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Highly Increased Plasma Concentrations of the Nicked Form of β_2 Glycoprotein I in Patients with Leukemia and with Lupus Anticoagulant: Measurement with a Monoclonal Antibody Specific for a Nicked Form of Domain V

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β_2-Glycoprotein I (β_2GPI) consists of five tandem repeated domains (I, II, III, IV, and V). The nicked form of β_2GPI (N-β_2GPI) which was cleaved by plasmin *in vitro* at Lys 317-Thr 318 in domain V, showed reduced affinity for the negatively charged phospholipids, especially cardiolipin (CL). Recently, the N-β_2GPI was detected in the plasma of patients with disseminated intravascular coagulation syndrome (DIC) by an immunological method. In the present study, we prepared monoclonal antibodies for the nicked form, and demonstrated that the concentrations of this form of β_2GPI, which were analyzed by a sandwich ELISA using two specially prepared monoclonal antibodies, were significantly increased in the plasma of patients with leukemia (n = 51, mean ± SD: 162.0 ± 118.3 ng/ml) and with lupus anticoagulant (LA) (n = 40, mean ± SD: 3,041.5 ± 16,579.7 ng/ml), compared to the normals (n = 33, mean ± SD: 1.04 ± 1.54 ng/ml). We found a significant correlation between the concentrations of N-β_2GPI and those of typical molecular markers for a fibrinolytic state such as plasmin-α_2 plasmin inhibitor complex (PIC) and D-dimer in patients with leukemia, but not in patients with LA. These results suggested that the generation of N-β_2GPI was caused by plasmin in the patients with leukemia, and by unknown proteases in the patients with LA. In the patients with LA, the levels of N-β_2GPI tended to be higher in those without thrombosis than in those with thrombosis.

Key words: anti-phospholipid antibody syndrome, β_2-glycoprotein I, lupus anticoagulant, monoclonal antibody, thrombosis.

β_2-Glycoprotein I (β_2GPI) is a highly glycosylated plasma protein consisting of a single polypeptide chain (326 amino acid residues) with a molecular mass of about 50 kDa. The plasma concentration of β_2GPI is approximately 200 μg/ml. Although the function of β_2GPI has been investigated, its physiological role is still unclear. However, it is known to bind negatively charged substances such as DNA, heparin, dextran sulfate, and negatively charged phospholipids (*1–4*). The most relevant physiological role of β_2GPI is supposed to be the regulation of the function of anionic phospholipids, *e.g.*, phosphatidylserine (PS) or cardiolipin (CL). For the binding of PS, it is suggested to regulate platelet-dependent thrombosis (*5*) and the clearance of PS-expressing cells (*6*). Anti-CL antibodies or LA are strongly associated with thrombosis. In these autoimmune diseases with anti-phopholipid antibody syndrome, β_2GPI is a cofactor in the recognition of the phospholipid antigen, CL, by anti-CL antibodies (*7–9*).

β_2GPI consists of five tandem repeated domains (I, II, III, IV, and V), which have a common motif known as the complement control protein or Sushi domain superfamily (*10–12*). Recent studies suggest that domain V plays important roles in the binding to CL and expression of cofactor activity. Steinkasserer *et al.* (*13*) found that recombinant human domain V (r-domain V) inhibited the interaction of β_2GPI with CL. Hunt *et al.* (*14*) reported that the commercial preparation of human β_2GPI contained a nicked form in domain V, which displayed a lower interaction with CL. Recently, Ohkura *et al.* reported that plasmin produced the N-β_2GPI by cleaving the Lys 317-Thr 318 peptide bond in domain V *in vitro* (*15*). Horbach *et al.* raised a monoclonal antibody against the N-β_2GPI, but the location of epitope for the antibody remains to be determined (*16*). By enzyme immunoassay using the monoclonal antibody, they found

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Abbreviations: APA, anti-phospholipid antibodies; β_2GPI, β_2-glycoprotein I; CL, cardiolipin; DIC, disseminated intravascular coagulation; domain V, C-terminal domain of β_2-glycoprotein I; ELISA, enzyme-linked immunosorbent assay; HRPO, horseradish peroxidase; I-β_2GPI, intact form of β_2-glycoprotein I; I-r-domain V, intact form of recombinant domain V; LA, lupus anticoagulant; mAb, monoclonal antibody; N-β_2GPI, nicked form of β_2-glycoprotein I; N-r-domain V, nicked form of recombinant domain V; PBS, phosphate buffered saline; PIC, plasmin-α_2 plasmin inhibitor complex; PS, phosphatidylserine; r, coefficient of correlation; r-domain V, recombinant domain V; TAT, thrombin–antithrombin III complex; TBS, tris buffered saline; Tween, polyoxyethylene sorbitan monolaurate.

that the N-β_2GPI was present in plasma of septic patients with disseminated intravascular coagulation (DIC) and in plasmas of patients treated with streptokinase. They also suggested that β_2GPI is cleaved by plasmin *in vivo* as well as *in vitro*.

In the present study, we describe monoclonal antibodies that recognize neo-epitopes exposed in the N-β_2GPI and discuss the epitope of these antibodies with reference to possible conformational change induced in the domains of β_2GPI. We also described the application of these antibodies to measurement of the N-β_2GPI in plasma samples derived from patients with leukemia and patients with LA.

MATERIALS AND METHODS

Subjects—We examined 51 patients with leukemia; 41 had acute promyelocytic leukemia (APL), 6 acute myeloblastic leukemia (AML), and 4 acute lymphoblastic leukemia (ALL).

We also examined 40 patients with LA. LA was considered to be positive if at least two of the following three criteria were fulfilled: (i) prolongation of activated partial thromboplastin time (aPTT), (ii) prolongation of kaolin clotting time (KCT) and mixing test in KCT, and (iii) prolongation of dilute Russell's viper venom time (DRVVT) and increased DRVVT/DRVVT with a high-lipid concentration (DRVVT-HCL) ratio. Eight of 40 patients presented symptoms of thrombosis. These patients were usually treated with anticoagulant, antiplatelet, or fibrinolytic agents. Blood samples were obtained from these patients after symptoms improved and after temporary withdrawal of the drug. None of the patients were being treated with anticoagulants at the time of blood sampling. Blood was collected by venipuncture, and nine parts of whole blood were mixed with one part of 3.8% trisodium citrate. APTT, KCT, and DRVVT were determined by methods described elsewhere (*17*). The levels of thrombin–antithrombin III complex (TAT), plasmin-α_2 plasmin inhibitor complex (PIC), and D-dimer were determined with commercially available ELISA kits, TAT Test-Teijin-F, PIC Test-Teijin-F, and D Dimer Test-Teijin-F (International Reagents, Hyogo), respectively. The anti-phospholipid antibodies (APA) in plasma of patients with LA were detected with a commercially available ELISA kit, Asserachrom APA (Diagnostica Stago, Asnieres-Sur-Seine, France).

Preparation of Human β_2GPI and r-Domain V—r-Domain V with a signal peptide, which consists of the extra amino acid sequence Tyr-Val-Glu-Phe-Met-Ile-Glu-Gly-Arg-Thr with the amino terminal Lys 242 of human β_2GPI, was expressed in methylotrophic yeast, *Pihia pastoris*, and purified by anion-exchange and reversed-phase chromatography, as described in a previous paper (*18*). r-Domain V containing an extra Thr at the N-terminus was obtained by a treatment with factor Xa (Enzyme Research Laboratories, South Bend, IN). The numbers of amino acid residues of intact β_2GPI were employed for the numbering of the amino acid residues of r-domain V, neglecting the extra Thr at the N-terminus. Human β_2GPI was prepared as described by Polz *et al.* (*2*). Briefly, fresh human plasma was treated with 1.3% perichloric acid at 4°C for 15 min and centrifuged at 5,000 $\times g$ for 10 min. The supernatant was neutralized by adding a 1/3 vol. of 1.0 M Tris-HCl, pH 8.0 and concentrated by Centricon-30 (Amicon Beverly, MA). The concentrate was applied to a column of HiTrap Heparin (Pharmacia Biotech, Tokyo). After the column had been washed with 10 mM Tris-HCl, pH 8.0, β_2GPI was eluted with a linear gradient of 10 mM Tris-HCl and the same buffer containing 1.0 M NaCl. Nicked forms of β_2GPI and r-domain V were prepared by plasmin treatment using the method of Ohkura *et al.* (*15*). The intact and nicked form of r-domain V thus prepared were found to have native disulfide bonds and a similar compactly folded conformation, judging from the circular dichroism spectrum. In this paper, we tentatively designated the intact form of β_2GPI and r-domain V as I-β_2GPI and I-r-domain V, and their nicked forms as N-β_2GPI and N-r-domain V, respectively.

Preparation of Monoclonal Antibodies—We immunized BALB/c mice (Clea Japan, Tokyo) with 50 μg of N-β_2GPI in complete Freund's adjuvant followed by 50 μg of the same antigen without the adjuvant essentially by a hybridoma technique of Köhler and Milstein (*19*) with minor modifications as described elsewhere (*20*). The culture supernatants were screened for monoclonal antibodies (mAbs) by direct binding enzyme-linked immunosorbent assay (ELISA) as described below. The subclass of mAbs was determined with an isotyping kit (Amersham, Tokyo).

Analysis of Binding of mAbs to Antigens by Direct Binding ELISA—Binding between the antigens and mAbs was studied by direct binding ELISA as described previously (*21*). Briefly, wells of polystyrene microtiter plates (Immulon-II, Dynatech, Chantilly, VA) were coated overnight at 4°C with 50 μl of respective antigens at 3 μg/ml in 50 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl (TBS). The antigen-coated wells were washed with 0.15 M NaCl containing 0.05% (w/v) Tween-20 (NaCl-Tween) and incubated with 50 μl of the culture supernatants or the purified mAbs at 5 μg/ml in 50 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl and 0.05% (w/v) Tween-20 (TBS-Tween) for 1 h at 25°C. After aspiration of the reaction mixture, the wells were washed with NaCl-Tween. Horseradish peroxidase (HRPO)–conjugated anti–mouse IgG rabbit antibody (DAKO, Glostrup, Denmark) diluted 200-fold with TBS-Tween was added to each well as the second antibody. The amounts of bound antibodies were determined by using 50 mM Tris-HCl, pH 7.5, containing 0.5 mM 4-aminoantipyrine, 10 mM phenol, and 0.005% hydrogen peroxide as substrate, and the color produced was read at 492 nm on an MPR-A4i microplate reader (Tosoh, Tokyo).

Binding of mAbs to Antigens Bound to Solid-Phase CL—For binding experiments, wells of polystyrene microtiter plates were coated with 30 μl of CL (from bovine heart, Sigma, St Louis, MO) at 300 μg/ml in ethanol, dried under vacuum, and blocked with 200 μl of a mixture of 1% (w/v) skim milk and 0.3% (w/v) gelatin in 50 mM sodium phosphate, pH 7.4, containing 0.15 M NaCl (PBS) for 1 h at 25°C, as described previously (*14*). The wells were washed three times with PBS, and then 50 μl of I-β_2GPI at 3 μg/ml in PBS containing 0.3% (w/v) gelatin was added to each well, and incubation was continued for 2 h at 25°C. The antigen-coated wells were washed with PBS and incubated with 50 μl of purified mAbs at 5 μg/ml in PBS for 1 h at 25°C. The bound antibodies were detected as described above.

Determination of the Dissociation Constant of mAbs—The binding between antigen and the established mAbs,

NGPI-23, N-GPI-59, and N-GPI-60, was studied by a solid-phase ELISA using microtiter plates coated with N-β_2GPI as described previously (*20*). The amounts of bound antibodies were calculated from calibration curves constructed with known amounts of the respective bound antibodies. The dissociation constant was calculated as described previously (*22*).

Immunoblotting Analysis—Reactivity of NGPI-59 and NGPI-60 to various antigens was analyzed by immunoblotting essentially as described elsewhere (*21, 23*).

Effect of the Ionic Strength on the Binding of Antibody to Antigen—Wells of polystyrene microtiter plates were coated overnight at 4°C with 50 μl of purified IgG of NGPI-59 or NGPI-60 in TBS (5 μg/ml). The IgG-coated wells were washed three times with TBS-Tween, then incubated for 2 h at 25°C with 50 μl of purified antigens diluted 10-fold with the buffer containing various concentrations of NaCl. The buffers were composed of 50 mM Tris-HCl, pH 7.5, containing 0.05%(w/v) Tween-20 and 0.15, 0.5, or 1.0 M NaCl. After aspiration of the reaction mixture, the wells were washed with TBS-Tween. Biotinylated $F(ab')_2$ fragment of NGPI-23 was added to each well at 2.5 μg/ml and incubated for 1 h at 25°C. The $F(ab')_2$ fragment was prepared as follows. The purified IgG of NGPI-23 was digested with pepsin at an enzyme/substrate ratio of 1/130 (w/w) for 3 h at 37°C in 0.1 M sodium citrate, pH 3.7, and the $F(ab')_2$ fragment was isolated by gel filtration on a Sephacryl S-300 HR column (Pharmacia Biotech, Tokyo). HRPO-conjugated avidin (DAKO, Glostrup, Denmark), diluted with TBS-Tween (3,000-fold dilution), was added to each well as the tag. After washing, the amounts of bound peroxidase were determined by using 4-aminoantipyrine-phenol-hydrogen peroxide as substrate and by reading the absorbance at 492 nm as described above.

Measurement of the Nicked Form of β_2GPI in Clinical Samples by Sandwich ELISA—Wells of polystyrene microtiter plates were coated overnight at 4°C with 50 μl of IgG of NGPI-59 or NGPI-60 in TBS (5 μg/ml). The IgG-coated wells were washed three times with 20 mM Tris-HCl, pH 7.6, containing 0.5 M NaCl, 0.1%(w/v) Tween-20 (TB-N-Tween). Subsequently, 50 μl of citrated plasma samples diluted 10-fold with TB-N-Tween were applied to the wells and incubated for 2 h at 25°C. After aspiration of the reaction mixture, the wells were washed with TB-N-Tween. Biotinylated $F(ab')_2$ fragment of NGPI-23 was added to each well at 2.5 μg/ml and incubated for 1 h at 25°C. HRPO-conjugated avidin diluted with TB-N-Tween (3,000-fold dilution) was added to each well as the tag. After washing, the amounts of bound peroxidase were determined by using 4-aminoantipyrine-phenol-hydrogen peroxide as substrate and by reading the absorbance at 492 nm as described above. The amounts of the N-β_2GPI in plasma samples were calculated from a calibration curve constructed with citrated plasma spiked with known amounts of the purified N-β_2GPI.

Measurement of Total β_2GPI in Clinical Samples by Sandwich ELISA—Total β_2GPI was measured by a sandwich ELISA utilizing $F(ab')_2$ fragment of NGPI-23 as the capture antibody and biotinylated anti-β_2GPI rabbit IgG (Cedarlane Laboratories Ontario, Canada) as the tag antibody. Plasma samples (7,500-fold diluted) were allowed to react with the immobilized antibody. Then HRPO-conjugated avidin was added, and the amounts of bound HRPO were determined as described above. The amounts of total β_2GPI in plasma samples were calculated from a calibration curve constructed with known amounts of purified I-β_2GPI.

Isolation of the Nicked Form of β_2GPI from a Patient's Plasma by Immunoaffinity Chromatography—The nicked form of β_2GPI was isolated from a patient's citrated plasma using an NGPI-60 immobilized HiTrap NHS-activated column. In brief, 10 mg of purified IgG of NGPI-60 was coupled to a HiTrap NHS-activated column (bed volume, 1 ml, Pharmacia Biotech, Tokyo) according to the manufacturer's instructions. After coupling, 0.35 ml of the citrated plasma sample (the nicked form of β_2GPI measured; 92.6 μg/ml) was directly applied to the NGPI-60 immobilized HiTrap NHS-activated column, which had been equilibrated with 50 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl and 1 mM disodium ethylenediaminetetraacetate (EDTA). After washing the column with 50 mM Tris-HCl, pH 7.5, containing 1.0 M NaCl and 0.05% (w/v) Tween-20 and subsequently with 10 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl, the adsorbed proteins were eluted with 0.2 M glycine-HCl, pH 2.5, and 0.2-ml fractions were collected. The eluted samples were neutralized immediately by adding a 1/5 volume of 1.0 M Tris-HCl, pH 8.0. The purity of the eluted samples was judged by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) (*24*). The N-terminal amino acid sequences of the eluted samples were analyzed by use of a protein sequencer, model 473A (Applied Bisystems, Foster City, CA).

Statistical Analysis—To compare the levels of the nicked form of β_2GPI in plasma between patients and the normal control, Student's *t*-test was used. A *p*-value of less than 0.05 was considered as significant.

RESULTS

Characterization of the mAbs for the Nicked Form of β_2GPI—By immunizing mice with N-β_2GPI as described in the Methods, we obtained three mAbs. The immunoglobulin subclasses of these antibodies were determined to be IgG_{2a} with κ type light chains for NGPI-59, IgG_1 with κ type light chains for NGPI-60, and NGPI-23. The reactivities of the mAbs with antigens were analyzed by direct binding ELISA, using the antigens directly immobilized to wells of polystyrene microtiter plates. NGPI-23 equally reacted with N-β_2GPI and I-β_2GPI. NGPI-59 and NGPI-60 specifically reacted with N-β_2GPI (Fig. 1A). The dissociation constant of these mAbs with N-β_2GPI was determined to be 7.8×10^{-10}, 9.5×10^{-10}, and 4.7×10^{-10} M, respectively.

To see whether the epitopes for NGPI-59 and NGPI-60 were exposed in the β_2GPI bound to negatively-charged phospholipids, we examined the binding activities of antibodies to antigens bound to solid-phase CL. We confirmed that I-β_2GPI bound to CL, because NGPI-23 reacted with the wells incubated with intact form (Fig 1B). However, neither NGPI-59 nor NGPI-60 reacted with the β_2GPI bound to CL. That is, the epitopes for NGPI-59 and NGPI-60 were not formed in the β_2GPI bound to CL (Fig. 1B).

To identify the location of the epitopes for NGPI-59 and NGPI-60, we performed immunoblot analysis of N-β_2GPI, I-β_2GPI, N-r-domain V, and I-r-domain V. All these proteins were stained with anti-β_2GPI rabbit IgG under both non-reducing and reducing conditions (Fig. 2C). When the anal-

ysis was conducted against non-reduced antigens, neither I-β_2GPI nor I-r-domain V were stained with NGPI-59, whereas N-β_2GPI and N-r-domain V were stained positively (Fig. 2A, lanes 1–4). When the analysis was conducted against reduced antigens, all of them were stained positively with NGPI-59 (Fig. 2A, lanes 5–8). The result indicates that the epitope for NGPI-59 had been exposed in domain V of N-β_2GPI, and hidden in the intact protein under non-reducing conditions. NGPI-60 reacted with N-β_2GPI under non-reducing conditions, but not under reducing conditions (Fig. 2B, lanes 1 and 5). This result indicates that the epitope for NGPI-60 had been located in the region containing domains I–IV of N-β_2GPI, and the disulfide-linked structure in the region was required for the epitope expression.

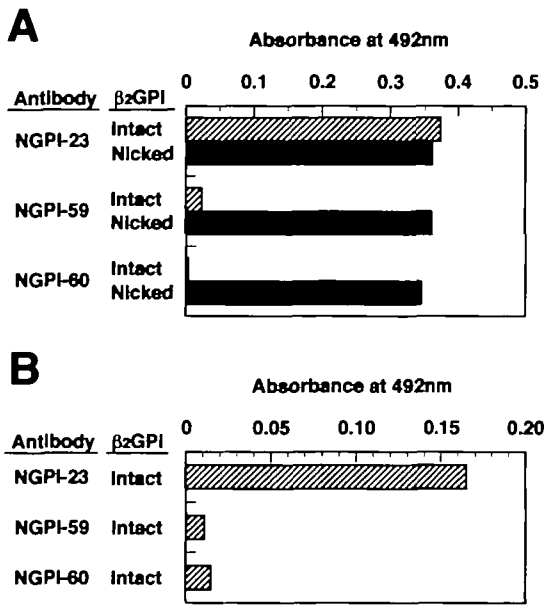

Fig. 1. **Specificity of monoclonal antibodies for immobilized antigens by ELISA.** Each monoclonal antibody was incubated in wells coated with intact or nicked form of β_2GPI as antigen, and the amounts of bound antibodies were determined. The antigens were immobilized directly (A) or through CL (B) to the wells.

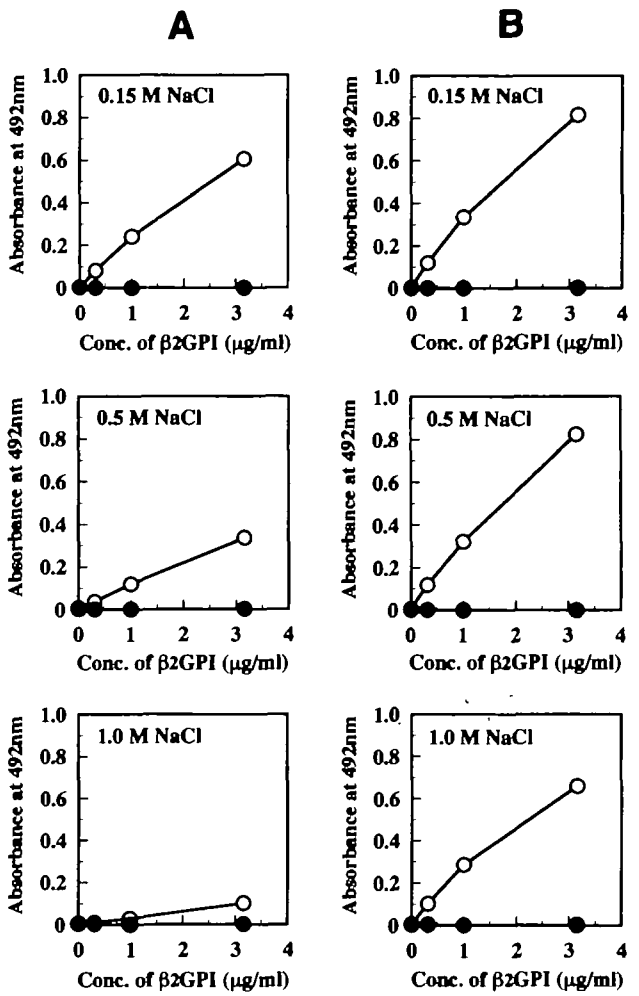

Fig. 3. **Effect of ionic strength on the binding of antibody.** Wells of polystyrene microtiter plates were coated with NGPI-59 (A) or NGPI-60 (B). The purified intact form (●) or nicked form (○) of β_2GPI, diluted 10-fold with the buffer containing various concentrations of NaCl, was added to the wells. The bound antigens were detected with the biotinylated NGPI-23.

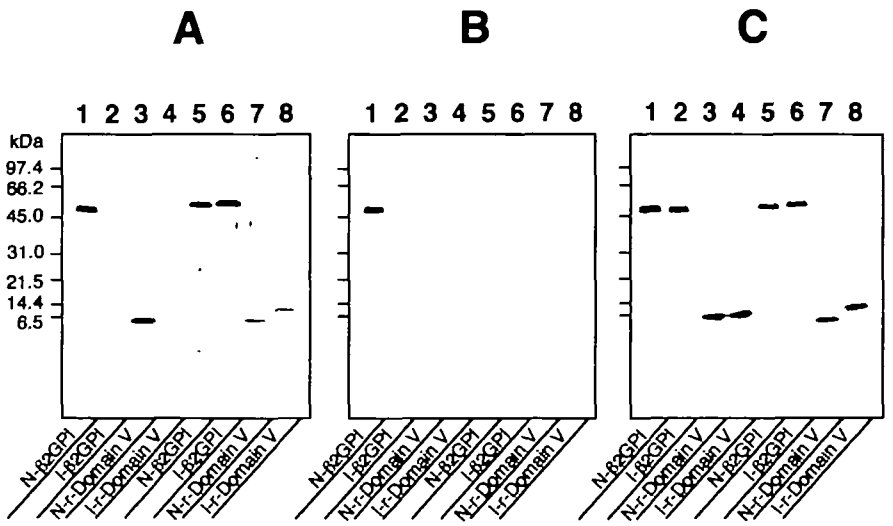

Fig. 2. **Immunoblot analysis of β_2GPI and r-domain V using NGPI-59 (A), NGPI-60 (B), and anti-β_2GPI rabbit IgG as a reference (C).** The proteins were resolved by SDS-PAGE using 10–20% gradient gels under non-reducing conditions (lanes 1–4 of each panel) or reducing conditions (lanes 5–8 of each panel) and subjected to immunoblotting. N-β_2GPI: nicked β_2GPI; I-β_2GPI: intact β_2GPI; N-r-Domain V: nicked recombinant domain V; I-r-Domain V: intact recombinant domain V.

NGPI-23 reacted with N-β_2GPI and I-β_2GPI, but not with N-r-Domain V and I-r-Domain V, under both non-reducing and reducing conditions (data not shown). That is, the epitope for NGPI-23 was located in the region containing domains I–IV of β_2GPI, and the conformations of the region were not required for the epitope expression.

We then examined the effect of ionic strength on the binding of the antibodies to antigens by sandwich-type ELISA. The reactivity of NGPI-59 was significantly affected by ionic strength (Fig. 3A), whereas the reactivity of NGPI-60, which recognized the region containing domains I–IV of N-β_2GPI, was not (Fig. 3B).

Application of NGPI-59 and NGPI-60 to the Measurement of the Nicked Form of β_2GPI in Clinical Samples by

TABLE I. **Levels of typical molecular markers for thrombotic and fibrinolytic states and of total β_2GPI.**

	Normal (n = 33)[a]	Leukemia (n = 51)[a]	LA (n = 40)[a]
TAT (ng/ml)[b]	1.3 ± 1.0	18.4 ± 19.8	4.5 ± 5.5
PIC (μg/ml)[b]	0.5 ± 0.3	4.8 ± 4.6	0.9 ± 0.7
D-dimer (μg/ml)[b]	0.2 ± 0.2	8.0 ± 9.1	0.4 ± 0.4
Total β_2GPI (μg/ml)[c]	193 ± 39	226 ± 68	182 ± 39

[a] Values are mean±SD. [b] The levels of TAT, PIC, and D-dimer were determined with commercially available EIA, TAT Test-Teijin-F, PIC Test-Teijin-F, and D Dimer Test-Teijin-F (International Reagents Hyogo), respectively. [c] The levels of total β_2GPI (intact form + nicked form) were determined by sandwich ELISA as described in "MATERIALS AND METHODS."

TABLE II. **Correlation between the levels of nicked β_2GPI and those of typical molecular markers and total β_2GPI.**

	Leukemia (n = 51)		LA (n = 40)	
	r	p-value	r	p-value
TAT	0.207	>0.05	−0.023	>0.05
PIC	0.297	<0.05	0.052	>0.05
D-dimer	0.785	<0.01	−0.146	>0.05
Total β_2GPI[a]	−0.043	>0.05	0.087	>0.05

[a] Total β_2GPI is composed of intact and nicked form of β_2GPI.

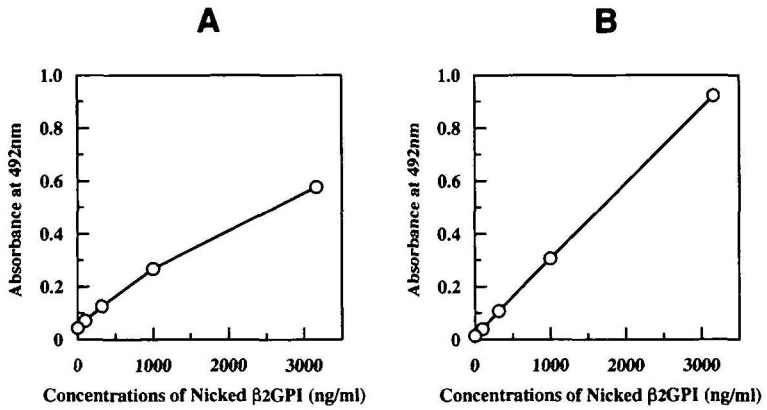

Fig. 4. **Sandwich ELISA on plasma spiked with various concentrations of the nicked form of β_2GPI, utilizing NGPI-59 (A) or NGPI-60 (B) as capture antibody.** Each antibody was able to measure the nicked form of β_2GPI up to 3,000 ng/ml dose-dependently, without any interference by coexisting intact β_2GPI in plasma.

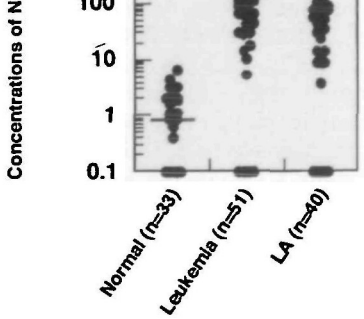

Fig. 5. **Concentrations of nicked β_2GPI in clinical samples measured by the sandwich ELISA utilizing NGPI-60 as capture antibody.** In each column, the horizontal bar indicates the average concentration of nicked β_2GPI. In the upper panel, the results of hypothesis testing by Student's t-test are indicated.

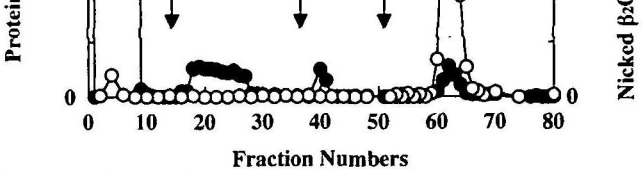

Fig. 6. **Isolation of the nicked form of β_2GPI from a patient's plasma by immunoaffinity chromatography.** An NGPI-60 immobilized HiTrap NHS-activated column was equilibrated with 50 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl and 1mM EDTA. A 0.35-ml volume of patient's plasma was applied onto the column, and adsorbed proteins were eluted with 0.2 M glycine-HCl, pH 2.5. The eluted proteins were neutralized immediately. SDS-PAGE profiles using 10% gel of eluted proteins are shown in the inset. Lanes 1 and 4: intact β_2GPI; lanes 2 and 5: nicked β_2GPI; lanes 3 and 6: eluted proteins. Lanes 1–3, run under non-reducing conditions, lanes 4–6, run under reducing conditions.

TABLE III. **Amino acid sequence analysis of the eluate from immunoaffinity chromatography.**

Assigned sequence	Amino acid recovered at each cycle[a]							
	1	2	3	4	5	6	7	8
Gly 1 – Asp 8	Gly	Arg	Thr	(Cys)	Pro	Lys	Pro	Asp
	14.4	22.0	6.7	nd[b]	12.6	16.2	11.6	12.4
Thr 318 – Pro 325	Thr	Asp	Ala	Ser	Asp	Val	Lys	Pro
	10.4	21.8	17.4	4.4	20.8	14.4	23.9	16.2

[a]Each value under the amino acid sequence identified shows the amount of PTH–amino acid (in picomoles) recovered using a gas-phase sequencer. [b]Cys residue was not detected.

Sandwich ELISA—The citrated plasma spiked with purified N-β_2GPI was examined by sandwich ELISA using NGPI-59 or NGPI-60 as the capture antibody and NGPI-23 as the tag antibody (Fig. 4). This assay was able to measure the nicked form of β_2GPI in normal citrated plasma at up to 3,000 ng/ml dose-dependently, without any interference from coexisting intact β_2GPI in plasma. One set of mAbs, NGPI-60 and NGPI-23, seemed to be superior in sensitivity to the other set, NGPI-59 and NGPI-23. Thus, we used the superior combination to determine the levels of N-β_2GPI in plasma samples. This ELISA showed good linearity even in the range of 0–100 ng/ml (data not shown).

The levels of TAT, PIC, D-dimer, and total β_2GPI in the plasma samples are summarized in Table I. The levels of TAT, PIC, and D-dimer were found to increase in patients with leukemia, but those of PIC and D-dimer increased only slightly in patients with LA. The level of the N-β_2GPI in normal citrated plasma was determined to be 0 to 6.5 ng/ml (n = 33, mean ± SD: 1.04 ± 1.54 ng/ml). However, the levels of the N-β_2GPI were mostly elevated in clinical samples from patients with leukemia (n = 51, mean ± SD: 162.0 ± 118.3 ng/ml) and patients with LA (n = 40, mean ± SD: 3,041.5 ± 16,579.7 ng/ml). We found a significant difference between the average of normal control and each average for clinical samples by Student's t-test (Fig. 5).

We also examined the correlation between the levels of the N-β_2GPI and those of typical molecular markers for thrombotic and fibrinolytic states or that of total β_2GPI. In the patients with leukemia, we found a significant correlation with fibrinolytic molecular markers, PIC and D-dimer. However, we did not find any correlation in the patients with LA (Table II).

Isolation of the Nicked Form of β_2GPI from a Patient's Plasma by Immunoaffinity Chromatography—To prove that NGPI-60, which recognized the epitope expressed in the region containing domains I-IV of N-β_2GPI, had specifically reacted to the N-β_2GPI in clinical samples, we attempted to isolate the proteins bound to NGPI-60 by immunoaffinity chromatography utilizing this antibody. We used a plasma sample derived from a patient with LA (92.6 μg/ml of the N-β_2GPI measured by sandwich ELISA). The protein eluted as a single peak with the acidic buffer was found to have the same mobility as the N-β_2GPI on SDS-PAGE with or without reduction (Fig. 6 and inset). Furthermore, the protein was found to have two N-terminal amino acid sequences (Table III). One started from Gly 1 assigned to the N-terminal amino acid sequence of β_2GPI, the other from Thr 318 assigned to that of the C-terminal peptide from plasmin-treated r-domain V (*15*). Thus, NGPI-60 specifically recognized the nicked form of β_2GPI in a clinical sample, of which the peptide bond of Lys 317-Thr 318 in domain V had been cleaved.

DISCUSSION

The most relevant physiological role of β_2GPI is supposed to be the regulation of the function of negatively-charged phospholipids, *e.g.*, PS or CL. The binding of β_2GPI to the negatively-charged phospholipids has been shown to result in conformational change of the protein (*25*), which expresses a new epitope (*26*, *27*). This new epitope in β_2GPI-cardiolipin complex has been reported to be the recognition site for anti-phospholipid antibodies in sera of patients with anti-phospholipid antibody syndrome (*7–9*). Recently, it has been reported that N-β_2GPI and the nicked domain V showed much lower affinity for negatively-charged phospholipids than I-β_2GPI (*16*, *18*). Therefore, the physiological function of β_2GPI seems to be modified by the cleavage of the protein.

In the present work, we produced two mAbs, NGPI-59 and NGPI-60, that specifically reacted with N-β_2GPI but not with I-β_2GPI. These mAbs could not recognize β_2GPI bound to solid-phase CL (Fig. 1B). Thus, the epitopes for these mAbs were distinct from the new epitope for anti-cardiolipine antibodies. These results indicate that a certain neo-epitope was exposed specifically in *N*-β_2GPI. Our result is consistent with the report by Horbach *et al.* (*16*). However, the location of the epitope for the mAb which specifically recognized N-β_2GPI remains to be determined. In contrast, we identified the location of the epitopes for our antibodies. NGPI-59 recognized the neo-epitope that was exposed in domain V of N-β_2GPI (Fig. 2A). Hagihara *et al.* examined the conformation of the nicked domain V by circular dichroism and fluorescence measurements, but did not detect any significant difference between them (*18*). Therefore, NGPI-59 was speculated to recognize the neo-epitope composed of a local area caused by slight conformational change. The finding that NGPI-59 reacted with intact β_2GPI and domain V under reducing conditions indicates that this local segment resides in the region of Lys 242–Lys 317 of β_2GPI, which was hidden in the intact protein under non-reducing conditions. Furthermore, the finding that the reactivity of NGPI-59 was significantly affected by ionic strength suggests that a highly positive charge in the region of domain V also influenced the expression of the epitope for NGPI-59 (Fig. 3A). The binding of NGPI-60 to the antigens seems to be dependent on the preservation of disulfide bonds in the primary structure of the epitope, because the antigens were no longer recognized after reduction by NGPI-60. The epitope for NGPI-60 was located in domains I–IV of the N-β_2GPI (Fig. 2B). This indicates that the cleavage by plasmin could induce conformational changes in domains I–IV as well as domain V of β_2GPI. This assumption is supported by a recent observation that

TABLE IV. **Levels of nicked β_2GPI in patients with lupus anticoagulant.**

Symptoms	*n*	Nicked β_2GPI (ng/ml)[a]	No. of patients with APA[b]	
With thrombosis	8	18.9 ± 34.7	7/8	(88%)
Without thrombosis	32	3,797.2 ± 16,579.7	9/32	(28%)

[a]Values of nicked β_2GPI are mean ± SD. [b]The anti-phospholipid antibodies (APA) in patients' plasmas were detected with a commercially available ELISA kit, Asserachrom APA.

PS-bound β_2GPI associated with the macrophage membrane through a region in domain II and/or III, which was hidden in unbound β_2GPI (*28*). β_2GPI might play an important role physiologically through the neo-epitope that was recognized by these antibodies.

By using these mAbs, we established an ELISA for specific measurement of N-β_2GPI. The levels of N-β_2GPI were significantly increased in the plasma of patients with leukemia (n = 51, mean ± SD: 162.0 ± 118.3 ng/ml) and with LA (n = 40, mean ± SD: 3,041.5 ± 16,579.7 ng/ml) (Fig. 5), compared to the normals (n = 33, mean ± SD: 1.04 ± 1.54 ng/ml). Ohkura *et al.* (*15*) demonstrated that the cleavage of β_2GPI in plasma could occur when α_2-plasmin inhibitor levels decreased. Therefore, the generation of N-β_2GPI was not found to occur in the plasma samples abundantly containing various proteinase inhibitors including α_2-plasmin inhibitor. Indeed, we have not found any differences in the levels of N-β_2GPI in preparations of plasma from patients with various diseases made with or without protease inhibitors, soybean trypsin inhibitor, benzamidine HCl, *N*-ethylmaleimide, and phenylmethylsulfonyl fluoride (data not shown). Therefore, the N-β_2GPI in the plasma of patients with leukemia and LA was found to be generated *in vivo*, but not *in vitro* after blood collection.

Nicked form of β_2GPI is probably generated on the surface of cells such as endothelial cells or platelets but not in the fluid phase, because various proteinase inhibitors are abundant in plasma. Plasmin is the most feasible candidate for the *in vivo* cleavage of β_2GPI, for the reasons described below. Plasminogen and plasminogen activator are associated with fibrin, and the activation of plasminogen occurs on fibrin (*29*, *30*). Plasminogen binds to extracellular matrix (ECM) synthesized by endothelial cell monolayers and is activated by t-PA or UK on the matrix (*31*, *32*). The plasmin generated on the extracellular matrix is protected from inhibition by α_2 plasmin inhibitor (*31*). The matrix produced by endothelial cells is a complex array of glycoproteins and glycosaminoglycans, and β_2GPI has been reported to bind to heparin (*2*). Therefore, it is conceivable that β_2GPI, which binds to heparin-like glycosaminoglycans, is cleaved by plasmin in the extracellular matrix.

We found a patient with LA in whom half of the plasma β_2GPI was N-β_2GPI (N-β_2GPI: 92.6 μg/ml, total β_2GPI: 202 μg/ml). We isolated the N-β_2GPI from the plasma, and detected the cleavage of Lys 317-Thr 318 in isolated proteins (Fig. 6 and Table III). Although this cleavage site was the same as that of N-β_2GPI produced with plasmin *in vitro*, other proteases such as Xa, trypsin, and elastase were reported to induce the same proteolytical cleavage in β_2GPI (*15*, *16*). The levels of the fibrinolytic molecular marker, PIC, in the patient's plasma were slightly elevated (1.2 μg/ml). And for patients with LA overall, the levels of PIC were slightly elevated in comparison with those of leukemia patients (Table I). Furthermore, no correlation between the level of N-β_2GPI and that of PIC was found in the patients with LA (Table II). These results suggested that certain proteases might contribute to the cleavage of β_2GPI rather than plasmin in patients with LA.

It is reported that LA and anti-phospholipid antibody syndrome are strongly associated with thrombosis. Indeed, of the 8 of 40 patients with LA who showed symptoms of thrombosis, 7 demonstrated anti-phospholipid antibodies, namely, anti-β_2GPI antibodies. Nevertheless, the levels of N-β_2GPI tended to be higher in the patients without thrombosis than in those with thrombosis (Table IV). Thus, it does not seem that β_2GPI bound to anti-β_2GPI antibodies in plasma from patients with LA is more sensitive to cleavage by unknown proteases. Although we have not yet identified the proteases that contribute to the cleavage of β_2GPI in the plasma of patients with LA, these proteases may control the manifestation of thrombosis in patients with LA or with anti-phospholipid antibody syndrome. The emergence of a negatively charged phosholipid such as phosphatidylserine (PS) on the cell surface results in binding of β_2GPI to PS. If β_2GPI bound to PS was digested by proteases and released into the blood circulation as the nicked form, the anticoagulant system, *e.g.*, the phosholipid-dependent activated protein C system (*33*, *34*), may mobilize normally, and the immune response to the new epitope expressed on the β_2GPI-phospholipids complex may decrease. To understand the clinical implications of higher plasma levels of N-β_2GPI in relation to symptoms of thrombosis, further investigation is needed.

In the present paper, we described two mAbs that react with N-β_2GPI but not with I-β_2GPI, and established methods for the detection of N-β_2GPI in plasma using these antibodies. These tools may clarify the significance of generation of N-β_2GPI *in vivo* and provide further insight into the mechanism behind anti-phospholipid antibody syndrome.

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