Simultaneous core 2 β1→6*N*-acetylglucosaminyltransferase up-regulation and sialyl-Le^X expression during activation of human tonsillar B lymphocytes

Mitsuru Nakamura[a,*], Takashi Ishida[b,1], Jiro Kikuchi[a,c], Yusuke Furukawa[a], Michio Matsuda[a]

[a] *Division of Molecular Hemopoiesis, Center for Molecular Medicine, Jichi Medical School, Minamikawachi, Tochigi 329-0498, Japan*
[b] *Department of Oto-naso-laryngology, Jichi Medical School, Minamikawachi, Tochigi 329-0498, Japan*
[c] *Katsuta Research Laboratory, Hitachi Koki Co., Ltd., Ibaraki 312-8502, Japan*

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Abstract We have investigated the regulation mechanism of the surface sialyl-Le^X (sLe^X) expression level in tonsillar B cells during activation. sLe^X antigen became strongly positive after activation, while resting B cells were weakly positive. sLe^X structures were mainly located on *O*-linked oligosaccharide chains of glycoprotein. Transcripts of FucT-VII and core 2 GlcNAc transferase (C2GnT) were up-regulated after activation, while those of ST3GalIV and β1→4GalT-I were expressed constitutively. However, the up-regulation of C2GnT was more dramatic than that of FucT-VII. These results suggest that sLe^X expression level is regulated by C2GnT during tonsillar B cell activation.

Key words: Sialyl-Le^X; B cell activation; Core 2 GlcNAc transferase; Tonsillar B cell

1. Introduction

Cell surface glycoproteins and glycosphingolipids play important roles on presenting functional oligosaccharide structures mostly as sugar chain termini. Hematopoietic cells express such terminal oligosaccharide structures as differentiation markers in a lineage-specific and stage-specific manner [4]. There are key glycosyltransferases for the control of lineage-specific sugar chain expression during differentiation. In some human and murine leukemia cell lines, the most upstream glycosyltransferases critically determine the level of terminal carbohydrate expression by modulating total metabolic flow of glycosphingolipid biosynthesis at upstream branching steps [5–8].

Sialyl-Le^X (sLe^X; CD15s) antigen determinant is biosynthesized by the action of sequential glycosyltransferases at the terminus of the type I poly-*N*-acetyllactosamine backbone of the oligosaccharide chain attached to glycoproteins or glycosphingolipids. Recently, we have reported that cell surface CD15s is expressed mostly on the *O*-linked oligosaccharide of glycoproteins and its expression level is regulated by a single glycosyltransferase, core 2 β1→6-*N*-acetylglucosaminyltransferase (C2GnT) [9], during differentiation of human pre-B lymphocytic leukemia cell lines [10,11]. Namely, the terminal α1→3-fucosyltransferase, FucT-VII [12], and α2→3-sialyltransferase, ST3GalIV [13], were constitutively expressed and did not show significant changes in their expression levels during pre-B cell differentiation. Instead, C2GnT expression was significantly down-regulated and the decrease was in good correlation with down-regulation of the surface sLe^X level. However, it was uncertain whether C2GnT plays such a regulatory role on sLe^X expression not only in pre-B cell differentiation but also in B cell activation. In the present study, we have investigated glycosyltransferase expression during human tonsillar B cell activation and obtained data suggesting that the cell surface CD15s level is regulated by C2GnT during tonsillar B cell activation as well as during pre-B cell differentiation.

2. Materials and methods

2.1. Cells

Human tonsils were surgically obtained in Jichi Medical School Hospital and used for research purposes according to the informed consent guideline of the hospital. Mononuclear cells were isolated by Ficoll–Conray gradient sedimentation, and tonsillar B cells were purified using Dyna-beads M450 Pan-B CD19 (Veritas, Oslo, Norway) after activation with 1% (v/v) pokeweed mitogen (PWM; Life Technology, Rockville, MD, USA). For inhibition of sugar chain biosynthesis, tonsillar mononuclear cells were cultured with inhibitors for 4 days in the presence of PWM as described [10,11]; 10 μg/ml swainsonine (Genzyme, Cambridge, MA, USA), 4 mM benzyl-α-GalNAc (Bz-α-GalNAc; Sigma, St. Louis, MO, USA), or 20 μM D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP; Seikagaku, Tokyo, Japan). Human pre-B NALL1 and Nalm1 cell lines (strongly positive for sLe^X), and the mature lymphoblastoid cell line KJM-LCL and Burkitt's lymphoma cell line Raji (only weakly positive for sLe^X) were obtained and cultured as described [10].

*Corresponding author. Fax: (81)-285-44 7501.
E-mail: owlmnaka@jichi.ac.jp

[1] Present address: Department of Oto-naso-laryngology, Iwate Medical University, School of Medicine, Uchimaru 19-1, Morioka, Iwate 020-8505, Japan.

Abbreviations: Fucosyltransferase, α2→3-sialyltransferase and β1→4-galactosyltransferases are designated according to the recommendations [1–3]; sLe^X, sialylated Lewis antigen with Galβ1→4GlcNAc backbone; C2GnT, core 2 β1→6GlcNAc transferase or UDP-GlcNAc:Galβ1→3GalNAc (GlcNAc to GalNAc) β1→6-*N*-acetylglucosaminyltransferase; PWM, pokeweed mitogen; Bz-α-GalNAc, benzyl-α-GalNAc; PDMP, D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; dThd, thymidine

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2.2. Flow cytometry analyses

Immunofluorescence analyses of cell surface differentiation antigen expression were carried out by FACScan (Becton Dickinson) as described previously [5]. The monoclonal antibodies against differentiation antigens were KM93 (CD15s; Seikagaku, Tokyo, Japan), J4.119 (CD19; Immunotech, Marseilles, France), OKBcALLa (CD10; Ortho Diagnostics Systems, Tokyo, Japan), and WEHI-B2 (CD21; Japan Turner, Suita, Japan). The second antibody was fluorescein isothiocyanate-conjugated goat $F(ab')_2$ anti-mouse IgG plus IgM (Tago, Inc., Burlingame, CA, USA). Mouse anti-IgG monoclonal antibody (IgM) was obtained from Sigma (St. Louis, MO, USA) and used as a control first antibody. R-phycoerythrin-conjugated SJ25-C1 (CD19; Sigma, St. Louis, MO, USA) monoclonal antibody was used for counter staining in two-color analysis.

2.3. [^{3}H]Thymidine (dThd) incorporation

Activation of B cells was characterized by [^{3}H]dThd uptake as described [14] with modification. Mononuclear cells were incubated with [^{3}H]dThd in the presence or absence of PWM. After 4 days incubation, CD19 positive cells were purified and [^{3}H]dThd incorporation into 5×10^5 cells was measured by liquid scintillation counting.

2.4. Semi-quantitative reverse-transcribed PCR analysis

Semi-quantitative reverse-transcribed PCR analyses of glycosyltransferases, FucT-VII, ST3GalIV, β1→4GalT-I [15], and C2GnT were conducted using the respective primer pairs as described [10,11].

2.5. Glycosyltransferase activity and protein assays

The total membranous fractions were prepared, aliquoted, and stored at −80°C until use for glycosyltransferase assays as described previously [6]. α1→3-Fucosyltransferase and C2GnT activities were determined using acceptor substrates, NeuAcα2→3Galβ1→4Glc-NAcβ1→3Galβ1→4Glc-pyridylamine and Galβ1→3GalNAcα1→*p*-nitrophenol, respectively, as described [10,11]. Protein was determined by an amido-Schwarz dye binding method [16] with bovine serum albumin as a standard.

3. Results

3.1. Expression of CD15s on human tonsillar B cells

Tonsillar B cells surgically obtained from volunteers were $CD19^+CD10^-CD21^+$. In the purified B cell fractions, there was little contamination of $CD10^+$ pre-B cells and $CD3^+$ T cells. Donors exhibited various degrees, from very weak to very strong, of positivity for CD15s in the B cell fractions even before activation by PWM. We considered that this may be caused by some acute or chronic infection or non-infectious status; tonsillar B cells of such donors may be activated to various grades by subclinical infection or by other stimuli before surgery. Consequently, we chose several cases whose B cell fractions were only weakly positive for CD15s. A typical example is presented in Table 1 and Fig. 1. When the tonsillar B cells were activated by PWM in vitro, increased [^{3}H]dThd uptake was observed (Table 1). As shown in Fig. 1, isolated $CD19^+$ cells were significantly converted from weakly positive for anti-CD15s KM93 monoclonal antibody to strongly positive after PWM stimulation. By contrast, tonsillar B cells from some patients with infectious and non-malignant disease were strongly positive with KM93 monoclonal antibody (data not shown). This positivity may be due to B cell activation in tonsils of the patients.

To investigate the location of the antigenic determinants, tonsillar B lymphocytes from healthy donors were activated in vitro by PWM in the presence of Bz-α-GalNAc, swainsonine, or PDMP, and analyzed by flow cytometry (Table 1). Bz-α-GalNAc treatment significantly reduced CD15s expression, while the expression was not influenced by swainsonine or PDMP. These results strongly suggest that tonsillar B cells express sLe^X antigenic determinant(s) not on glycosphingolipids or *N*-glycans but on *O*-linked oligosaccharide chains of glycoproteins.

3.2. Expression of glycosyltransferases involved in sLe^X biosynthesis

To investigate the enzymatic background of sLe^X synthesis during tonsillar B cell activation, expression of glycosyltransferase gene transcripts involved in sLe^X biosynthesis was analyzed by semi-quantitative reverse-transcribed PCR analyses. FucT-VII, ST3GalIV, β1→4GalT-I, and C2GnT transcripts were examined using total RNA from activated tonsillar B lymphocytes, as shown in Fig. 2 (two right-hand lanes). Standard GAPDH was constitutively amplified and its radioactivity levels were used for normalization of glycosyltransferase expression. The resting tonsillar B lymphocytes did not express C2GnT transcript, while FucT-VII, ST3GalIV, and β1→4GalT-I transcripts were strongly detected. By contrast, PCR product of C2GnT was significantly amplified in PWM-stimulated tonsillar B lymphocytes. Although the FucT-VII PCR product expression level in the activated tonsillar B cells was enhanced compared with that in the resting cells, the enhancement was not as significant as in C2GnT. However, the expression level of ST3GalIV and β1→4GalT-I transcripts was constitutive throughout the activation. When we compare B cell activation with pre-B cell differentiation, the strong and constitutive expression of ST3GalIV and β1→4GalT-I during B cell activation was more remarkable than that in pre-B cell differentiation (Fig. 2, four left-hand lanes).

Subsequently, glycosyltransferase activities of α1→3-fucosyltransferase and C2GnT were examined using the resting

Table 1
Expression of differentiation markers and [^{3}H]dThd incorporation in tonsillar B cells during activation

PWM treatment	CD10	CD19	CD21	CD15s				[^{3}H]dThd incorporation[b]
				no inhibitor[a]	*N*-glycan[a]	*O*-glycan[a]	glycolipid[a]	cpm/5×10^5 cells
Tonsillar B (−)	−	++++	++	+	N.D.[c]	N.D.[c]	N.D.[c]	351 ± 57.8*
Tonsillar B (+)	−	++++	++	+++	+++	+	+++	82 263 ± 4307*

Tonsillar mononuclear cells were cultured in the presence or absence of PWM for 4 days and CD19 positive cells were purified by Dyna-beads M450 Pan-B and then analyzed by flow cytometry. The expression was analyzed by flow cytometry and shown in a semi-quantitative manner: ++++, more than 80% positive cells; +++, 40–80% positive; ++, 20–40% positive; +, 5–20% positive; −, less than 1% positive cells.

[a]Tonsillar mononuclear cells were cultured for 4 days with or without treatment by sugar chain processing inhibitors, swainsonine (*N*-glycan), Bz-α-GalNAc (*O*-glycan), or PDMP (glycolipid) in the presence of PWM. CD15s expression was analyzed by flow cytometry.

[b]Tonsillar mononuclear cells were labeled with [^{3}H]dThd and cultured in the presence or absence of PWM. After 4 days culture, CD19 positive cells were purified using Dyna-beads M-450 Pan-B CD19 and then radioactivity was counted with a liquid scintillation counter. The values are expressed as means of triplicate assays ± S.D. Statistical analyses were conducted with Student's *t*-test; $*P<0.01$.

[c]N.D.; not done.

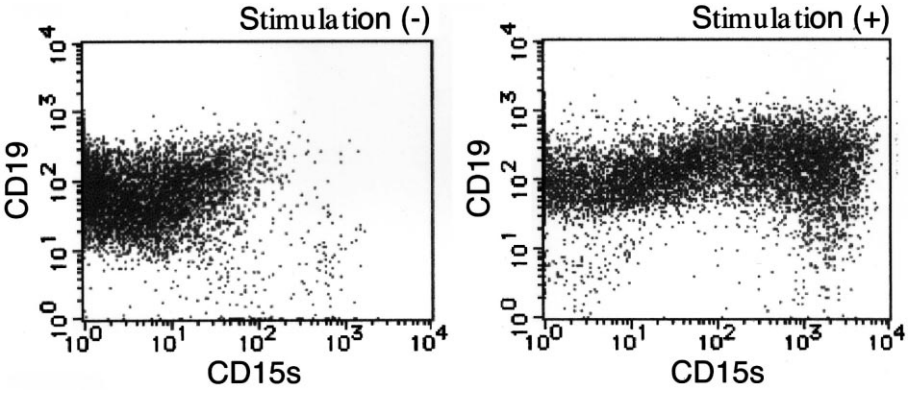

Fig. 1. CD15s expression in activated human tonsillar B cells from a healthy donor. Tonsillar mononuclear cells were cultured in the presence or absence of PWM for 4 days. $CD19^+$ cells were then purified and analyzed by two-color immunofluorescence flow cytometry. Left panel: no stimulation; right panel: stimulated by PWM.

and stimulated tonsillar B lymphocytes (Fig. 3). α1→3-Fucosyltransferase activities in the PWM-activated tonsillar B cells were about 60% higher than those in the resting cells. On the other hand, C2GnT activities in the stimulated B lymphocytes from tonsils were about 10 times higher than those in the non-activated cells.

4. Discussion

According to the present results, expression level of surface sLe^X in mature tonsillar B cells is regulated by C2GnT during cell activation as well as during pre-B cell differentiation. For differentiation of pre-B cells, we confirmed the role of C2GnT not only by comparing a set of pre-B lymphoid cell lines with that of mature B cell lines [10] but also by gene transfection and overexpression using a pre-B lymphoid cell line that is capable of differentiating into a more mature stage [11]. For mature B cell activation, however, we used tonsillar B cells in the present study and could not prove the C2GnT role by gene transfection and overexpression. If we use any cell line to be activated in vitro, we would be able to demonstrate the key involvement of C2GnT in a deeper level. However, this remains to be elucidated.

During T cell activation, it was reported that FucT-VII is the key regulatory glycosyltransferase for determination of sLe^X expression [17] and that both FucT-VII and C2GnT are up-regulated [18]. That is, in addition to FucT-VII, an increase in C2GnT message and activity is required for surface sLe^X expression in T cell activation. Regarding B cell activation, both FucT-VII and C2GnT were up-regulated (Figs. 2 and 3) as well as in T cells. However, FucT-VII up-regulation in B cells did not seem to be as significant as that in T cells [18]. Namely, while the FucT-VII message and enzyme activity level is high enough before activation, B cell surface sLe^X expression seems to be weak due to little C2GnT activity. After up-regulation of C2GnT, sLe^X is significantly expressed on the activated B cell surface. It may be true that the difference between T and B cells is derived from that of materials: peripheral blood T cells and tonsillar B cells, respectively. Because the percentage of B cell fraction is too low, we did not use peripheral blood in the present study. However, to compare B cells with T cells using the same peripheral blood would be of interest to elucidate and compare sLe^X expression mechanisms during activation of T and B lymphocytes.

Gene families for all glycosyltransferase that form the same sugar linkage are reported. Among them, the C2GnT and β1→4-galactosyltransferase gene families have recently been demonstrated [3,19] and are closely related to the present work. For C2GnT, Yeh et al. described mucin-type C2GnT [19] in addition to conventional leukocyte-type C2GnT [9]. Regarding the β1→4-galactosyltransferase gene family, Ujita et al. [20] reported that poly-*N*-acetyllactosamine in core 2 branched *O*-glycans is synthesized by β1→3-*N*-acetylglucosaminyltransferase [21] in combination not with β1→4GalT-I [15] but with β1→4GalT-IV [3]. On the other hand, Kotani et al. revealed that β1→4GalT-I is predominantly responsible for β1→4-galactosylation of core 2 *O*-glycan branches by analyzing erythrocytes from β1→4GalT-I knock-out mice [22]. According to the latter results, we analyzed only the β1→4GalT-I transcript level in the present report. However,

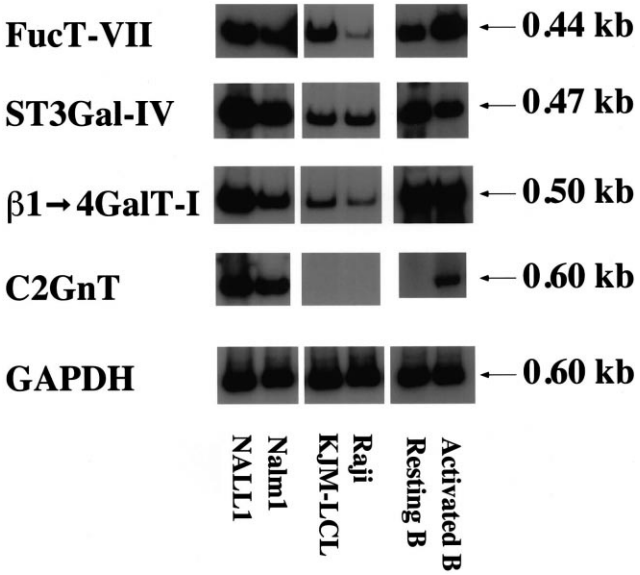

Fig. 2. Semi-quantitative reverse-transcribed PCR analyses of glycosyltransferases involved in sLe^X synthesis in tonsil B cells. For comparison, data using pre-B and mature B lymphoid cell lines are presented in the left four lanes. RNA from the cells was reverse-transcribed and cDNA was subjected to PCR reaction. Polyacrylamide gel electrophoresis was conducted, and the products were visualized by autoradiography (FucT-VII, 0.44 kb; ST3GalIV, 0.47 kb; β1→4GalT-I, 0.5 kb; C2GnT, 0.6 kb; GAPDH, 0.6 kb).

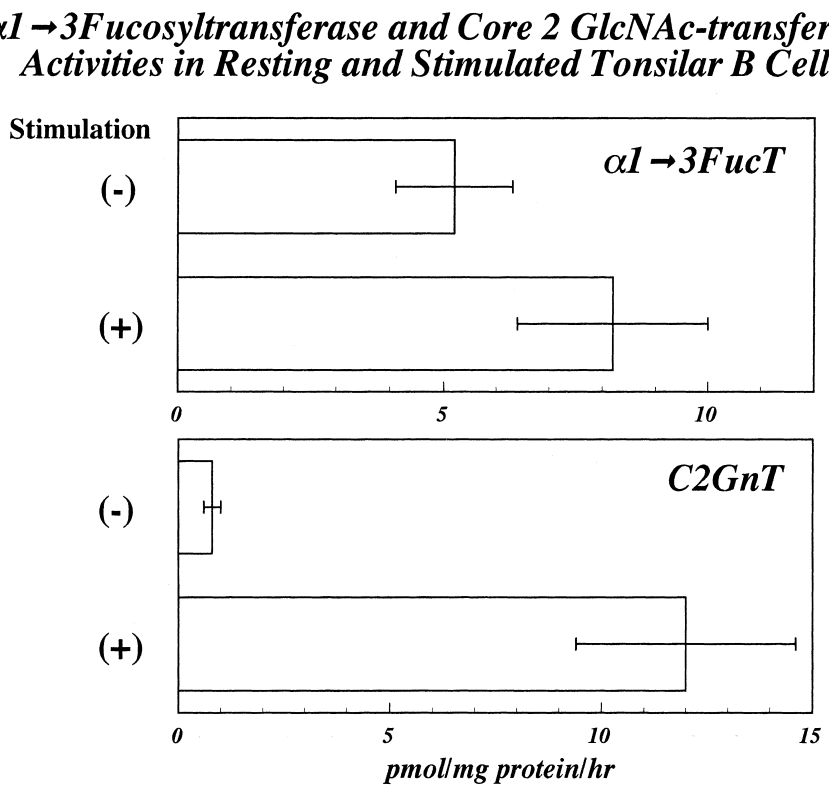

Fig. 3. α1→3-Fucosyltransferase and C2GnT activities in tonsillar B cells after stimulation by PWM. The activities were measured using respective pyridylaminated or *p*-nitrophenyl oligosaccharides as acceptors and total membranous fractions as enzyme preparations. α1→3FucT, α1→3-fucosyltransferase. The values are expressed as pmol/mg protein/h and as means of triplicate assays ± S.D.

re-evaluation by analyzing expression levels of such gene families would be required for further elucidation of sLe^X expression mechanism in lymphocytes.

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