussion

TGF-β1 is a multifunctional cytokine, which affects a variety of biological processes, including extracellular matrix accumulation, embryonic development, tumorigenesis, inflammation, wound healing, differentiation and immunoregulation (29). Extensive clinical and experimental evidence shows that the expression of TGF-β1 is increased in human patients with IgAN, compared with normal control groups (30-33). Although these studies demonstrated that TGF-β1 mediates the progression of IgAN by inducing extracellular matrix accumulation, the effect of TGF-β1 on IgA in the context of IgAN has not been investigated.

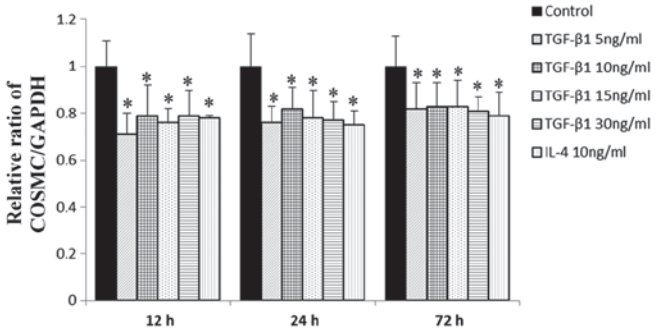

Figure 4. Effects of TGF-β1 on the mRNA expression of Cosmc. Compared with the negative control, TGF-β1 markedly downregulated the mRNA expression of Cosmc. No significant difference was found between incubation durations or concentrations. *P<0.05, compared with the control. TGF-β1, transforming growth factor-β1; IL-4, interleukin-4.

TGF-β1 is a known physiological mediator of IgA isotype switching (34,35). In the present study, it was found that low concentrations of TGF-β1 (5 and 10 ng/ml) promoted the production of IgA1 in the DAKIKI human IgA1-producing B cell line. By contrast, high concentrations of TGF-β1 (15 and 30 ng/ml) inhibited the production of IgA1. TGF-β1 inhibits the proliferation, differentiation and activity of various immune cells, including B cells (36). The B cell surface presents receptors for TGF-β1, the stimulation of which leads to the inhibition of B cell proliferation (37). The degree of inhibition of cell proliferation by TGF-β1 depends on the cell type, cytokine concentration and interactions with biologically active substances (38). In the present study, TGF-β1 had an effect on the production of IgA1 in IgA^+ B cells, which may be due, in part, to an increase in cell numbers following proliferation. Thus, the inhibited production of IgA1 by high doses of TGF-β1 may have been caused by the suppression of B cell proliferation.

It has been suggested that aberrant glycosylation of IgA1, for example galactose deficiency in the hinge-region of O-linked glycans, is directly involved in the pathogenesis of IgAN (39-41). Lectin binding assays have been extensively used to examine the glycosylation patterns of circulating glycoproteins with O-glycans. In the present study, the degree of glycoslylation and sialylation were assessed by measuring the binding activities of VVL and PNA. Although different concentrations of TGF-β1 induced different levels of IgA1, they had a consistent effect on the glycosylation of IgA1. The results indicated that TGF-β1 promoted the under-glycosylation of IgA1, which may not be accounted for by the overproduction of IgA1. The effect of TGF-β1 on IgA1 sialylation was not confirmed in the present study.

C1GalT1 and its chaperone, Cosmc, are key in protein glycosylation (42). Abnormal expression of C1GalT1 and

Cosmc can result in aberrant protein glycosylation. Functional abnormality of C1GALT1, which is responsible for the O-glycosylation of IgA1, has been suggested as a mechanism for the altered O-glycosylation observed in IgAN (43). Cosmc is essential for the folding and stability of C1GalT1 (11). In PBMCs, tonsil tissue and tonsillar B cells of patients with IgAN, the expression levels of C1GalT1 and Cosmc are significantly downregulated (44-46). In addition, the expression level of C1GalT1 in the tonsillar B cells of patients with IgAN is correlated with the estimated glomerular filtration rate, proteinuria and the histological injury score (46). Thus, aberrant IgA1 O-glycosylation in patients with IgAN may be a consequence of reduced expression levels of C1GalT1 and Cosmc in B lymphocytes (26). The results of the present study demonstrated that the mRNA expression levels of C1GalT1 and Cosmc in DAKIKI cells were reduced by TGF-β1 treatment, which may have contributed to the under-glycosylation of IgA1.

In conclusion, the results of the present study showed that certain concentrations of TGF-β1 stimulated the production of IgA1 in DAKIKI cells. In addition, TGF-β1 promoted the under-glycosylation of IgA1 through the downregulation of C1GalT1 and Cosmc. Further investigation is required to clarify the mechanisms by which TGF-β1 downregulates the mRNA levels of C1GalT1 and Cosmc, and whether the same results are observed in other B cell lines. The findings of the present study indicated a novel role for TGF-β1 in IgAN, which may form the basis of IgAN therapies.

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