Specificity of Human Natural Antibody to Recombinant Tissue-Type Plasminogen Activator (t-PA) Expressed in Mouse C127 Cells

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A natural antibody with binding specificity for recombinant tissue-type plasminogen activator (t-PA) expressed in mouse C127 cells was present in almost all disease-free humans and patients with thrombotic disease examined. This antibody was specific for a carbohydrate, α1–3-linked galactose residue, and was isolated by affinity chromatography using Synsorb 90 coupled with the glycosidic epitope Galα1–3Galβ1–4Glc–R as an immunoadsorbent. The evaluation of various glycoproteins for ability to bind the purified antibody in ELISA demonstrated that not only recombinant t-PA from C127 cells but also recombinant erythropoietin (EPO) and recombinant protein C produced in C127 cells have α1–3-linked galactose residues on their sugar side chains. This anti-α-galactosyl antibody also interacted with natural t-PA from human vascular trees (vascular t-PA) and placenta (placenta t-PA), but not to melanoma t-PA, recombinant t-PA, EPO or protein C expressed in Chinese hamster ovary (CHO) cells.

Keywords α-galactose; natural antibody; recombinant; tissue plasminogen activator; erythropoietin; protein C; enzyme-linked immunosorbent assay

Tissue-type plasminogen activator (t-PA) is a serine protease which converts plasminogen into plasmin and can thereby induce thrombolysis. Because t-PA specifically binds fibrin[1] and its ability to activate plasminogen is markedly increased in the presence of fibrin,[2] it is expected to offer therapeutic advantages over the conventional thrombolytic agents, streptokinase and urokinase, for the treatment of thrombo-embolic disorders.

Small quantities of natural human t-PA were isolated from the uterus,[3] vascular trees of cadavers[4] and placenta.[5] Relatively large quantities of purified material were obtained from the culture media of a human melanoma cell line.[1,6–8] Human t-PA can be produced in large amounts by using recombinant deoxyribonucleic acid (DNA) technology.[9,10] A recombinant t-PA expressed in mouse C127 cells (t-PA(C127)) is currently being developed by our institute.

In clinical studies on t-PA(C127), we found that almost all individuals tested had a natural antibody to t-PA(C127), even without any prior exposure to this protein. This natural antibody also reacted with natural t-PA, *i.e.* vascular t-PA and placental t-PA, but not recombinant t-PA from Chinese hamster ovary (CHO) cells. Several glycoproteins other than t-PA were also examined for reactivity against this natural antibody. Erythropoietin (EPO) and protein C expressed in C127 cells reacted with the natural antibody, while their counterparts from CHO cells showed no such reactivity.

We isolated and characterized this natural antibody, and examined the nature of the epitope on recombinant glycoproteins and natural t-PA recognized by it.

Experimental

Proteins and Glycoproteins Recombinant t-PA and recombinant EPO expressed in mouse C127 cells and recombinant t-PA expressed in Chinese hamster ovary (CHO) cells were obtained from Toyobo Research Institute (Shiga, Japan). Recombinant EPO expressed in CHO cells and two kinds of recombinant protein C produced in C127 and CHO cells were kindly supplied by Integrated Genetics, Inc. (Framingham, MA). In this paper, recombinant t-PA, EPO and protein C expressed in C127 and CHO cells are designated t-PA(C127), t-PA(CHO), EPO(C127), EPO(CHO), protein C(C127) and protein C(CHO), respectively. Human vascular plasminogen activator (vt-PA) was a gift from Dr. H. Saitoh (Nagoya Univ., Japan). Placental plasminogen activator (pt-PA) was extracted from human placenta[5] and purified by affinity chromatography on anti-t-PA monoclonal antibody. Melanoma t-PA (mt-PA) was purchased from American Diagnostica, Inc. (NY). Ovalbumin (OVA) and bovine thyroglobulin were obtained from Sigma Chemical Co. (St. Louis, MO), and murine laminin was from Collaborative Res. (Bedford, MA).

Carbohydrates All monosaccharides, lactose and melibiose were purchased from Nakarai Chemicals, Ltd. (Kyoto, Japan) and Galα1–3Galα–OMe, Galβ1–3GlcNAcβ–OMe and Galα1–4Galβ–OEt were from Janssen Biochimica (Belgium). A series of Synsorb was obtained from Chembiomed, Ltd. (Edmonton, Canada).

Human Sera Sera were obtained from male and female healthy donors and patients with thrombotic diseases in clinical studies of t-PA(C127).[11] All serum samples were collected between 1986 and 1988 and were stored at −80 °C.

ELISA 1) Determination of the Antibody Titers in Human Sera: Microtitration plates (Costar, Cambridge, MA) were coated with 100 μl of t-PA(C127) in 0.1 M $NaHCO_3$ at a concentration of 10 μg/ml at room temperature (r.t.) overnight. Plates were washed five times with 0.01 M phosphate-buffered saline (PBS), pH 6.7 containing 0.1% Tween-20 (Bio-Rad, Richmond, CA), then human sera diluted 100-fold in 0.01 M phosphate buffer, pH 6.7 containing 1% casein (Oxoid Ltd., Hampshire, England), 0.05% ethylenediaminetetraacetic acid (EDTA), 0.1% Tween-20 and 2.6% NaCl were added and the plates wre incubated for 2 h at 37 °C. After 5 washings, a 1/100000 dilution of peroxidase-conjugated anti-human immunoglobulin G (IgG) (Tago, Burlingame, CA) was incubated for 2 h at 37 °C. Peroxidase activity was revealed by the use of 3 mg/ml orthophenylene diamine dihydrochloride and 0.02% H_2O_2 in 0.1 M citrate–phosphate buffer, pH 5.7 for 15 min at room temperature. The reaction was stopped by the addition of 2 N H_2SO_4. Absorbance at 490 nm was read with a multiscan reader.

The quantities of antibody in the sera were calculated reference to known quantities of affinity-purified rabbit anti-t-PA antibody. The OD value at 1 μg/ml of standard rabbit anti-t-PA was defined as 1 arbitrary unit/ml. Rabbit anti-t-PA antibody was measured by using a 1/500000 dilution of peroxidase-conjugated anti-rabbit IgG (Cappel, West Cheser, PA).

2) Specificity Evaluation by Competitive ELISA: This method consists of preincubating human serum with various proteins, carbohydrates or Synsorb immunoadsorbents, before adding the mixture to wells coated with antigens. Diluted human serum was preincubated with the same volume of antigens for 16 h at 4 °C. Plates coated with antigen were incubated with the pre-incubated mixture for 2 h at 37 °C. After washing, peroxidase-conjugated anti-human IgG was added.

3) Examination of the Existence of 1-3-Linked α-Galactosyl Epitopes on Various Glycoproteins: For coating, the glycoproteins were used at 3 μg/ml, except for thyroglobulin and laminin, used at a concentration of 30 μg/ml. After coating, the plates were incubated with human anti-Gal or anti-t-PA(C127) antibody. The human antibodies were used at a dilution of 100-fold. After 2 h of incubation at 37 °C, the plates were washed and peroxidase-conjugated anti-human IgG was added. Results are shown as

the mean of OD_{490} values. Blank OD_{490} values obtained in reactions without anti-Gal or anti-t-PA(C127) were no more than 0.05.

Isolation of Antibodies from Normal Sera Anti-Gal antibody was purified from normal human sera by affinity chromatography, using Synsorb 90 coupled with the glycosidic epitope Galα1-3Galβ1-4Glc-R, according to Galili *et al.*[12]

Anti-t-PA(C127) antibody was isolated from sera of normal donors by affinity chromatography, using protein A-Sepharose 4B and t-PA(C127)-Sepharose 4B. The amounts of antigen immobilized were 2 mg for protein A and 4 mg for t-PA (C127) per 1 ml of beads. Heat-inactivated sera were first loaded onto a column of protein A-Sepharose 4B. The column was then extensively washed with PBS. The absorbed IgG was eluted with 0.1 M HCl–glycine buffer, pH 3.0 and immediately neutralized with 2 M Tris. After dialysis, the eluate was passed through a column of t-PA(C127)-Sepharose 4B. The absorbed protein was eluted with 0.5 M melibiose. The antibody preparations were brought to a concentration of 0.5 mg/ml in PBS.

Results

Occurrence of Natural Antibody to t-PA(C127) in Human Sera Human sera from 118 disease-free Japanese individuals and 103 patients with thrombotic diseases, which were obtained before treatment with t-PA(C127), were provided for titration of IgG antibodies to t-PA(C127). The results of this survey are summarized in Fig. 1. Natural anti-t-PA(C127) antibodies were detected in about 80% of both the normal volunteers and the patients. Moreover, with respect to the mean value of antibody titers there was no significant difference between the normal volunteers (0.91 unit/ml) and the patients (0.82 units/ml).

Specificity Evaluation by Competitive ELISA These experiments were carried out using sera from several individual donors. Only representive results obtained from experiments using serum from a healthy volunteer are presented, because results obtained for each individual donor were similar. Anti-t-PA(C127) titer in the serum from the healthy volunteer was 2.45 units/ml.

1) Competition with Proteins The serum from the healthy volunteer contained natural antibodies to not only t-PA(C127), but also EPO(C127) and OVA, at various titers, but not to melanoma t-PA(mt-PA). The binding specificities of these natural antibodies were determined by preincubating the antibodies with free antigens before the reaction with immobilized antigens (Table I). Antibody capable of binding to OVA appeared to be specific for the antigen, because it was inhibited by the free antigen. On the other hand, binding activities to t-PA-(C127) and EPO(C127) were inhibited by t-PA(C127) as well as by EPO(C127).

2) Competition with Carbohydrates The results of Table I can be interpreted as suggesting that a common epitope on t-PA and EPO from C127 cells, as recognized by the natural antibodies, exists other than on the peptide region, since the amino acid sequence of t-PA is quite different from that of EPO, and the natural antibodies cannot react with mt-PA, which has the almost same amino acid sequence as t-PA(C127). Thus, the natural antibodies seem to be directed against a common antigenic determinant in the sugar chains of those molecules. To better define the specificity of the natural antibody, human sera were preincubated with various carbohydrates before the reaction with antigens. Antibody reactivities to t-PA(C127) and EPO(C127) were inhibited by D-galactose, lactose and melibiose, but not by D-glucose, D-mannose, GalNAc and GlcNAc (data not shown), indicating that the natural antibody might have specificity directed to galactosyl residues. Synsorb immunoadsorbents containing galactose were used as a competitor to test this hypothesis (Table II). Synsorb 90 with non-reducing terminal α1-3 galactose

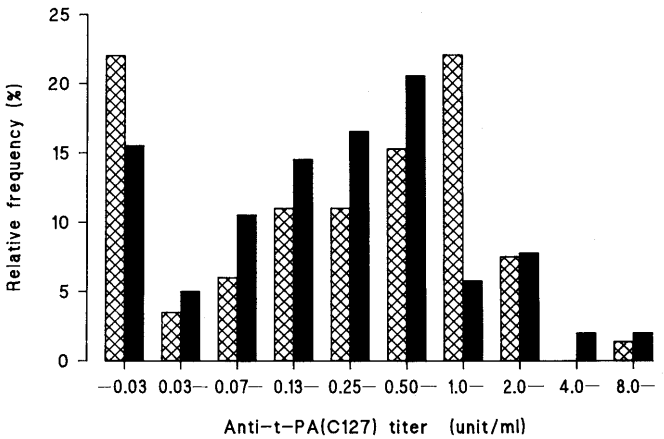

Fig. 1. Distribution of Natural Antibody Titers to t-PA(C127) in Human Sera

Antibody titers were determined, by ELISA, in sera obtained from 118 disease-free humans (hatched bar) and 103 patients (solid bar) with thrombotic diseases. All sera were obtained before the injection of t-PA(C127).

TABLE I. Inhibition of Antibody Binding by Various Glycoproteins

Free Ag	Coated Ag			
	t-PA (C127)	Melanoma t-PA	EPO (C127)	OVA
—	0.535	0.036	0.297	0.550
t-PA (C127)	0.308	0.034	0.096	0.570
Melanoma t-PA	0.493	0.032	n.d.	n.d.
EPO (C127)	0.238	n.d.	0.063	0.615
OVA	0.554	n.d.	0.350	0.072

Human serum (1/100 dilution) was preincubated with the same volume of various free antigens (100 μg/ml) for 16 h at 4 °C before adding the mixture to wells coated with antigens (3 μg/ml). Results are means of triplicate OD_{490} values. n.d.; not done.

TABLE II. Absorption of Antibodies with Various Synsorb Immunoadsorbents

Synsorb immunoadsorbent		Coated Ag (%)		
		t-PA (C127)	EPO (C127)	OVA
	—	100	100	100
A	Fucα1 | 2 GalNAcα1-3Gal-R	94	92	94
B	Fucα1 | 2 Galα1-3Gal-R	51	39	93
H	Fucα1 | 2 Galβ1-3GlcNAc-R	93	89	94
Lewis C	Galβ1-3GlcNAc-R	94	90	97
T	Galβ1-3GalNAc-R	88	85	95
Lactosamine	Galβ1-4GlcNAc-R	92	89	94
P1	Galα1-4Gal-R	71	60	96
90	Galα1-3Galβ1-4Glc-R	9	9	97
Forssman	GalNAcα1-3GalNAc-R	93	90	94

Human serum (1 ml) was preincubated with 50 mg of various Synsorb immunoadsorbents for 3.5 h at room temperature. The supernatants (1/100 dilution) were added to wells coated with antigens (3 μg/ml). The values obtained without preincubation with Synsorb immunoadsorbents were taken as 100%.

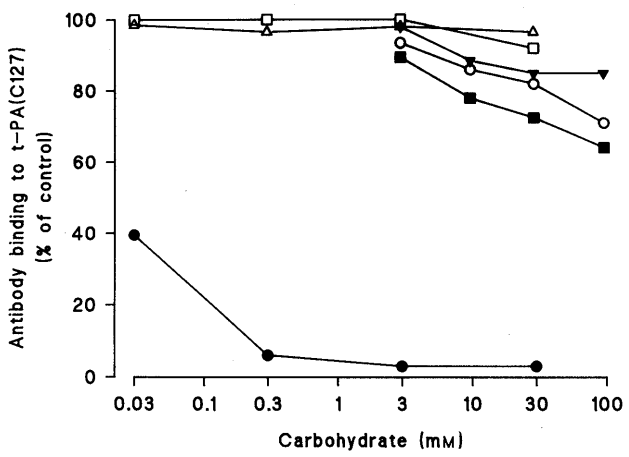

Fig. 2. Inhibition of Antibody Binding to t-PA(C127) by Galactosyl-Containing Carbohydrates

Human serum (1/50 dilution) was preincubated with various concentrations of galactosyl-containing carbohydrates for 16 h at 4 °C, before adding the mixture to wells coated with t-PA(C127) (3 μg/ml). The values obtained without preincubation with carbohydrates were taken as 100%. Open circles, Gal; solid circles, Galα1–3Galα–OMe; open triangles, Galβ1–3GlcNAcβ–OMe; solid triangles, Galβ1–4Glc; open squares, Galα1–4Galβ–OEt; solid squares, Galα1–6Glc.

TABLE III. Analysis of the Expression of 1–3-Linked α-Galactosyl Epitopes on Various Glycoproteins with Antibodies Isolated from Normal Human Sera

Coated Ag	From normal human sera	
	Anti-Gal	Anti-t-PA (C127)
t-PA (C127)	0.745	0.599
t-PA (CHO)	0.044	0.036
mt-PA	0.039	0.022
vt-PA	0.632	0.631
pt-PA	0.593	n.d.
EPO (C127)	0.222	0.207
EPO (CHO)	0.022	0.020
Protein C (C127)	0.269	n.d.
Protein C (CHO)	0.047	n.d.
Thyroglobulin	0.556	0.410
Laminin	0.684	0.526
Ovalbumin	0.031	0.019

Plates coated with a given antigen were incubated with isolated antibodies from normal human sera and the amounts of antibodies retained by the immobilized antigen were determined. The results are means of triplicate OD_{490} values. mt-PA, melanoma t-PA; vt-PA and pt-PA, t-PA isolated from human vascular trees and placenta, respectively; n.d., not done.

completely inhibited the binding activity of antibody to t-PA(C127) and EPO(C127). The other α-galactosyl containing Synsorbs, B with non-reducing terminal α1-3 galactose and branched fucose, and P1 with non-reducing terminal α1-4 galactose, inhibited the binding activity much less strongly than did Synsorb 90. The Synsorb with a non-reducing terminal β1-3 or β1-4 galactose or α1-3 GalNAc failed to prevent the antibody from binding to t-PA(C127) and EPO(C127). The binding to OVA was not inhibited by any type of Synsorb.

As shown in Fig. 2, the saccharide Galα1–3Gal was the most effective inhibitor. It completely inhibited antibody reactivity to t-PA(C127), at a concentration of 3 mM. Melibiose (Galα1–6Glc) inhibited the antibody reactivity far less potently (more than 3000-fold) than did Galα1–3Gal. D-Galactose was more inhibitory than lactose (Galβ1–4Glc) and was less effective than melibiose. Galβ1–3GlcNAc was completely inactive up to 30 mM, and Galα1–4Gal exerted only a slight inhibition, at this concentration.

Analysis of the Expression of 1-3-Linked α-Galactosyl Epitopes on Various Glycoproteins with Isolated Antibodies from Normal Human Sera We attempted to isolate, by affinity chromatography, the natural antibodies from pooled serum of normal donors. We used Synsorb 90 with the glycosidic epitope Galα1–3Galβ1–4Glc–R or t-PA(C127)-Sepharose 4B as an immunoadsorbent. The natural antibodies purified by Synsorb 90 and t-PA(C127)-Sepharose 4B were designated as anti-Gal and anti-t-PA(C127), respectively.

Various glycoproteins were evaluated in ELISA for their ability to bind these purified antibodies. The objective was to elucidate the nature of the glycosidic antigenic epitope expressed on glycoproteins of various origins. These results are shown in Table III. Anti-Gal and anti-t-PA(C127) from normal human sera reacted with all of the glycoproteins produced in C127 cells, *i.e.* t-PA(C127), EPO(C127) and protein C(C127), but not with any glycoproteins from CHO cells. Both human antibodies were also bound to naturally occurring t-PA, vt-PA from vascular trees of human cadavers and pt-PA from human placenta, but not to mt-PA from a human melanoma cell line. These isolated antibodies interacted with bovine thyroglobulin and mouse laminin, which were previously demonstrated to possess Galα1–3Gal residues.[13,14] Immunoblotting of t-PA(C127) and the naturally occurring t-PA(pt-PA, vt-PA) with anti-Gal revealed the presence of an immunoreactive 6 kilodalutors (kDa) polypeptide, corresponding to rabbit anti-t-PA staining of t-PA(C127) and the natural t-PA. Antibody reactivities to the same samples were completely inhibited by the preincubation of anti-Gal with Galα1–3Gal (data not shown).

Discussion

We obtained evidence that the natural antibody to recombinant t-PA expressed in mouse C127 cells is present in sera from almost all the individuals tested and is specific to oligosaccharide side chains with the 1-3-linked α-galactose residue. The presence of the anti-α-galactosyl antibody in normal human sera has also been demonstrated by other investigators. Anti-melibiose (Galα1–6Glc) antibody was reported by Bird and Roy[15] and Lalezari *et al.*[16,17] Natural antibody to the saccharide Galα1–3Gal in normal sera was described Suzuki and Naiki[18] and Galili *et al.*[19,20] They assessed the reactivity of the antibody in terms of its interaction with rabbit red blood cell glycolipids having Galα1–3 terminal residues. Galili *et al.* have also reported that this antibody, designated anti-Gal, is a polyclonal antibody present in every individual examined, and constitutes as much as 1% of the circulating IgG.[19,20] In addition, they isolated the antibody by affinity chromatography with a chemically defined antigen, Synsorb 90 with non-reducing terminal α1-3 galactose, and showed that anti-Gal had a distinct specificity for glycosphingolipids with a Galα1–3Gal glycosidic epitope. In our study, we found that the natural antibody had this same narrowly defined specificity for Galα1–3Gal structures as determined by competitive ELISA with various oligosaccharides and Synsorb immunoadsorbents (Table II and Fig. 2). The

reactivity of affinity-purified anti-t-PA(C127) against various glycoproteins paralleled that of purified anti-Gal (Table III), and so the two appear to be identical. It is also very likely that the natural anti-t-PA(C127) IgG described in the present study is the same antibody as that of Galili *et al.*

The results of our current study also indicate that the Galα1–3Gal epitope has been expressed on all bioengineered glycoproteins from C127 cells tested and on naturally occurring t-PA from human vascular trees and placenta (Table III). Recently, chemical structural analysis confirmed the presence of α-galactose which is 1–3-linked to lactosamine of the carbohydrate side chain on the interferon-β1 molecule from C127 cells.[21] Their results are in good accord with our results obtained by immunological analysis. As far as we are aware, this is the first report to show the occurrence of the 1–3-linked α-galactosyl residues on a natural human glycoprotein. Since naturally occurring t-PA exists in extremely low concentration (about 5 ng/ml) in normal plasma,[22] detailed studies on the carbohydrate structures of this natural glycoprotein have been hampered mainly by difficulties in obtaining a sufficient amount. Human anti-Gal antibody proved to be a valuable tool for studies of natural t-PA obtainable only in minute quantities.

The enzyme activity of t-PA(C127) was not inhibited by anti-Gal (data not shown). This result suggests that the epitopes for anti-Gal are not close to the catalytic site or fibrin-binding site of t-PA(C127), and/or the affinity constant for the binding between fibrin and its binding site on the t-PA molecule is greater than that for the binding between anti-Gal and the epitopes on the oligosaccharide side chain.

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