Nicked β₂-glycoprotein I: a marker of cerebral infarct and a novel role in the negative feedback pathway of extrinsic fibrinolysis

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β₂-Glycoprotein I (β₂-GPI) is proteolytically cleaved by plasmin in domain V (nicked β₂-GPI), being unable to bind to phospholipids. This cleavage may occur in vivo and elevated plasma levels of nicked β₂-GPI were detected in patients with massive plasmin generation and fibrinolysis turnover. In this study, we report higher prevalence of elevated ratio of nicked β₂-GPI against total β₂-GPI in patients with ischemic stroke (63%) and healthy subjects with lacunar infarct (27%) when compared to healthy subjects with normal findings on magnetic resonance imaging (8%), suggesting that nicked β₂-GPI might have a physiologic role beyond that of its parent molecule in patients with thrombosis. Several inhibitors of extrinsic fibrinolysis are known, but a negative feedback regulator has not been yet documented. We demonstrate that nicked β₂-GPI binds to Glu-plasminogen with Kᵥ of 0.37 × 10⁻⁶ M, presumably mediated by the interaction between the fifth domain of nicked β₂-GPI and the fifth kringle domain of Glu-plasminogen. Nicked β₂-GPI also suppressed plasmin generation up to 70% in the presence of tissue plasminogen activator, plasminogen, and fibrin. In fact, β₂-GPI lacks these properties. These data suggest that β₂-GPI/plasmin-nicked β₂-GPI controls extrinsic fibrinolysis via a negative feedback pathway loop. (Blood. 2004; 103:3766-3772)

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Introduction

β₂-Glycoprotein I (β₂-GPI), also known as apolipoprotein H, is a phospholipid-binding plasma protein. Phospholipid-bound β₂-GPI is one of the major target antigens for antiphospholipid antibodies present in patients with antiphospholipid syndrome (APS), an autoimmune disorder characterized by arterial/venous thrombosis and pregnancy morbidity. β₂-GPI has 5 homologous short consensus repeats, designated as domains I to V. Domains of β₂-GPI structurally resemble each other, except that domain V has an extra C-terminal loop and a positively charged lysine cluster. In 1993, Hunt et al reported that β₂-GPI is proteolytically cleaved between Lys317 and Thr318 in domain V (nicked β₂-GPI), being unable to bind to phospholipids. This cleavage is generated by factor Xa or plasmin, with plasmin being more effective.

A large number of reports have detailed the in vitro properties of β₂-GPI as a natural anticoagulant/procoagulant regulator by inhibiting phospholipid-dependent reactions, such as prothrombinase and tenase activity on platelets or phospholipid vesicles, factor XII activation, and anticoagulant activity of activated protein C. Apart from specific hemostatic functions, β₂-GPI activates lipoprotein lipase, lowers the triglyceride level, binds to oxidized low-density lipoprotein to prevent the progression of atherosclerosis, and binds to nontissue fibrin or apoptotic bodies to allow their clearance. Little attention has been given to the functions of the nicked form of β₂-GPI because its phospholipid-binding activity was thought to exert the physiologic or pathologic functions of β₂-GPI.

Fibrinolytic reactions involve the formation of plasmin from the zymogen plasminogen and the hydrolytic cleavage of fibrin to fibrin degradation products by plasmin. Plasminogen, a 92-kDa glycoprotein, is present in plasma at a concentration of approximately 2 μM. Plasminogen consists of 7 domains: one N-terminal peptide, 5 kringle domains bearing a lysine-binding site (LBS) with the capacity to bind fibrin as well as antifibrinolytic proteins carrying lysine, and one serine protease domain. Plasmin conversion from plasminogen by tissue plasminogen activator (tPA) is a key event in extrinsic fibrinolysis for the thrombolysis against intravascular blood clots. Plasmin is one of the most potent enzymes and has a variety of biologic activities; thus, the regulation of plasmin generation and activity is important to maintain the homeostatic balance in vivo. In particular, an excess of fibrinolytic activity can lead to life-threatening bleeding events. Physiologic inhibitors of extrinsic fibrinolysis include α₂-antiplasmin (α₂-AP) and plasminogen activator inhibitor 1 (PAI-1). These inhibitors regulate fibrinolysis through different mechanisms.

Nicked β₂-GPI has been identified by sandwich enzyme-linked immunosorbent assay (ELISA) in plasma of patients with disseminated intravascular coagulation (DIC) or leukemia, both conditions characterized by massive thrombin generation and fibrinolytic...
turnover. To investigate the biologic and clinical significance of nicked β2-GPI in a disease characterized by a lower level of thrombin generation and fibrin turnover than DIC, we evaluated the cleavage ratio of β2-GPI in plasma of patients with ischemic stroke and the results are presented herein. Further, we investigated the role of nicked β2-GPI in extrinsic fibrinolysis and demonstrate for the first time that nicked β2-GPI binds to plasminogen. We also describe the inhibitory effect of nicked β2-GPI on the fibrin surface where plasminogen is proteolytically activated into plasmin. Because β2-GPI may be cleaved in vivo by plasmin during thrombus formation and thrombolysis, these phenomena represent a novel negative feedback loop in extrinsic fibrinolysis where β2-GPI plays a key role.

Patients, materials, and methods

Study patients

The study population comprised 62 patients with history of ischemic stroke diagnosed by magnetic resonance imaging (MRI) performed at the time of admission to the Azabu Neurosurgical Hospital (female-to-male ratio, 12:50; mean age, 68 ± 9 years). Blood samples were obtained from the patients at least 6 months after their last occlusive event.

We also investigated 130 age- and sex-matched apparently healthy subjects with no history of cerebral infarct who consented to join the study. All subjects underwent a cerebral MRI at the Neuroradiology Department at Mitsui Memorial Hospital and images were analyzed by an experienced neuroradiologist. According to the MRI findings the healthy subjects were divided into two groups: 52 with lacunar infarcts (female-to-male ratio, 20:32; mean age 67 ± 9 years) and 78 without any abnormality (female-to-male ratio, 26:52; mean age, 66 ± 6 years). Blood sampling was performed at the same time of the MRI scan. All the patients and healthy volunteers provided informed consent according to Declaration of Helsinki principles.

Blood collection

Venous blood was collected in tubes containing one-tenth volume of 0.105 M sodium citrate and was centrifuged immediately at 4 °C. Plasma samples were depleted of platelets by filtration then stored at −70 °C until use.

Materials

Monoclonal antibodies. To measure the plasma levels of nicked or total β2-GPI, we used 2 monoclonal antibodies, 1 monoclonal anti-nicked β2-GPI antibody (NGP1-60) that specifically reacts against nicked β2-GPI and the other monoclonal anti-β2-GPI antibody (NGP2-23) that equally reacts with nicked and intact β2-GPI.21 An IgG mouse monoclonal antihuman β2-GPI antibody directed to domain III of human β2-GPI (Cof-22) was used for the purification of nicked β2-GPI and evaluation of the binding of nicked β2-GPI to immobilized Glu-plasminogen.24 Cleavage of β2-GPI by plasmin did not affect the binding of Cof-22 to β2-GPI because the epitope of Cof-22 antibody on β2-GPI molecule resides on domain III (data not shown).

Antihuman plasminogen antibodies directed to kringle 1 to 3 or against kringle 4 were obtained from American Diagnostica (Greenwich, CT).

Proteins. β2-GPI was purified from human plasma, as described.25 Nicked β2-GPI was prepared as reported25 with slight modifications that included an additional purification step. β2-GPI was treated with human plasmin (Calbiochem Novabiochem, La Jolla, CA) at 37 °C for 3 hours, at a molar ratio of β2-GPI/plasmin of 8:1. Plasmin-treated β2-GPI was first purified on a Cof 22-Sepharose column and subsequently on a heparin-Sepharose column. The heparin nonbinding fraction was collected and further purified by ion-exchange chromatography using Mono-Q column (Pharmacia Biotech, Uppsala, Sweden). Purified β2-GPI was reduced using 2-mercaptoethanol and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), appearing as a single band smaller than that of the intact one (data not shown).

The domain V–deleted mutant protein (domains I-IV) of β2-GPI was expressed using a baculovirus system as reported.24 This mutant β2-GPI does not include the cleavage site for plasmin.

Glu-plasminogen was purified from the plasma of healthy Japanese donors using chromatography on lysine-Sepharose 4B (Pharmacia Biotech) and diethylaminoethyl (DEAE) Sephadex A-50 (Pharmacia Biotech). Plasminogen kringle 1 to 3 fragment, plasminogen kringle 4 fragment, and mini-plasminogen, which consists of the kringle 5 and serine protease domain of plasminogen, were obtained from Technoclone (Vienna, Austria). Recombinant t-PA (2-chain, Duteplase) was obtained from Sumitomo Pharmaceutical (Osaka, Japan). 3-Aminopropionic acid (EACA) was purchased from Sigma Chemical (St Louis, MO).

Methods

Measurement of plasma levels of nicked β2-GPI. Plasma levels of nicked β2-GPI were determined by a sandwich ELISA as previously described with slight modifications.25 Briefly, polystyrene microtiter plates were coated with 100 μL monoclonal anti-nicked β2-GPI antibody (NGP1-60) in 50 mM Tris (trishydroxymethylaminomethane)–HCl, pH 7.5, containing 0.15 M NaCl and incubated overnight at 4 °C. Wells were washed 3 times with 0.5 M NaCl containing 0.05% Tween 20 and 100 μL citrated plasma samples diluted 5-fold in 20 mM Tris-HCl, pH 7.5, containing 0.5 M NaCl and 0.05% Tween 20 (sample buffer) were added. After 2 hours of incubation at room temperature and washing 3 times, 100 μL biotinylated F(ab')2 fragment of monoclonal anti-β2-GPI (NGP2-23; 2 μg/mL) was added to each well, followed by 1 hour of incubation. Then, 100 μL alkaline phosphatase (ALP)–conjugated streptavidin (Zymed, San Francisco, CA) at a 1:1000 dilution in sample buffer was added to each well. After another 1 hour of incubation and 3 times washing, 200 μL substrate (1 mg/mL 3-nitrophenylphosphate disodium [Sigma Chemical] in 1 M diethanolamine buffer [pH 9.8]) was added. Optical density (OD) was read at 492 nm with reference at 620 nm using an ELISA plate reader. The plasma levels of nicked β2-GPI were determined from a standard curve constructed with citrated plasma spiked with known amounts of purified nicked β2-GPI.

Measurement of plasma levels of total β2-GPI. Plasma levels of total β2-GPI were determined by a sandwich ELISA using F(ab')2 fragment of NGP1-60 as the capture antibody and biotinylated antihuman β2-GPI rabbit IgG as the tag antibody as previously reported.23 Plasma samples of 50 μL (8000-fold diluted) were added to the wells containing the immobilized antibody. The ALP-conjugated streptavidin (Zymed) was then added and bound ALP was determined as described (“Measurement of plasma levels of nicked β2-GPI”). The amounts of total β2-GPI in plasma were calculated from a calibration curve constructed with known amounts of purified total β2-GPI. A nicked β2-GPI ratio was calculated in all samples using the formula: (plasma nicked β2-GPI/plasma total β2-GPI) × 1000.

Other laboratory investigations. The same plasma samples were tested for thrombin-antithrombin (TAT) complexes, plasmin-antiplasmin (plasmin inhibitor) complex (PPI), and D-dimers (DDs) by latex agglutination assay using commercial kits LIAPACE TAT, LIAACE PPI, LIAPACE D-D dimer (Dia-Iatron, Tokyo, Japan), according to the manufacturer’s instructions.

ELISA for binding of intact or nicked β2-GPI to plasminogen. The binding of nicked or intact β2-GPI was investigated by ELISA. Fifty microliters of Glu-plasminogen (10 μg/mL) in phosphate-buffered saline (PBS), pH 7.4, was distributed in each well of a 96-well format microtiter ELISA plate (Sumitomo Bakelite, Tokyo, Japan) and incubated overnight at 4 °C. After washing twice with PBS and blocking with 2% gelatin–PBS for 1 hour at 37 °C, 50 μL of serial dilutions of intact or nicked β2-GPI in 1% bovine serum albumin (Sigma Chemical)–PBS (1% BSA–PBS) were placed in each well. Plates were incubated for 1 hour at room temperature and washed 3 times with PBS containing 0.05% Tween 20 (PBS-Tween), then 50 μL/well Cof-22 (100 ng/mL) in 1% BSA–PBS was distributed. After incubation and washing as above, 50 μL/well of ALP-conjugated antihuman IgG (Sigma Chemical), diluted 1:2000 in 1% BSA–PBS, was put into each well, followed by incubation. Substrate (100 μL) was distributed after washing 4 times with PBS–Tween and incubated. OD was read at 405 nm with reference at 620 nm.
The inhibition of mini-plasminogen with that of kringle 4. After incubation antikringle 1 to 3 antibody (American Diagnostica) was used to compare mini-plasminogen, a monoclonal antikringle 4 antibody (American Diagnostica) for 30 minutes at room temperature, and plates were washed 3 times with PBS-Tween and blocking with 2% gelatin-PBS at 37°C overnight, followed by washing with plate (Sumitomo Bakelite) was coated with soluble fibrin. Glu-plasminogen to immobilized fibrin. The inhibition of Glu-plasminogen binding by fragments of plasminogen was examined. Fifty microliters of nicked Glu-plasminogen (0.2 μM) diluted in PBS was put into each well of a MaxiSorp microtiter plate (Nalge Nunc International, Roskilde, Denmark) and incubated overnight at 4°C. After washing twice with PBS and blocking with 2% gelatin-PBS for 1 hour at 37°C, serial dilutions of inhibitor (BSA, plasminogen kringle 1-3, plasminogen kringle 4, or mini-plasminogen) were added (50 μL/well) followed by overnight incubation at 4°C. After washing with PBS-Tween, 10 μg/mL Glu-plasminogen was then added (50 μL/well) and incubated for 30 minutes at room temperature, and plates were washed 3 times with PBS-Tween. To compare the inhibitory effect between kringle 1 to 3 and mini-plasminogen, a monoclonal antikringle 4 antibody (American Diagnostics) was used to detect bound Glu-plasminogen, whereas a monoclonal antikringle 1 to 3 antibody (American Diagnostics) was used to compare the inhibition of mini-plasminogen with that of kringle 4. After incubation with these monoclonal antibodies, bound Glu-plasminogen on nicked β2-GPI was evaluated by ALP-conjugated antimonous IgG, followed by substrate addition as described (“ELISA for binding of intact or nicked β2-GPI to plasminogen”).

Inhibitory effect of nicked β2-GPI on the binding of plasminogen to fibrin. To investigate whether nicked β2-GPI interferes with the binding of Glu-plasminogen to immobilized fibrin in a liquid phase or not, the following experiment was done. Each well of a 96-well U-bottom microplate (Sumitomo Bakelite) was coated with soluble fibrin monomer (5 μg/mL) and incubated at 4°C overnight, followed by washing with PBS-Tween and blocking with 2% gelatin-PBS at 37°C. Biotinylated Glu-plasminogen (5 μg/mL, in 1% BSA-PBS) was preincubated with different concentrations of intact or nicked β2-GPI for 1 hour at room temperature and added to the wells in triplicate. After incubation for 1 hour at room temperature, each well was washed with PBS-Tween. ALP-conjugated streptavidin was diluted to 3000 times in PBS and distributed to the wells. After 1 hour of incubation and washing, substrate was added and absorbance was measured as described.

Effects of intact or nicked β2-GPI on tPA activity: chromogenic assay. In the presence of fibrin, tPA can effectively activate plasminogen to plasmin. Because we speculated that nicked β2-GPI might interfere with this activation step by binding to plasminogen, chromogenic assay measuring plasmin generation was introduced in the presence of tPA, Glu-plasminogen, fibrin monomer, and β2-GPI. The effect of intact/nicked β2-GPI on the activity of plasmin generated was evaluated using a parabolic rate assay. The activity of tPA was measured in a chromogenic assay as described27 with some modifications. A mixture of the same volume of 50 U/mL tPA in PBS and 1 M acetic acid buffer (pH 3.9) was incubated for 5 minutes at room temperature, then diluted 1:160 with assay buffer (50 mM Tris-HCl, pH 8.8, 100 mM NaCl, and 0.01% Triton X-100). Then 100 μL of the diluted tPA solution was incubated in a Sulfinol Type S microtiter plate with 100 μL detection reagents consisting of Glu-plasminogen and plasmin-sensitive substrate (Glu-plasminogen [70 μg/mL] and 0.6 mM chromogenic substrate S-2251 [Chromogenix, Mönland, Sweden] in assay buffer) with intact or nicked β2-GPI and 2 μL/well soluble fibrin monomer (3.3 mg/mL, in 3.5 M urea). The final concentrations of intact/nicked β2-GPI were 0.25, and 0.5 μM. Domain I to IV of β2-GPI mutant or BSA served as the negative control. After incubation at 37°C for 12 hours, the activity of plasmin generated was determined by measuring absorbance at 405 nm using a microplate reader (model 3550; BioRad, Hercules, CA). A standard curve was generated using serial dilutions of tPA. The plasmin generation in this system was expressed as corresponding tPA activity (U/mL).

Effects of intact or nicked β2-GPI on tPA activity: fibrin plate assay. To exclude the possibility that nicked β2-GPI affected S-2251 cleavage without interacting with fibrinolytic factors, fibrinolysis was evaluated by conventional fibrin plate assays. Fibrin was layered on a plastic plate 10 cm in diameter, using the same volumes of 0.2% plasminogen-free fibrin (Sigma Chemical), 1% agarose, and 200 U/plate thrombin, 20 U/mL. Then, 6 μL of the diluted tPA solution ("Effects of intact or nicked β2-GPI on tPA activity: chromogenic assay") was incubated with the same volume of Glu-plasminogen (70 μg/mL) in assay buffer, with intact or nicked β2-GPI (up to 0.5 μM). After 36 hours of incubation at 37°C, the area of lysis rings was measured. A standard curve was generated from serial dilutions of tPA.

Statistical analysis. Statistical evaluation was performed by the t test, Fisher exact test, x2 test, or Spearman rank correlation as appropriate. P values less than .05 were considered statistically significant.

Results
Levels of nicked β2-GPI in plasma samples
The plasma levels of nicked β2-GPI ratio were shown in Figure 1. A normal level of nicked β2-GPI ratio was derived from the apparently healthy subjects without any MRI abnormality, the mean plus 1 SD representing the upper limit of normal. A higher prevalence of elevated nicked β2-GPI ratio was found in patients with ischemic stroke (63%, 39 of 62) and healthy subjects with lacunar infarct (27%, 14 of 52) when compared to healthy subjects with normal MRI findings (8%, 6 of 78). Relative risks of having stroke or asymptomatic lacunar infarction were approximated by odds ratio (95% CI), 20.3 (7.6-54.2) and 4.4 (1.6-12.4), respectively.

The prevalence of elevated levels of markers of thrombin generation and fibrinolytic turnover in our population are shown in Figure 2. A statistically significant correlation was observed between levels of PPI and nicked β2-GPI ratio in plasma of healthy subjects with lacunar infarct (r2 = 0.31, P = .02). No correlations were found between nicked β2-GPI ratio and DDs or TAT complexes in any of the groups.

Figure 1. Plasma levels of nicked β2-GPI. Total and nicked β2-GPI plasma levels were determined by ELISA. A nicked β2-GPI ratio (plasma total β2-GPI/plasma total β2-GPI) × 1000, was established in all the samples. The dashed line indicates the mean + 1 SD of the ratio in healthy subjects without lacunar infarct. P values were calculated using t test.

P < .0001
P = .0002
P = .042

Figure 2. A statistically significant correlation was observed between levels of PPI and nicked β2-GPI ratio in plasma of healthy subjects with lacunar infarct (r2 = 0.31, P = .02). No correlations were found between nicked β2-GPI ratio and DDs or TAT complexes in any of the groups.
In the apparently healthy subjects group \((n = 130)\), plasma nicked \(\beta_2\)-GPI ratio significantly correlated with age \((r^2 = 0.483, P < .0001; \text{Figure 3})\). Therefore, plasma measurement of nicked \(\beta_2\)-GPI might be a useful screening tool in the assessment of patients at risk of ischemic stroke.

### Binding of nicked \(\beta_2\)-GPI to Glu-plasminogen

The binding of up to 0.4 \(\mu\)M nicked \(\beta_2\)-GPI to solid-phase Glu-plasminogen occurred in a dose-dependent manner, whereas the same concentrations of intact \(\beta_2\)-GPI did not bind to Glu-plasminogen (Figure 4A). The binding of Cof-22 to \(\beta_2\)-GPI was not affected by the cleavage of \(\beta_2\)-GPI. Molecular interaction between intact or nicked \(\beta_2\)-GPI and plasminogen was investigated using an optical biosensor. Nicked \(\beta_2\)-GPI showed a large extent of binding to immobilized Glu-plasminogen, whereas intact \(\beta_2\)-GPI did not show any specific binding (Figure 4B). The data of \(k_\text{on}\) at different concentrations of nicked \(\beta_2\)-GPI were fitted using linear regression, determining \(k_\text{on}\) as \(0.0006 \text{ M}^{-1}\text{s}^{-1}\) and \(k_\text{off}\) as \(0.0022 \text{ s}^{-1}\) (Figure 4C). Accordingly, \(k_\text{D}\) and \(k_\Lambda\) were determined as \(0.37 \times 10^{-6} \text{ M}^{-1}\) and \(2.70 \times 10^6 \text{ M}^{-2}\) respectively.

### Inhibition of binding of Glu-plasminogen to nicked \(\beta_2\)-GPI by the fragments of plasminogen or by EACA

The binding of Glu-plasminogen (10 \(\mu\)g/mL) to immobilized nicked \(\beta_2\)-GPI, but not to native \(\beta_2\)-GPI, was demonstrated by ELISA. For the inhibition assay, the fragments of plasminogen (mini-plasminogen or kringle 4) as the inhibiting factors were added to the wells coated with nicked \(\beta_2\)-GPI, and bound Glu-plasminogen was detected using a monoclonal antikringle 1 to 3 antibody. Mini-plasminogen, but not kringle 4, inhibited the binding between Glu-plasminogen and nicked \(\beta_2\)-GPI (Figure 5A). Kringle 1 to 3 fragment or mini-plasminogen was added as inhibitor and bound Glu-plasminogen was detected using a monoclonal antikirgine 4 antibody. Glu-plasminogen binding to nicked \(\beta_2\)-GPI was dose dependently inhibited by mini-plasminogen but not by kringle 1 to 3 fragment (Figure 5B). The fifth domain or the catalytic domain of Glu-plasminogen, therefore, was predicted to mediate its binding to nicked \(\beta_2\)-GPI.

When the binding of nicked \(\beta_2\)-GPI (10 \(\mu\)g/mL) to solid-phase Glu-plasminogen was tested in the presence of different concentrations of EACA, the binding between nicked \(\beta_2\)-GPI and immobilized Glu-plasminogen was abolished in a dose-dependent manner (Figure 5C). Accordingly, LBS on plasminogen might mediate the binding of nicked \(\beta_2\)-GPI to Glu-plasminogen.

### Binding of plasminogen to fibrin interfered with by nicked \(\beta_2\)-GPI

We also investigated whether nicked \(\beta_2\)-GPI has an effect on the binding of Glu-plasminogen to immobilized fibrin monomer using an ELISA system. After preincubation with nicked \(\beta_2\)-GPI, but not with intact \(\beta_2\)-GPI, Glu-plasminogen showed decreased binding activity to soluble fibrin monomer (Figure 5D).

### Effects of nicked \(\beta_2\)-GPI on extrinsic fibrinolysis

The amidolytic activity of newly generated plasmin was evaluated as tPA activity (U/mL) in a chromogenic assay. The activity increased with the concentration of tPA (data not shown). When nicked \(\beta_2\)-GPI was added, the tPA activity decreased in a dose-dependent manner (Figure 6A). Intact \(\beta_2\)-GPI at 0.25 \(\mu\)M did not suppress the fibrinolytic activity, whereas intact \(\beta_2\)-GPI in a higher concentration (0.50 \(\mu\)M) slightly suppressed the fibrinolytic activity. The same amount of BSA or the recombinant domain I to IV of \(\beta_2\)-GPI did not affect the tPA activity.

The fibrinolytic activity of generated plasmin was measured as tPA activity (U/mL) in a fibrin plate assay. Fibrinolytic activity was suppressed by nicked \(\beta_2\)-GPI at 0.25 and 0.50 \(\mu\)M. Intact \(\beta_2\)-GPI at 0.50 \(\mu\)M also slightly inhibited the fibrinolytic activity. However, 0.25 \(\mu\)M intact \(\beta_2\)-GPI did not affect the fibrinolytic activity of tPA (Figure 6B).

### Discussion

In the first part of this study, we demonstrated that plasma levels of nicked \(\beta_2\)-GPI were elevated in patients with ischemic stroke, indicating an elevated degree of fibrin turnover, but lower than that of DIC where thrombin and plasmin are massively generated.

In fact, nicked \(\beta_2\)-GPI was detected in large quantities in plasma of patients with DIC, a pathologic state characterized by marked increase of plasma PPl.\(^2\) We observed a strong correlation between plasma levels of nicked \(\beta_2\)-GPI and those of PPI in the healthy individuals showing lacunar infarcts on MRI, suggesting that nicked \(\beta_2\)-GPI may rather reflect “minor” plasmin generation. In the presence of larger plasmin generation, the correlation between nicked \(\beta_2\)-GPI and PPI may be lost,\(^3\) presumably due to the consumption of \(\alpha_2\)-AP. In individuals with MRI abnormalities the prevalence of increased nicked \(\beta_2\)-GPI ratio was higher than that of PPI, DDs, and TAT complexes (46%, 27%, 19%, and 11%, respectively). Thus, the detection of nicked \(\beta_2\)-GPI may...
Figure 4. Binding of intact/nicked β2-GPI to Glu-plasminogen. (A) Binding of intact or nicked β2-GPI to immobilized Glu-plasminogen was evaluated by ELISA using mouse monoclonal anti-β2-GPI antibody Cof-22. Closed circles indicate the dose-dependent binding of nicked β2-GPI to Glu-plasminogen, whereas open circles indicate that intact β2-GPI is unable to bind to Glu-plasminogen. (B-C) Kinetic plot showing molecular interaction between Glu-plasminogen and intact or nicked β2-GPI. Intact β2-GPI or nicked β2-GPI binding to Glu-plasminogen was detected using iAsys, an optical biosensor as described in “Materials, patients, and methods.” Binding extent (arc sec) was compared between intact and nicked β2-GPI. A formula for determining the association rate constant (k_{on}) and dissociation rate constant (k_{off}) is as follows: k_{on} = k_{on} + k_{off} [Igand]. Error bars indicate SDs.

Figure 5. Identification of the binding site of Glu-plasminogen to β2-GPI by inhibition ELISA using plasminogen fragments. (A) Binding of Glu-plasminogen to immobilized nicked β2-GPI was tested by ELISA in the presence of possible inhibitors. After nicked β2-GPI immobilization onto microtiter plates, different concentrations of kringle 4 of plasminogen (C) or mini-plasminogen (that consists of kringle 5 and catalytic domain of plasminogen) were added as inhibitors. BSA (B) served as control. After incubation and washing, Glu-plasminogen (10 μg/mL) was added and bound Glu-plasminogen was determined using kringle 1- to 3-specific mouse monoclonal antiplasminogen antibody. (B) For the inhibition ELISA kringle 1 to 3 of plasminogen (C) or mini-plasminogen (D) served as inhibitors. Glu-plasminogen bound to immobilized β2-GPI was detected using kringle 4-specific mouse monoclonal antiplasminogen antibody. Assays were run in triplicate. (C) Competitive ELISA using EACA, a lysine homologue. Binding of nicked β2-GPI (0.2 μM) to immobilized Glu-plasminogen was tested by ELISA using Cof-22 antibody in the presence of various concentrations of EACA (0-0.2 μg/mL). (D) Soluble fibrin monomer (5 μg/mL) was coated on the surface of a microtiter plate and blocked. Biotinylated Glu-plasminogen (5 μg/mL) was preincubated with intact or nicked β2-GPI and added to the wells. After incubation and washing, ALP-conjugated streptavidin was used for detection. Assays were run triplicate. Error bars indicate SDs. K indicates kringle; mini-plg, mini-plasminogen.

represent a more sensitive marker of vascular lesions than PPI, DDs, or TAT complexes.

In support of this concept is the correlation between nicked β2-GPI ratio and age in the apparently healthy subjects, suggesting that “minor” plasmin generation might be associated with subclinical or early clinical atherosclerosis. It is widely accepted that atherosclerosis is associated with endothelial cell activation and minor plaque rupture leading to small thrombus formation, secretion of t-PA, and plasmin generation, ultimately cleaving minor plaque rupture leading to small thrombus formation, secreting a more sensitive marker of vascular lesions than PPI, DDs, or TAT complexes.

Under clinical conditions characterized by massive plasminogen-mediated plasmin generation and plasmin generation, ultimately cleaving minor plaque rupture leading to small thrombus formation, secreting a more sensitive marker of vascular lesions than PPI, DDs, or TAT complexes.

Indeed, nicked β2-GPI can be generated on the surface of activated endothelial cells or platelets.23

In the second part of this study, we investigated the properties of nicked β2-GPI in vitro to evaluate the biologic significance of our observations. We showed that nicked β2-GPI specifically binds to Glu-plasminogen and inhibits extrinsic fibrinolysis in vitro. In contrast, neither domain I to IV of β2-GPI nor intact β2-GPI revealed such functions. The administration of intact β2-GPI in higher concentrations also suppressed plasmin generation, perhaps owing to the nicked β2-GPI produced by the newly generated plasmin. Under clinical conditions characterized by massive plasmin generation such as DIC or acute thrombosis, plasmin is generated by tPA released from activated endothelial cells with thrombus formation, and plasmin cleaves β2-GPI on the thrombus, changing the properties of β2-GPI. We propose that β2-GPI is a precursor of plasmin-nicked β2-GPI, a physiologic inhibitor of fibrinolysis.

The crystal structure of human β2-GPI has been defined.28,29 Bouma et al.28 proposed that a large positively charged patch in domain V binds to anionic surfaces with a flexible and partially hydrophobic loop inserted into the lipid layer. According to the conformation of the nicked domain V, as predicted from the x-ray structure of the intact domain V and confirmed by heteronuclear magnetic resonance, the nicked C-terminal loop is tightly fixed by electrostatic interaction with enhanced stability, the result being neutralization of the positive charge of the lysine cluster.26,30

Glu-plasminogen, a full-length protein, is the naturally circulating form of plasminogen. Kringle 5 of Glu-plasminogen has a higher affinity for intact fibrin.31,32 LBS in kringle 5 of Glu-plasminogen mediates its binding to N-terminal lysine on fibrin, an event essential to initiate fibrinolysis reactions. This initial binding of Glu-plasminogen to fibrin induces a conformational change from a “closed” to an “open” form, thus promoting activity to plasminogen activators such as tPA or urokinase.19 On the fibrin surface, generated plasmin cleaves the single-chain tPA into the 2-chain tPA, a more active form, providing a positive feedback for plasmin generation. Plasmin simultaneously degrades fibrin and makes C-terminal lysine of fibrin more accessible to plasminogen via kringle 1,33,34 2, and 3,39 thus accelerating fibrinolysis.

According to the results of the inhibition studies using plasminogen fragments or EACA (Figure 5), and comparison of the effect on plasmin generation between nicked β2-GPI and domain I to IV of β2-GPI (Figure 6A), it would be indicated that the binding of nicked β2-GPI to Glu-plasminogen is mediated by the interaction between the lysine-cluster patch in domain V of the nicked β2-GPI and LBS on the plasminogen kringle 5,36 although it still may be possible that an excess amount of EACA interacts with the catalytic domain of Glu-plasminogen. The conformational difference between intact and nicked β2-GPI is critical for its binding to

A formula for determining the association rate constant (k_{ass}) and dissociation rate constant (k_{diss}) is as follows: k_{ass} [ligand]. Error bars indicate SDs.
phospholipid or plasminogen. The lysine-cluster patch in domain V of nicked \( \beta_2 \)-GPI may gain accessibility for the LBS of Glu-plasminogen, whereas the C-terminal loop of intact \( \beta_2 \)-GPI may interfere with interactions of LBS and the Glu-plasminogen kringle 5.

The fibrinolytic system is regulated at different levels, either at plasminogen activation or at enzymatically active plasmin. Many factors, including \( \alpha_2 \)-AP, \( \alpha_2 \)-macroglobulin, \( \alpha_1 \)-antitrypsin, inactivated C1, PAI-1, and PAI-2, prevent the overactivation of the fibrinolytic system. The most potent inhibitors are \( \alpha_2 \)-AP and PAI-1\(^{17} \); the former binds to a component of kringle 1 to 3 of plasminogen\(^{18} \) and can neutralize the generated plasmin more rapidly than \( \alpha_2 \)-macroglobulin.

Fibrinolysis initiates on binding of kringle 5 of plasminogen to lysine residues on fibrin followed by the binding of kringle 1 to 3 of plasminogen to lysine residues on the cleaved fibrin. \( \alpha_2 \)-AP does not bind to kringle 5 of plasminogen, hence, does not seem to affect the first interaction. Based on the observation that nicked \( \beta_2 \)-GPI interferes the binding between Glu-plasminogen and fibrin monomer (Figure 5D), it is likely that the binding of nicked \( \beta_2 \)-GPI to Glu-plasminogen affects the first step of fibrinolysis at least and exerts an inhibitory function in the fibrinolytic system via different mechanisms from that of \( \alpha_2 \)-AP.

In conclusion, first we have demonstrated that plasma levels of nicked \( \beta_2 \)-GPI can be a sensitive marker of cerebral ischemic events and we suggest that plasma measurement of nicked \( \beta_2 \)-GPI might be a useful screening tool in the assessment of patients at risk of ischemic stroke. Second, we propose that nicked \( \beta_2 \)-GPI is a physiologic inhibitor of fibrinolysis and that plasmin cleavage of \( \beta_2 \)-GPI is part of the negative feedback pathway of extrinsic fibrinolysis.

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