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Functional Sialylated *O*-Glycan to Platelet Aggregation on Aggrus (T1α/Podoplanin) Molecules Expressed in Chinese Hamster Ovary Cells*

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Aggrus, also called T1α and podoplanin, is a novel platelet aggregation-inducing factor that is expressed in various carcinoma cells. Aggrus/T1α/podoplanin is known to be expressed in lung type I alveolar cells or lymphatic endothelial cells. However, its physiological role has not been clarified. To assess the attribution of glycosylation to Aggrus platelet aggregation activity, recombinant molecules were stably expressed in a series of Chinese hamster ovary (CHO) cell mutants, *N*-glycan-deficient Lec1, CMP-sialic acid transporter-deficient Lec2, and UDP-galactose transporter-deficient Lec8. A new anti-human Aggrus monoclonal antibody, YM-1, was established to detect the expression of human Aggrus on these CHO cell mutants. Aggrus on Lec1 cells induced platelet aggregation, but those on Lec2 and Lec8 cells did not. Further, the glycans on Aggrus were analyzed by lectin blotting. Aggrus expressed in CHO and Lec1 cells showed *Wheat-germ* agglutinin, Jacalin, and *Vicia villosa* lectin bindings. Lectin blotting results indicated that sialylated core 1 structures, sialic acid plus Galβ1,3GalNAc-Ser/Thr, were critical for the platelet aggregation activity. This oligosaccharide structure is known as tumor-associated antigen, which is potentially related to the metastasis process of cancer cells.

The concept has been accepted that tumor metastasis is associated with platelet-aggregation activity possessed by human and other mammalian cancer cells (1, 2). A previous study has clarified that membranous 44- and 36-kDa sialoglycoproteins in the cancer cells of mice and humans, respectively, aggregated platelets with no relation to plasma components (3–5). Ectopic expression of these molecules was observed in a variety of cancer cells including colorectal and testicular tumors, in which up-regulated expression was evident (3, 6). The molecules, designated as Aggrus, belong to a type-I transmembrane sialomucin-like glycoprotein that consisted of an extracellular domain with abundant Ser and Thr residues as potential *O*-glycosylation sites, a single transmembrane portion, and a short cytoplasmic tail with putative sites for protein kinase C and cAMP phosphorylation (3, 7).

Homologous molecules to Aggrus have been identified independently in several mammalians; T1α (7, 8), podoplanin (9), PA2.26 (10), gp40 (11), RANDAM-2 (12) are isolated from rat alveolar type-I cells in lung, rat glomerular epithelial cell (podocyte), mouse keratinocytes, Madin-Darby canine kidney cells (type I), and glutamatergic neuronal cells in mouse cerebrum, respectively. Podoplanin (gp36) is utilized as a specific marker for lymphatic endothelium in histopathology (13, 14). Ramirez and co-workers (15, 16) showed that *T1α* null mice, generated by a targeted dysfunction of the gene, died at birth from lethal respiratory failure accompanied by immature lymphatic vessel formation (15, 16). T1α/podoplanin conceivably plays an important role in regulating peripheral lung cell proliferation and lymphatic vascular development.

In the experiment with respect to the 8F11 antibody that neutralized the platelet aggregating activity, it was evident that the segment of ED*XX*VTPG in the extracellular domain, designated as platelet aggregation-stimulating domain, was critical for the activity of Aggrus (3). In particular, this motif, which is highly conserved among species, is tandemly triplicated in gp40 of dog. The occurrence of *O*-glycosylation to Thr residues was proved by Edman degenerative microsequencing (17). Furthermore, in the study of targeted mutagenesis to mouse and human Aggrus molecules, we obtained evidence that glycans on the platelet aggregation-stimulating domain play an important role for platelet aggregation (3, 5).

The present study examined the association of glycosylation with the platelet aggregating activity; recombinant Aggrus molecules were expressed in a set of three defective cell lines from Pro-5 Chinese hamster ovary (CHO)[1] cells. Those mutant cells have defects in distinct steps of glycosylation. Lec1 CHO cells lack the glycosyltransferase termed GlcNAc-TI, which produces incomplete intermediates of *N*-linked carbohydrates (18–20). Lec2 cells are CMP-sialic acid transporter-deficient. In addition, both glycoproteins and glycolipids lack 90% of sialic acids (SA). Lec8 cells exhibit a remarkable reduction of the ability to transport UDP-galactose (Gal) into the Golgi compartment (22, 23). Platelet aggregation-inducing activity was examined in those stably transfected cells. Moreover, the structure of active glycans was examined by the lectin-blot analysis. In addition, a monoclonal antibody to human Aggrus (hAggrus) was obtained by immunizing rat with the synthetic hAggrus peptide.

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[1] The abbreviations used are: CHO, Chinese hamster ovary; SA, sialic acid; Gal, galactose; WGA, *Wheat-germ* agglutinin; s-WGA, succinylated WGA; VVA, *Vicia villosa* lectin; mAb, monoclonal antibody; h, human; m, mouse.

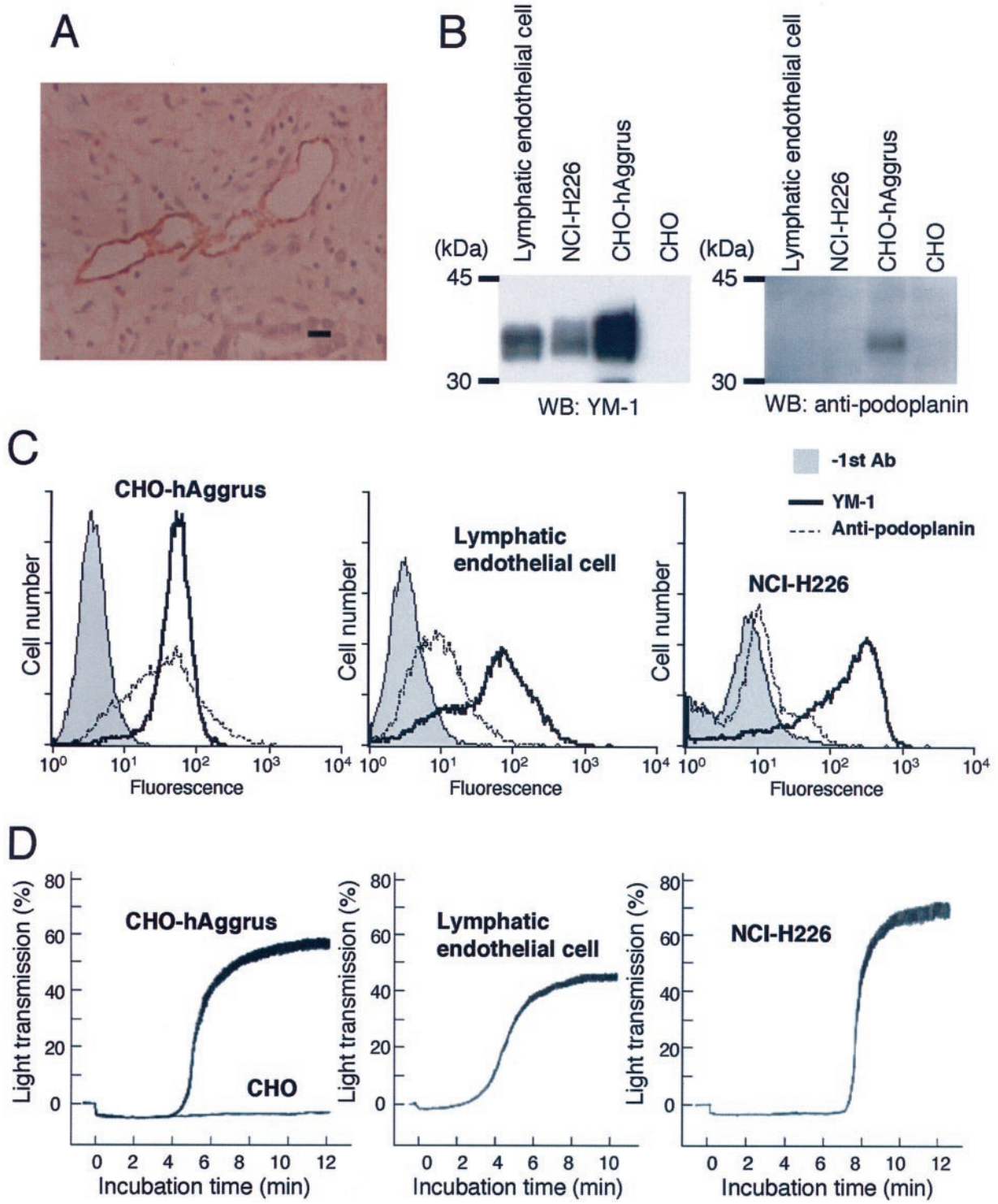

FIG. 1. **Characterization of YM-1 and platelet aggregation-inducing activity of cell lines.** *A*, immunohistochemical staining with YM-1 on human specimens derived from prostate. The *scale bar* represents 10 μm. *B*, the cell lysate of lymphatic endothelial cell, NCI-H226, CHO-hAggrus, and CHO were electrophoresed and immunoblotted with YM-1 and anti-podoplanin mAb. *C*, flow cytometric analysis of YM-1 and anti-podoplanin reactivity to CHO-hAggrus, lymphatic endothelial cell, and NCI-H226. *D*, CHO-hAggrus, lymphatic endothelial cell, and NCI-H226 were incubated with mouse platelet-rich plasma, and platelet aggregation activity was confirmed. Parental CHO served a control. *WB*, Western blot.

EXPERIMENTAL PROCEDURES

Materials—Lymphatic endothelial cell was purchased from AngioBio (Del Mar, CA). Lung carcinoma cells, NCI-H226, and NCI-H520 were obtained from the American Type Culture Collection (ATCC). The anti-mouse Aggrus rat monoclonal antibody (mAb), 8F11, was prepared previously (24). Mouse anti-human podoplanin (gp36) mAb was obtained from Research Diagnostics Inc. (Flanders, NJ). Anti-FLAG antibody (M2) was purchased from Sigma-Aldrich. Horseradish peroxidase-conjugated secondary antibodies were obtained from DakoCytomation Co., Ltd. (Glostrup, Denmark). Fluorescein-isothiocyanate-conjugated anti-rat IgG was obtained from Cappel (Cochranville, PA). Fluorescein-isothiocyanate-conjugated anti-mouse IgG was from MC Biomedicals (Irvine, CA). Horseradish peroxidase-conjugated streptavidin was obtained from Amersham Biosciences. The following biotinylated lectins were purchased from Vector Laboratories Inc. (Peterborough, UK), concanavalin A, *Dolichos biflorus* agglutinin, peanuts agglutinin, *Ricinus*

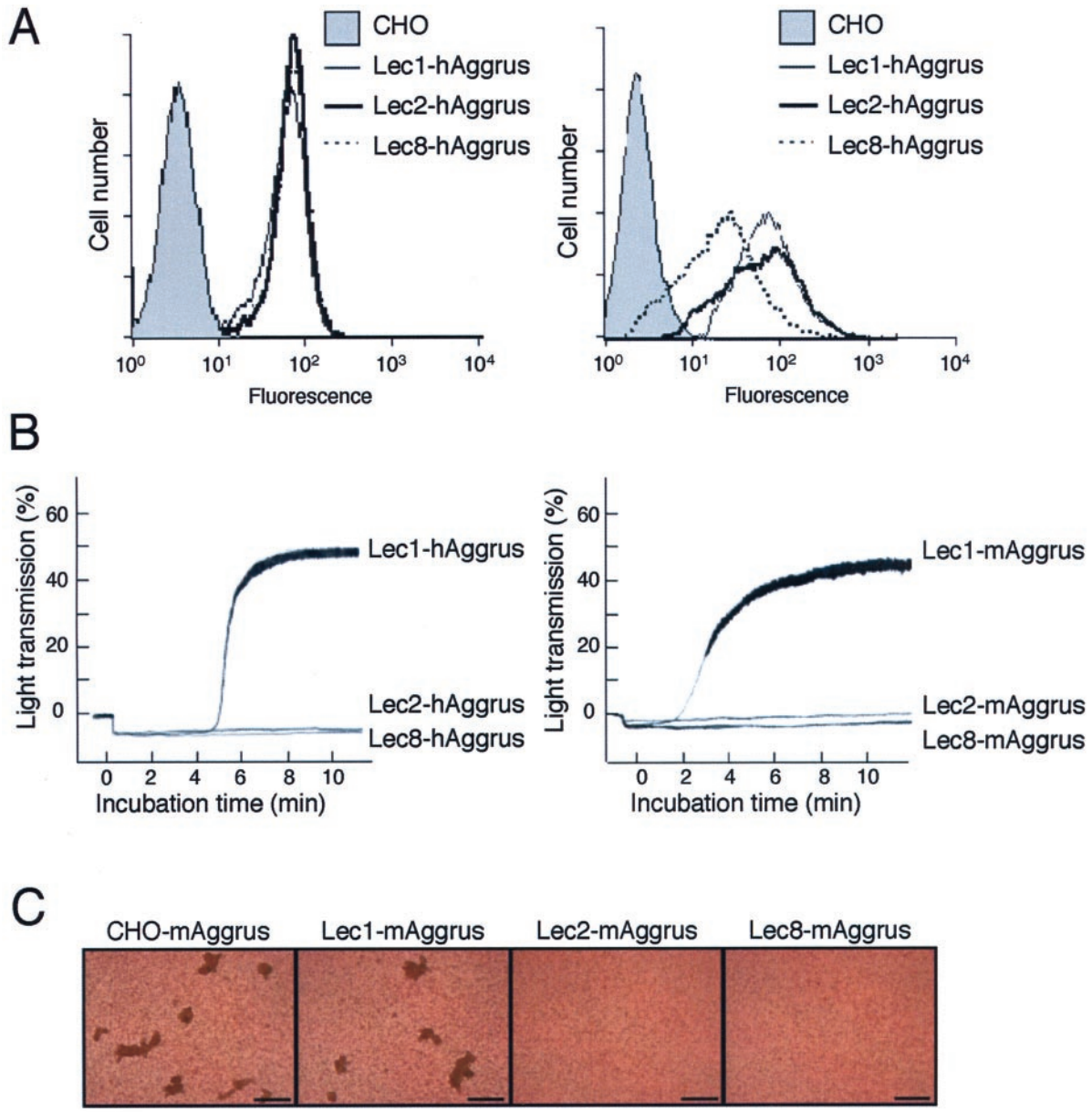

FIG. 2. **Flow cytometry of transfected Lec1, Lec2, and Lec8 cells and platelet aggregation-inducing activity of transfected cells.** *A*, the Lec1 (*thin line*), Lec2 (*thick line*), and Lec8 (*dotted line*) cells were transfected with pcDNA-hAGR and pcDNA-mAGR. Cells were incubated with YM-1 and 8F11, followed by fluorescein isothiocyanate-labeled secondary antibodies. Parental CHO (*shaded*) was involved as a control. *B*, platelet-rich plasma derived from mouse blood was incubated with the transfected Lec cells that expressed hAggrus and mAggrus. *C*, microscopic features of the induced platelet aggregation are also indicated. The *scale bar* represents 100 μm.

communis agglutinin I, soybean agglutinin, *Wheat-germ* agglutinin (WGA), succinylated WGA (s-WGA), *Griffonia simplicifolia* lectin I and II, *Sophora japonica* agglutinin, *Datura stramonium* lectin, Jacalin, *Lycopersicon esculentum* lectin, *Solanum tuberosum* lectin, *Vicia villosa* lectin (VVA), *Sambucus nigra* lectin, and *Maackia amurensis* lectin I and II.

Hybridoma Production—Two Sprague-Dawley rats were immunized with the synthetic peptide, CEGGVAMPGAEDDVV, corresponding to amino acids 38–51 of hAggrus plus an N-terminal cysteine. The spleen cells were fused with mouse myeloma P3U1 cells. Then the culture supernatants of the hybridomas were screened by enzyme-linked immunosorbent assay for the binding activity to the Aggrus peptide. The established mAb was designated YM-1.

Immunohistochemistry—YM-1 was added to the deparaffinized and rehydrated specimens for 4 h at 23 °C. Biotin-conjugated secondary anti-rat IgG antibody was added, followed by incubation with peroxidase-conjugated avidin-streptavidin complex (Vectastain ABC Kit, Vector Laboratories Inc.). Color was developed with 3,3-diaminobenzidine tetrahydrochloride tablet sets (DakoCytomation Co. Ltd.) used for 10 min. The human specimens were derived from prostate cancer (25).

Production of Aggrus in CHO/Lec Cells—Lec1, Lec2, and Lec8 cells of CHO mutant cell lines were obtained from ATCC (18–23). Cells were cultured at 37 °C in 5% CO_2 with α-minimal essential medium (Sigma-Aldrich) containing 10% fetal calf serum (Moregate Biotech, Queensland, Australia), supplemented with L-glutamine (2 mM), L-proline (0.04 mg/ml), penicillin (100 units/ml), and streptomycin (100 μg/ml). Human and mouse aggrus cDNAs (AB127958, AJ297944), incorporated with FLAG tag sequence, were subcloned into a vector of pcDNA3 (Invitrogen), which were named as pcDNA-hAGR and pcDNA-mAGR, respectively. Lec1, Lec2, and Lec8 cells were transfected with pcDNA-hAGR and pcDNA-mAGR by a procedure using LipofectAMINE reagent (Invitrogen) as described previously (3). Stable production was obtained using a selective culture of the transfectants in a medium containing Geneticin (G418; Sigma-Aldrich) at a concentration of 1.0 mg/ml for a couple of weeks.

Flow Cytometry—The expression levels of hAggrus and mouse Aggrus (mAggrus) were confirmed comparatively using by flow cytometry, as described previously (26). Briefly, lymphatic endothelial cells, NCI-H226 cells, parental CHO cells, and Lec cells, collected by trypsin-EDTA treatment, were incubated with antibodies for 1 h at room temperature, YM-1 culture supernatant, and 8F11 in phosphate-buffered saline at a concentration of 1 μg/ml. Then the cells were incubated

with fluorescein isothiocyanate-conjugated antibodies for 30 min. Flow cytometry was performed using FACS Caliber (BD Biosciences).

Platelet Aggregation—Platelet aggregation was examined by the procedures of Toyoshima *et al.* (5). Briefly, mouse platelet-rich plasma was prepared from fresh heparinized blood extracted from BALB/c mice. An aliquot of 200 μl of platelet-rich plasma was incubated in a cuvette at 37 °C with continuous stirring. After 5 min, 5 μl of phosphate-buffered saline-washed cells were added. Then light transmittance was monitored at 660 nm for 10 min. This assay was performed with a NKK HEMA Tracer I (Niko Bioscientific Co., Tokyo, Japan). A photograph was taken using a phase contrast microscope (100×, microphoto Fxa; Nikon Corp.) at the same time.

Western Blot Analyses and Lectin Blotting—Cultured cell pellets were lysed with a buffer for 30 min on ice. The buffer consisted of 25 mM Tris, 50 mM sodium chloride, 0.2% SDS, 0.5% sodium deoxycholate, 2% Nonidet P-40, 50 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride, pH 7.4. The lysate supernatants were centrifuged for 15 min at 15,000 rpm to remove cellular debris and nuclei. Cell lysates containing 5 μg of total protein were prepared for Western blot analysis by boiling in SDS sample buffer (50 mM Tris, 2% SDS, 5% glycerol, 10% 2-mercaptoethanol, pH 6.8). For lectin blotting analysis, cell lysates were immunoprecipitated with anti-FLAG antibody conjugated agarose for 1 h at 4 °C. After the complexes were washed, elution was performed with a SDS sample buffer by boiling for 5 min, followed by polyacrylamide gel electrophoresis in 10–20% gradient gels. After proteins were transferred onto an immobilon polyvinylidene difluoride membrane (Millipore Corp., Billerica, MA), the strips were incubated with YM-1 antibody or biotinylated lectins (20 μg/ml) for 1 h at room temperature followed by incubation with horseradish peroxidase-conjugated anti-rat IgG or streptavidin coupled to peroxidase. The enzyme activity was developed using an enhanced chemiluminescence kit (ECL, Amersham Biosciences).

RESULTS

Establishment of Anti-human Aggrus Monoclonal Antibody—We generated a mAb to hAggrus (YM-1) as described in the previous section. The reactivity of YM-1 was confirmed by immunohistochemical analysis, Western blot analysis, and flow cytometric analysis (Fig. 1, *A–C*). YM-1 clearly recognized lymphatic vessel via immunostaining (Fig. 1*A*). Approximately 36 kDa of Aggrus expressed on human lymphatic endothelial cells and lung cancer cell line NCI-H226 were detected by YM-1 but not anti-podoplanin mAb (Fig. 1*B*). This result indicated that the YM-1 exhibit more intense immunoreactivity for Aggrus than that of the anti-podoplanin mAb. Furthermore, flow cytometric analysis showed that the YM-1 could react more strongly than anti-podoplanin (Fig. 1*C*).

Platelet Aggregation by Lymphatic Endothelial Cells and NCI-H226 Cells—As shown in Fig. 1*D*, lymphatic endothelial and NCI-H226 cells also induced platelet aggregation as hAggrus on CHO cells did. However, another lung cell carcinoma NCI-H520, which did not express hAggrus, did not induce platelet aggregation (data not shown). These results indicated that the spontaneous hAggrus also induced platelet aggregation.

Platelet Aggregation by Lec Transfectants—Recombinants were generated in the three mutant cells and parental CHO cells by transfection of pcDNA-hAGR and pcDNA-mAGR to assess the physiological importance of glycosylation on Aggrus molecules. The stable transfectants were established by the Geneticin-selective culture. Expression of recombinant molecules was comparatively confirmed by flow cytometry using the specific antibodies of YM-1 and 8F11 (Fig. 2*A*). Judging from the shift of peaks, expressional levels of Aggrus were high and equal among these cell transfectants.

When the platelet aggregation activity of the established transfectants was examined, both hAggrus and mAggrus in Lec1 cells induced complete platelet aggregation as well as recombinants in parental CHO cells (Fig. 2*B*). The aggregation was confirmed using varying cell numbers in three independent experiments (data not shown). Real features of induced platelet aggregation are also demonstrated in Fig. 2*C* as a photograph. In contrast, aggregation was absent in Aggrus molecules expressed in Lec2 and Lec8 cells.

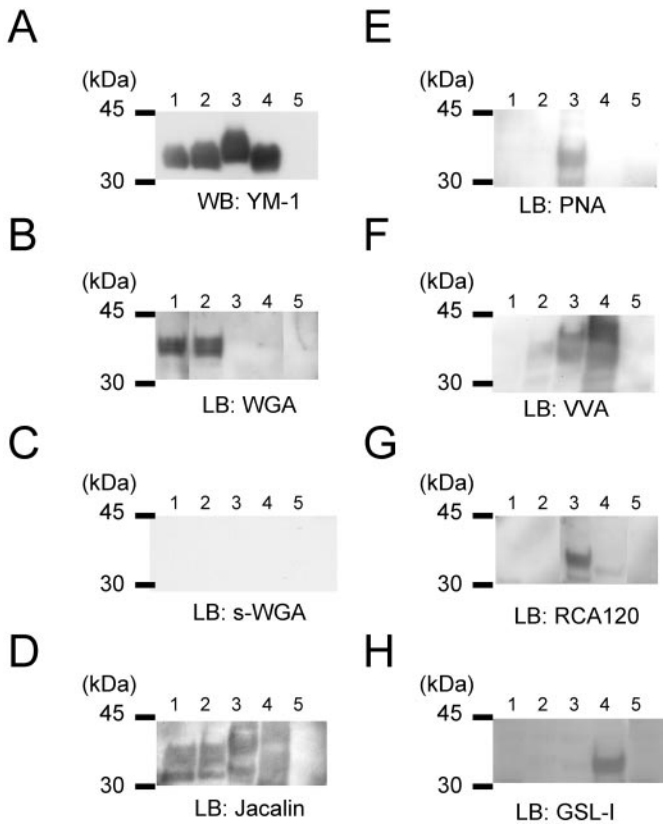

FIG. 3. **Lectin blot analysis to immunoprecipitated human Aggrus.** Immunoprecipitated hAggrus was applied to SDS-PAGE. The transferred membranes were incubated with YM-1 (*A*) and lectins including WGA (*B*), s-WGA (*C*), Jacalin (*D*), peanut agglutinin (*PNA*) (*E*), VVA (*F*), *Ricinus communis* agglutinin I (*RCA120*) (*G*) and *Griffonia simplicifolia* lectin I (*GSL-I*) (*H*). *Lane 1*, CHO-hAggrus cells; *lane 2*, lec1-hAggrus cells; *lane 3*, lec2-hAggrus cells; *lane 4*, lec8-hAggrus cells; *lane 5*, parental CHO cells as a negative control. *WB*, Western blotting; *LB*, lectin blotting.

Lec1 CHO cells are incapable of synthesizing *N*-glycan complexes (18–20). The amino acid sequence of mAggrus contained one consensus site for *N*-glycosylation but did not in hAggrus. Because Lec1 cells lacked *N*-glycosylation, platelet aggregation activity was not associated with this type of modification. Furthermore, CMP-SA transporter-deficient Lec2 cells lack 90% of common SA decoration in both glycoproteins and glycolipids (21). Maturation of sialylated structures is inhibited in Lec8 cells, because Gal is the major acceptor for SA in glycoconjugates (22, 23). Molecules that are modified by these two cell lines form insufficient SA decoration. Based on these results and interpretation, the presence of SA on *O*-glycans was conclusively critical for the induction of platelet aggregation.

Glycans on Aggrus Expressed in CHO and Lec Cells—To type the *O*-glycan structure on active hAggrus molecules in the CHO cells, lectin blotting using the 18 lectins, was further performed with immunoprecipitates of the cell lysates (Fig. 3). Table I summarizes this result. The hAggrus that was expressed in Lec1 and parental CHO cells reacted with WGA but not s-WGA. The aggregation-inducing molecules carried SA at their termini because s-WGA recognizes naked GlcNAc in contrast to the interaction of WGA to SA-bound GlcNAc. Jacalin, which recognizes core 1 structure (Galβ1,3GalNAc), reacted with hAggrus molecules expressed in parental, Lec1, and Lec2 CHO cells. This result implies that active molecules possess a sialylated core 1 structure. Furthermore, hAggrus from Lec 8

TABLE I
Summary of lectin blot analysis to immunoprecipitated hAggrus

For concanavalin A, *D. biflorns* agglutinin, *S. japonica* agglutinin, *L. esculentum* lectin, *S. tuberosum* lectin, *D. stramonium* lectin, *M. amurensis* lectins I and II, and *S. nigra* lectin, lectin did not react toward recombinant hAggrus. −, negative; +, positive; w/o, without.

Lectin	Specificity	CHO/hAggrus	Lec1/hAggrus	Lec2/hAggrus	Lec8/hAggrus
WGA	$(GlcNAc)_n$, SA	+	+	−	−
s-WGA	$(GlcNAc)_n$	−	−	−	−
Jacalin	Galβ1,3GalNAc	+	+	+	−
Peanut agglutinin	Galβ1,3GalNAc (w/o SA)	−	−	+	−
VVA	GalNAcα, β	−	+	+	+
R. communis agglutinin I	Gal, GalNAc (w/o SA)	−	−	+	−
G. simplicifolia lectin	GalNAcα, Galα	−	−	−	+
Soybean agglutinin	GalNAcα, β	−	−	−	+

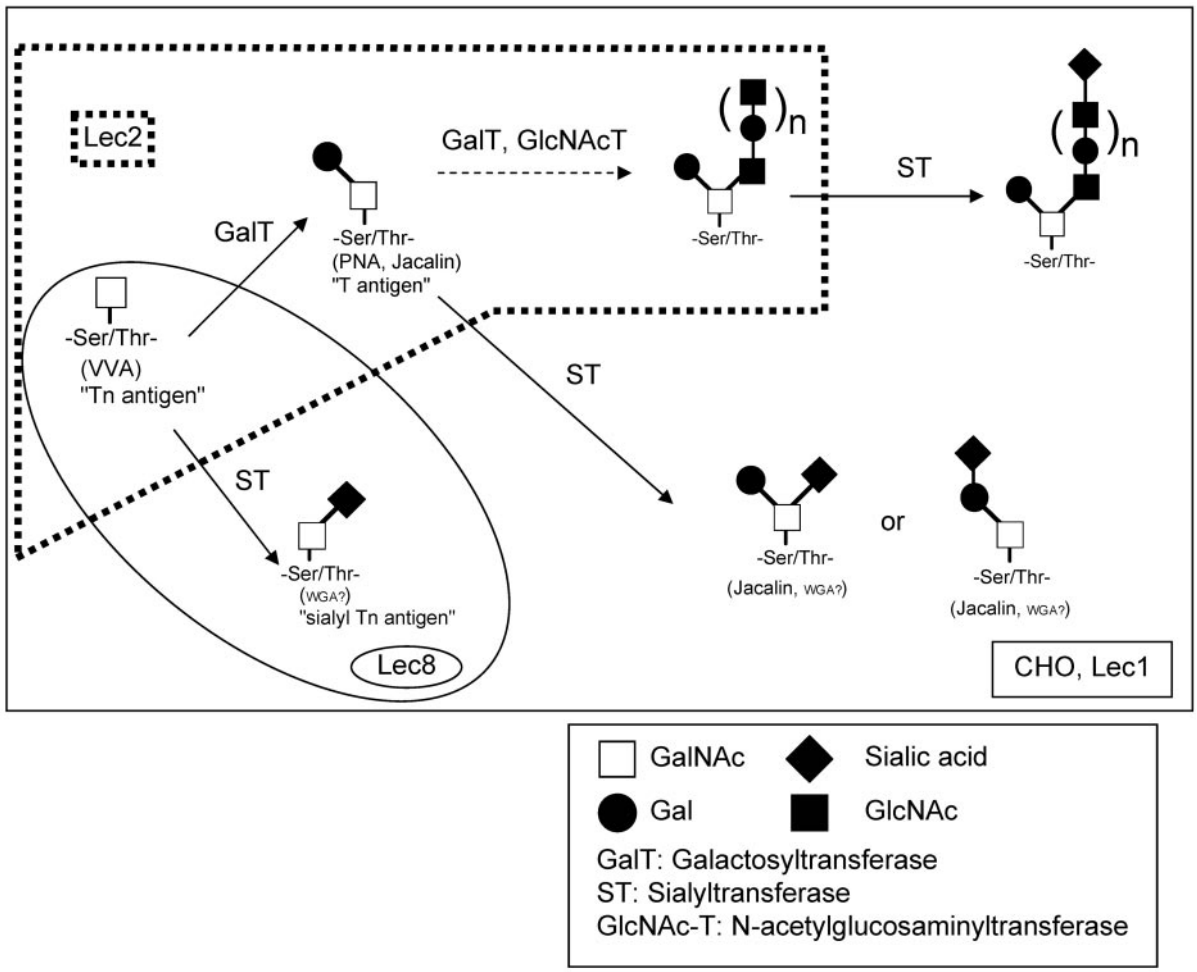

FIG. 4. **Scheme representing a possible pathway for *O*-glycans of Aggrus expressed in Lec mutant cells.** The initiating structure of *O*-glycan is the addition of the monosaccharide GalNAc to serine and threonine residues (Tn antigen). Subsequently, particular glycosyltransferases form T or sialyl Tn. Possible glycan structures formed by Lec mutant cells appear at intermediate products. Lectins that recognized the oligosaccharide were shown in *parentheses*.

cells was detected by VVA (to GalNAc-Ser/Thr, called Tn-antigen), *Griffonia simplicifolia* lectin I, and soybean agglutinin (to GalNAc residue), indicating that the major glycan on hAggrus from Lec8 was Tn-antigen. In addition, hAggrus molecules from Lec2 cells showed slower movement in electrophoresis compared with others. This lower mobility might result from the reduction of negative charges or elongation of *O*-glycan chain because of the lack of SA. Fig. 4 schematically illustrates the theoretically possible glycosylation structures of Aggrus expressed in the parental CHO and Lec mutant cells.

DISCUSSION

We generated anti-hAggrus mAb by immunizing rats with the synthetic Aggrus peptide. Recently, Aggrus/podoplanin has been utilized as a lymphatic endothelium marker in histopathology. The results of YM-1 immunostaining indicated that this antibody has superior sensitivity to lymphatic endothelium (Fig. 1*A*). Western blot and flow cytometric analyses indicated that the hAggrus protein is expressed in the lymphatic endothelial cell line and lung cancer cell line, NCI-H226 (Fig. 1, *B* and *C*). Furthermore, these results showed that the YM-1 antibody reacted more strongly than the anti-podoplanin antibody.

The present study utilized the advantage of unique characteristics of CHO mutant cell lines to show the sialylated *O*-glycan was critical for the platelet aggregation-inducing activity of Aggrus. *O*-Glycan profiles of MUC1 expressed in CHO cell lines showed an identical pattern to those produced in the breast carcinoma T47D cells (27), suggesting that recombinant

Aggrus in CHO cells, which provoked platelet aggregation, might be decorated as well as glycosylation in cancer cells. Although the physiological importance of the platelet-aggregating activity remains obscure, the fact that Aggrus is expressed in many tumor cells indicated that cancerous glycosylation of Aggrus is attributable to the tumor-induced activity of platelet aggregation during metastasis (3, 6).

Lectin blot analysis showed that active Aggrus molecules expressed in CHO cells bore a sialylated core 1 structure (SA-Galβ1,3GalNAc). This structure was basically consistent with those reported in the PA2.26 antigen (28) and gp40 (17). In addition to sialylated and non-sialylated GalNAc-Ser/Thr, called sTn and Tn antigen, respectively, sialylated and non-sialylated core 1 structures are also known as tumor-associated carbohydrate antigens (29, 30). These antigens are distributed widely in glycolipids, membranous mucins, and mucous plasma components in cancer patients. MUC1 mucin has been studied extensively as a target antigen for the immunotherapy of breast and other carcinomas, because the cancer-associated mucin is over-expressed and aberrantly glycosylated. In normal cells, *O*-glycans expressed on MUC1 typically consist of long and branched, mainly core 2 (GlcNAcβ1–6(Galβ1–3)GalNAc-Ser/Thr)-based, oligosaccharides (31, 32). In contrast, the glycans of MUC1 expressed in breast carcinomas transform to increased contents of the shorter non-branched core 1-based structure with enhanced SA addition (33–35). From this point of view, Aggrus in carcinoma cells is thought to be distinct from T1α/podoplanin in normal cells in glycosylation. However, Aggrus in lymphatic endothelial cells and Aggrus in NCI-H226 showed almost the same mobility in Western blot analysis, and both cells induced platelet aggregation (Fig. 1*B*). The different mobilities of Aggrus from different organs were indicated in previous studies with mouse Aggrus/PA2.26 (3, 28). The different mobilities may indicate different glycosylation or conformational changes that were caused by different modifications. Further studies are necessary to analyze the glycan structure on Aggrus molecules from cancer cells and normal cells, especially the glycan in the platelet aggregation-stimulating domain, to clarify whether the different glycosylation has an important correlation with the platelet aggregation activity of Aggrus or not.

Recent progress in research has demonstrated that the glycosyl modification is relevant to various physiological functions such as the intracellular signaling pathway (36). Notch functions in highly conserved intercellular signaling pathways. It plays essential roles in a wide variety of developmental events. A series of studies with deficient CHO cells, including Lec2 and Lec8, have been applied for the Fringe-dependent glycosylation of Notch (36, 37). Fringe is fucose-specific β1,3 *N*-acetylglucosaminyl transferase that adds GlcNAc to *O*-fucose on certain epidermal growth factor repeats of Notch (36, 38). The change in sugar structure affects ligand binding (38, 39).

Similarly, exploiting deficient CHO cells, we found that sialylated glycans on Aggrus promotes platelet aggregation. It is intriguing to speculate that the change in glycosylation modification affect platelet aggregation-inducing activity of Aggrus *in vivo*. Based on our findings that sialylated glycans on Aggrus induced platelet aggregation, Aggrus interacted with a ligand on the platelet surface. We have preliminarily investigated the association of Aggrus with three SA-binding Ig-like lectins (Siglec) of Siglec-3, Siglec-5, and Siglec-7, which are expressed in platelets (40). However, no positive evidence has been obtained by Western blotting and flow cytometry to date (data not shown). In the next step, it will be important to determine which acceptant component on platelets is attributable to the potent activity toward aggregation by Aggrus.

In conclusion, we generated a new anti-hAggrus antibody, YM-1. Then we showed that the decoration of SA on *O*-glycan is essential for platelet aggregation by membranous Aggrus molecules. Such data seem particularly important in light of the fact that the platelet aggregation activity is related to tumor metastasis.

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