Nicked beta2-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 (β2GPI) is proteolytically cleaved by plasmin in domain V (nicked β2GPI), being unable to bind to phospholipids. This cleavage may occur \textit{in vivo} and elevated plasma levels of nicked β2GPI were detected in patients with massive plasmin generation and fibrinolysis turnover. In this study, we reported higher prevalence of elevated ratio of nicked β2GPI against total β2GPI in patients with ischemic stroke (63%) and healthy subjects with lacunar infarct (27%) when compared to healthy subjects with normal MRI findings (8%), suggesting that nicked β2GPI might have a physiological 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 demonstrated that nicked β2GPI binds to Glu-plasminogen with $K_D$ of $0.37 \times 10^{-6}$ M, presumably mediated by the interaction between the fifth domain of nicked β2GPI and the fifth kringle domain of Glu-plasminogen. Nicked β2GPI also suppressed plasmin generation up to 70% in the presence of tissue plasminogen activator, plasminogen, and fibrin. Intact β2GPI lacks these properties. These data suggest that β2GPI/plasmin-nicked β2GPI control extrinsic fibrinolysis via a negative feedback pathway loop.
Introduction

β2-glycoprotein-I (β2GPI), also known as apolipoprotein H, is a phospholipid-binding plasma protein. Phospholipid-bound β2GPI is one of the major target antigens for antiphospholipid antibodies \(^1\)-\(^3\) present in patients with antiphospholipid syndrome (APS), an autoimmune-disorder characterized by arterial/venous thrombosis and pregnancy morbidity \(^4\). β2GPI has five homologous short consensus repeats, designated as domains I-V. Domains of β2GPI 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. \(^5\) reported that β2GPI is proteolytically cleaved between Lys-317 and Thr-318 in domain V (nicked β2GPI), being unable to bind to phospholipids. This cleavage is generated by factor Xa or by plasmin, the latter being more effective \(^6\).

A large number of reports have detailed the \textit{in vitro} properties of β2GPI as a natural procoagulant/anticoagulant regulator by inhibiting phospholipid dependent reactions, such as prothrombinase and tenase activity on platelets or phospholipid vesicles \(^7\)-\(^8\), factor XII activation \(^9\) and anticoagulant activity of activated protein C \(^10\)-\(^11\). Apart from specific haemostatic functions, β2GPI activates lipoprotein lipase \(^12\), lowers triglyceride level \(^13\), binds to oxidized low-density lipoprotein to prevent the progression of atherosclerosis \(^14\), and binds to non-self particles or apoptotic bodies to allow their clearance \(^15\)-\(^17\). Little attention has been given to the functions of the nicked form of β2GPI since its phospholipid-binding activity was thought to exert the physiological or pathological functions of β2GPI.

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 \(^{18}\). Plasminogen consists of seven domains; one N-terminal peptide, five kringle domains bearing a lysine-binding site (LBS) with the capacity to bind fibrin as well as anti-fibrinolytic proteins carrying lysine, and one serine protease domain \(^{19}\). 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 biological activities, thus the regulation of plasmin generation and activity is important to maintain the homeostatic balance \textit{in vivo}. In particular, an excess of fibrinolytic activity can lead to life threatening bleeding events. Physiological inhibitors of extrinsic fibrinolysis include \(\alpha_2\)-antiplasmin (\(\alpha_2\)AP) \(^{20}\) and plasminogen activator inhibitor-1 (PAI-1) \(^{21}\). These inhibitors regulate fibrinolysis through different mechanisms.

Nicked \(\beta_2\)GPI has been identified by sandwich enzyme-linked immunosorbent assay (ELISA) in plasma of patients with disseminated intravascular coagulation (DIC) \(^{22}\) or leukemia \(^{23}\), both conditions characterized by massive thrombin generation and fibrinolytic turnover. To investigate the biological and clinical significance of nicked \(\beta_2\)GPI in a disease characterized by a lower level of thrombin generation and fibrin turnover than DIC, we evaluated the cleavage ratio of \(\beta_2\)GPI in plasma of subjects with ischemic stroke and the results are presented herein. Further, we investigate the role of nicked \(\beta_2\)GPI in extrinsic fibrinolysis and demonstrate for the first time that nicked \(\beta_2\)GPI binds to plasminogen. We also describe the inhibitory effect of nicked \(\beta_2\)GPI on the fibrin surface where plasminogen is proteolytically activated into plasmin. As \(\beta_2\)GPI may be cleaved \textit{in vivo} by plasmin during thrombus formation and thrombolysis, these
phenomena represent a novel negative feedback loop in extrinsic fibrinolysis where β2GPI plays a key role.
Patients and Methods

Study subjects

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: male 12:50, mean age 68 ± 9 years). Blood samples were obtained from the patients at least six months after their last occlusive event.

We also investigated 130 age/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: male 20:32, mean age 67 ± 9 years) and 78 without any abnormality (female: male 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 1/10 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 β2GPI, we used two monoclonal antibodies, one monoclonal anti-nicked β2GPI antibody (NPGI-60) that specifically reacts against nicked β2GPI and the other monoclonal anti-β2GPI antibody (NGPI-23) that equally reacts with nicked and intact β2GPI 

An IgG mouse monoclonal anti-human β2GPI antibody directed to domain III of human β2GPI (Cof-22) was employed for the purification of nicked β2GPI and evaluation of the binding of nicked β2GPI to immobilized Glu-plasminogen 

Cleavage of β2GPI by plasmin did not affect the binding of Cof-22 to β2GPI because the epitope of Cof-22 antibody on β2GPI molecule resides on domain III (data not shown).

Anti-human plasminogen antibodies directed to kringle 1-3 or against kringle 4 were obtained from American Diagnostica Inc. (Greenwich, CT, USA)

Proteins

β2GPI was purified from human plasma, as described 
Nicked β2GPI was prepared as reported with slight modifications that included an additional purification step; β2GPI was treated with human plasmin (Calbiochem Novabiochem Corp., La Jolla, CA, USA) at 37°C for 3 hours, at a molar ratio of β2GPI: plasmin of 8:1. Plasmin-treated β2GPI was first purified on a Cof 22-Sepharose column and subsequently on a heparin-Sepharose column. The heparin non-binding fraction was collected and further purified by ion-exchange chromatography using Mono-Q column (Pharmacia-Biotech, Uppsala, Sweden).
Purified β2GPI was reduced using 2-mercaptoethanol and subjected to SDS-PAGE, appearing as a single band smaller than that of the intact one (data not shown).

The domain V-deleted mutant protein (domain I-IV) of β2GPI was expressed using a baculovirus system as reported 24. This mutant β2GPI 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) and DEAE Sephadex A-50 (Pharmacia). Plasminogen kringle 1-3 fragment, plasminogen kringle 4 fragment and mini-plasminogen, that consists of the kringle 5 and serine protease domain of plasminogen, were obtained from Technoclone (Vienna, Austria). Recombinant tissue plasminogen activator (two-chain, Duteplase) was obtained from Sumitomo Pharmaceutical (Osaka, Japan). ε-aminocaproic acid (EACA) was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Methods

Measurement of plasma levels of nicked β2GPI

Plasma levels of nicked β2GPI were determined by a sandwich ELISA as previously described with slight modifications 23. Briefly, polystyrene microtiter plates were coated with 100 µl of monoclonal anti-nicked β2GPI antibody (NGPI-60) in 50 mM Tris-HCl, pH 7.5, containing 0.15 M sodium chloride (NaCl) and incubated overnight at 4°C. Wells were washed three times with 0.5 M NaCl containing 0.05% Tween 20 and 100 µl of 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 three times, 100 µl of biotinylated F(ab')2 fragment of monoclonal anti-β2GPI
(NGPI-23) (2 µg/ml) was added to each well, followed by 1 hour of incubation. One hundred microliters of alkaline phosphatase (ALP)-conjugated goat anti-rabbit Ig (Zymed, San Francisco, CA, USA) at a 1:1000 dilution in samples' buffer were added to each well. After one further hour of incubation and three times washing, 200 µl of substrate [1mg/ml p-nitrophenylphosphate disodium (Sigma Chemical Co.) in 1 M diethanolamine buffer (pH 9.8)] were added. Optical density (OD) was read at 492nm with reference at 620nm using an ELISA plate reader. The plasma levels of nicked β2GPI were determined from a standard curve constructed with citrated plasma spiked with known amounts of purified nicked β2GPI.

**Measurement of plasma levels of total β2GPI**

Plasma levels of total β2GPI were determined by a sandwich ELISA using F(ab’) fragment of NGPI-23 as the capture antibody and biotinylated anti-human β2GPI rabbit IgG as the tag antibody as previously reported. Fifty microliters of plasma samples (8000-fold diluted) were added to the wells containing the immobilized antibody. The ALP-conjugated streptavidin (Jackson Immuno Research Laboratories Inc., Bar Harbor, MA, USA) was then added and bound ALP was determined as described above. The amounts of total β2GPI in plasma were calculated from a calibration curve constructed with known amounts of purified β2GPI. A nicked β2GPI ratio was [(plasma nicked β2GPI/ plasma total β2GPI) x 1000] was calculated in all samples.

**Other laboratory investigations**

The same plasma samples were tested for thrombin-antithrombin-complexes (TAT), plasmin-antiplasmin (plasmin inhibitor)
complex (PPI) and D-dimers (DD) by latex agglutination assay using commercially kits LPIAACE TAT, LPIAACE PPI, LPIAACE D-D dimer (Dia-Iatron Co., LTD. Tokyo, Japan), according to the manufacturer instructions.

**ELISA for binding of intact or nicked β2GPI to plasminogen**

The binding of nicked or intact β2GPI 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 Sumilon Type S 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 one hour at 37 °C, 50 µl of serial dilutions of intact or nicked β2GPI in 1% bovine serum albumin (Sigma Chemical Co.)-PBS (1% BSA-PBS) were placed in each well. Plates were incubated for one hour at room temperature and washed 3 times with PBS containing 0.05% Tween 20 (PBS-Tween), then 50 µl/well of Cof 22 (100 ng/ml) in 1% BSA-PBS was distributed. After incubation and washing as above, 50 µl/well of ALP-conjugated anti-mouse IgG (Sigma Chemical Co.), diluted 1:2000 in 1% BSA-PBS, was put into each well, followed by incubation. One hundred microliters of substrate was distributed after washing 4 times with PBS-Tween and incubated. OD was read at 405 nm with reference at 620 nm.

The role of plasminogen LBS in binding to nicked β2GPI was evaluated by a competitive ELISA adding serial dilutions of EACA, a lysine analogue, into the nicked β2GPI solution.

**Kinetic assay for molecular interaction between nicked β2GPI and plasminogen**
Real time analysis for molecular interaction between nicked β2GPI and Glu-plasminogen was performed using an optical-biosensor, IAsys system (Affinity Sensors, Paramus, NJ, USA). Biotinylated Glu-plasminogen was immobilized on the wall of a Biotin Cuvette (Affinity Sensors) via streptavidin (Sigma). After blocking with 0.01% BSA-PBS and washing with PBS, various concentrations (up to 4µM) of native or nicked β2GPI were placed in the cuvette and ligand bound to the plasminogen-coated surface was detected. Obtained data were fitted using linear regression to find the intercept and gradient. This analysis was used to determine the association rate constant (k_{ass}) and dissociation rate constant (k_{diss}), from the variation of the on-rate constant (k_{on}) with ligand concentration. According to the equation; k_{on} = k_{diss} + k_{ass} [ligand], K_D and K_A are determined as follows; K_D = k_{diss}/k_{ass} and K_A = k_{ass}/k_{diss}.

**Inhibition ELISA**

To identify the nicked β2GPI-binding site on Glu-plasminogen, the inhibition of Glu-plasminogen binding by fragments of Glu-plasminogen was examined. Fifty microliters of nicked β2GPI (0.2 µM) diluted in PBS were 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 wash with PBS-Tween, 10 µg/ml of Glu-plasminogen were 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 1-3 and mini-plasminogen, a
monoclonal anti-kringle 4 antibody (American Diagnostica Inc.) was used to detect bound Glu-plasminogen, whereas a monoclonal anti-kringle 1-3 antibody (American Diagnostica Inc.) 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 β2GPI was evaluated by ALP-conjugated anti-mouse IgG, followed by substrate addition as described above.

Inhibitory effect of nicked β2GPI on the binding of plasminogen to fibrin

To investigate whether nicked β2GPI interferes the binding of Glu-plasminogen to immobilized fibrin in a liquid phase or not, the following experiment was done. Each well of a Sumilon Type S microtiter plate (Sumitomo Bakelite) was coated with soluble fibrin monomer (5µg/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 (5µg/ml in 1% BSA-PBS) was pre-incubated with different concentrations of intact or nicked β2GPI for one hour at room temperature and added to the wells in triplicate. After incubation for one 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 one hour incubation and washing, substrate was added and absorbance was measured as described above.

Effects of intact or nicked β2GPI on tPA activity: chromogenic assay

In the presence of fibrin, tPA can effectively activate plasminogen to plasmin. As we speculated that nicked β2GPI might interfere this activation step by binding to plasminogen, chromogenic assay measuring plasmin generation was
introduced in the presence of tPA, Glu-plasminogen, fibrin monomer, and β2GPI. The effect of intact/nicked β2GPI on the activity of plasmin generated was evaluated using a parabolic rate assay. The activity of tPA was measured in a chromogenic assay as described with some modifications. A mixture of the same volume of 50 U/ml of tPA in PBS and 1M acetate buffer (pH 3.9) was incubated for five minutes at room temperature, then diluted 1 in 160 with assay buffer [50 mM tris-HCl (pH 8.8), 100 mM NaCl and 0.01% Triton X-100]. One hundred microliters of the diluted tPA solution was incubated in a Sumilon Type S microtiter plate with 100 µl of detection reagents consist of Glu-plasminogen and plasmin-sensitive substrate [Glu-plasminogen (70 µg/ml) and 0.6 mM of chromogenic substrate S-2251 (Chromogenix, Möndal, Sweden) in assay buffer] with intact or nicked β2GPI and 2 µl/well of soluble fibrin monomer (3.3 mg/ml, in 3.5 M urea). The final concentrations of intact/nicked β2GPI were 0, 0.25 and 0.5 µM. Domain I-IV of β2GPI 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 (BioRad, Model 3550). A standard curve was generated using serial dilutions of tPA. The plasmin generation in this system was expressed as corresponding tPA activity (Unit/ml)
diluted tPA solution described above were incubated with the same volume of Glu-plasminogen (70 µg/ml) in assay buffer, with intact or nicked β2GPI (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 T-test, Fisher’s exact test, χ² test or Spearman rank correlation as appropriate. P-values < 0.05 were considered statistically significant.

_Results_

_Levels of nicked β2GPI in plasma samples_

The plasma levels of nicked β2GPI ratio are shown in Figure 1. A normal level of nicked β2GPI ratio was derived from the apparently healthy subjects without any MRI abnormality, the mean plus one standard deviation representing the upper limit of normal. A higher prevalence of elevated nicked β2GPI ratio was found in patients with ischemic stroke (63%, 39/62) and healthy subjects with lacunar infarct (27%, 14/52) when compared to healthy subjects with normal MRI findings (8%, 6/78). Relative risks of having stroke or asymptomatic lacunar infarction were approximated by Odds ratio [95% Confidential Interval], 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 β2GPI ratio in plasma of healthy subjects with lacunar infarct (r² = 0.31, p = 0.02). No
correlations were found between nicked β2GPI ratio and DD or TAT in any of the groups.

In the apparently healthy subjects group (n =130), plasma nicked β2GPI ratio significantly correlated with age ($r^2 = 0.483$, $p < 0.0001$) (Figure 3).

Therefore plasma measurement of nicked β2GPI might be a useful screening tool in the assessment of patients at risk of ischemic stroke.

**Binding of nicked β2GPI to Glu-plasminogen**

The binding of up to 0.4 μM of nicked β2GPI to solid phase Glu-plasminogen occurred in a dose-dependent manner, whereas the same concentrations of intact β2GPI did not bind to Glu-plasminogen (Figure 4-A). The binding of Cof-22 to β2GPI was not affected by the cleavage of β2GPI. Molecular interaction between intact or nicked β2GPI and plasminogen was investigated using an optical biosensor. Nicked β2GPI showed a large extent of binding to immobilized Glu-plasminogen, whereas intact β2GPI did not show any specific binding (Fig. 4-B). The data of $k_{on}$ at different concentrations of nicked β2GPI were fitted using linear regression, determining $k_{abs}$ as 0.0006 M$^{-1}$s$^{-1}$, and $k_{diss}$ as 0.0022 s$^{-1}$ (Fig. 4-C). Accordingly, $K_D$ and $K_A$ was determined as 0.37 x 10$^{-6}$ M and 2.70 x 10$^{6}$ M$^{-1}$, respectively.

**Inhibition of binding of Glu-plasminogen to nicked β2GPI by the fragments of plasminogen or by EACA.**

The binding of Glu-plasminogen (10 μg/ml) to immobilized nicked β2GPI, but not to native β2GPI, 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 β2GPI, and bound Glu-plasminogen was detected using a monoclonal anti-Kringle 1-3 antibody. Mini-plasminogen, but not kringle 4, inhibited the binding between Glu-plasminogen and nicked β2GPI (Figure 5-A). Kringle 1-3 fragment or mini-plasminogen was added as inhibitor and bound Glu-plasminogen was detected using a monoclonal anti-kringle 4 antibody. Glu-plasminogen binding to nicked β2GPI was dose dependently inhibited by mini-plasminogen but not by Kringle 1-3 fragment (Figure 5-B). The 5th domain or the catalytic domain of Glu-plasminogen, therefore, was predicted to mediate its binding to nicked β2GPI.

When the binding of nicked β2GPI (10 µg/ml) to solid phase Glu-plasminogen was tested in the presence of different concentrations of EACA, the binding between nicked β2GPI and immobilized Glu-plasminogen was abolished in a dose-dependent manner (Figure 5-C). Accordingly, LBS on plasminogen might mediate the binding of nicked β2GPI to Glu-plasminogen.

**Binding of plasminogen to fibrin interfered by nicked β2GPI**

We also investigated whether nicked β2GPI has an effect on the binding of Glu-plasminogen to immobilized fibrin monomer using an ELISA system. After pre-incubation with nicked β2GPI, but not with intact β2GPI, Glu-plasminogen showed decreased binding activity to soluble fibrin monomer (Fig. 5D).

**Effects of nicked β2GPI on extrinsic fibrinolysis**

The amidolytic activity of newly-generated plasmin was evaluated as tPA activity (Unit/ml) in a chromogenic assay. The activity increased with the concentration of tPA (data not shown). When nicked β2GPI was added, the tPA
activity decreased in a dose dependent manner (Figure 6-A). Intact β2GPI at 0.25 μM did not suppress the fibrinolytic activity, whereas intact β2GPI in a higher concentration (0.50 μM) slightly suppressed the fibrinolytic activity. The same amount of BSA or the recombinant domain I-IV of β2GPI did not affect the tPA activity.

The fibrinolytic activity of generated plasmin was measured as tPA activity (Unit/ml) in a fibrin plate assay. Fibrinolytic activity was suppressed by nicked β2GPI at 0.25 and 0.50 μM. Intact β2GPI at 0.50 μM also slightly inhibited the fibrinolytic activity. However, 0.25 μM of intact β2GPI did not affect the fibrinolytic activity of tPA (Figure 6-B).
Discussion

In the first part of this study, we demonstrated that plasma levels of nicked β2GPI 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 β2GPI were detected in large quantities in plasma of patients with DIC, a pathologic state characterized by marked increase of plasma PPI. We observed a strong correlation between plasma levels of nicked β2GPI and those of PPI in the healthy individuals showing lacunar infarcts on MRI, suggesting that nicked β2GPI may rather reflect "minor" plasmin generation. In the presence of larger plasmin generation, the correlation between nicked β2GPI and PPI may be lost, presumably due to the consumption of α2AP. In subjects with MRI abnormalities the prevalence of increased nicked β2GPI ratio was higher than that of PPI, DD and TAT (46%, 27%, 19%, 11% respectively). Thus, the detection of nicked β2GPI may represent a more sensitive marker of vascular lesions than PPI, DD or TAT.

In support of this concept is the correlation between nicked β2GPI ratio and age in the apparently healthy subjects, suggesting that "minor" plasmin generation might be associated with sub-clinical 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 β2GPI. Indeed nicked β2GPI can be generated on the surface of activated endothelial cells or platelets.
In the second part of this study, we investigated the properties of nicked \( \beta_2 \text{GPI} \) \textit{in vitro} to evaluate the biological significance of our observations. We showed that nicked \( \beta_2 \text{GPI} \) specifically bind to Glu-plasminogen and inhibits extrinsic fibrinolysis \textit{in vitro}. In contrast, neither domain I-IV of \( \beta_2 \text{GPI} \) nor intact \( \beta_2 \text{GPI} \) revealed such functions. The administration of intact \( \beta_2 \text{GPI} \) in higher concentrations also suppressed plasmin generation, perhaps owing to the nicked \( \beta_2 \text{GPI} \) 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, plasmin cleaves \( \beta_2 \text{GPI} \) on the thrombus, changing the properties of \( \beta_2 \text{GPI} \). We propose that \( \beta_2 \text{GPI} \) is a precursor of plasmin-nicked \( \beta_2 \text{GPI} \), a physiological inhibitor of fibrinolysis.

The crystal structure of human \( \beta_2 \text{GPI} \) 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 accessibility to plasminogen activators such as tPA or urokinase. On the fibrin surface, generated plasmin cleaves the single-chain tPA into the two-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 kringles 1, 2, and 3, thus accelerating fibrinolysis.

According to the results of the inhibition studies using plasminogen fragments or EACA (Fig. 5), and comparison of the effect on plasmin generation between nicked β2GPI and domain I-IV of β2GPI (Fig. 6A), it would be indicated that the binding of nicked β2GPI to Glu-plasminogen is mediated by the interaction between the lysine-cluster patch in domain V of the nicked β2GPI and LBS on the plasminogen kringle 5, although it still may be possible that excess amount of EACA interacts with the catalytic domain of Glu-plasminogen. The conformational difference between intact and nicked β2GPI is critical for its binding to phospholipid or plasminogen. The lysine-cluster patch in domain V of nicked β2GPI may gain accessibility for the LBS of Glu-plasminogen, whereas the C-terminal loop of intact β2GPI 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 α2AP, α2 macroglobulin, α1 antitrypsin, inactivated C1, PAI-1, and PAI-2, prevent the over-activation of the fibrinolytic system. The most potent inhibitors are α2AP and PAI-1, the former binds to a component of kringle 1-3 of plasminogen and can neutralize the generated plasmin more rapidly than α2 macroglobulin.
Fibrinolysis initiates upon binding of kringle 5 of plasminogen to lysine residues on fibrin followed by the binding of kringle 1-3 of plasminogen to lysine residues on the cleaved fibrin. α2AP does not bind to kringle 5 of plasminogen, hence, does not seem to affect the first interaction. Based on the observation that nicked β2GPI interferes the binding between Glu-plasminogen and fibrin monomer (Fig. 5-D), it is likely that the binding of nicked β2GPI to Glu-plasminogen affects the first step of fibrinolysis at least, and exert an inhibitory function in the fibrinolytic system via different mechanisms from that of α2AP.

In conclusion, firstly we have demonstrated that plasma levels of nicked β2GPI can be a sensitive marker of cerebral ischemic events and we suggest that plasma measurement of nicked β2GPI might be a useful screening tool in the assessment of patients at risk of ischemic stroke. Secondly, we propose that nicked β2GPI is a physiological inhibitor of fibrinolysis and that plasmin-cleavage of β2GPI is part of the negative feedback pathway of extrinsic fibrinolysis.
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References


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Figure legends

Figure 1  Plasma levels of nicked β2GPI.

Total and nicked β2GPI plasma levels were determined by enzyme-linked immunosorbent assay (ELISA). A nicked β2GPI ratio [(plasma nicked β2GPI/plasma total β2GPI) X 1000] was established in all the samples. The dashed line indicates the mean plus one standard deviation of the ratio in healthy subjects without lacunar infarct. p-values were calculated using T-test.

Figure 2  Prevalence of abnormally elevated plasma levels of nicked β2GPI and of markers of thrombin generation/ fibrinolytic turnover in our population.

Plasma levels of D-dimers (DD), plasmin-antiplasmin complex (PPI) and thrombin-antithrombin-complexes (TAT) were determined in all the subjects as described in methods' section.

Figure 3  Correlation between plasma levels of nicked β2GPI and age in apparently healthy subjects.

Nicked β2GPI was measured by a sandwich ELISA.

Figure 4  Binding of intact/nicked β2GPI to Glu-plasminogen.

(A) Binding of intact or nicked β2GPI to immobilized Glu-plasminogen was evaluated by ELISA using mouse monoclonal anti-β2GPI antibody Cof-22. Closed circles indicate the dose-dependent binding of nicked β2GPI to Glu plasminogen, whereas open circles indicate that intact β2GPI is unable to bind
to Glu-plasminogen. (B, C) Kinetic plot showing molecular interaction between Glu plasminogen and intact or nicked β2GPI. Intact β2GPI or nicked β2GPI binding to Glu-plasminogen was detected using IAsys, an optical biosensor as described in Method section. Binding extent (arc sec) was compared between intact and nicked β2GPI (B). Obtained on-rate constant (k_{on}) for nicked β2GPI was plotted and fitted using linear regression to find the intercept and gradient (C). A formula for determining the association rate constant (k_{ass}) and dissociation rate constant (k_{diss}) is as follows; k_{on} = k_{diss} + k_{ass} [ligand]. Error bars indicate standard deviations. β2GPI: β2-glycoprotein I

**Figure 5** Identification of the binding site of Glu-plasminogen to β2GPI by inhibition ELISA using plasminogen fragments.

(A) Binding of Glu-plasminogen to immobilized nicked β2GPI was tested by ELISA in the presence of possible inhibitors. After nicked β2GPI immobilization onto microtiter plates, different concentrations of kringle 4 of plasminogen (open circles) or mini-plasminogen (that consists of kringle 5 and catalytic domain of plasminogen) (closed circles) were added as inhibitors. Bovine serum albumin (BSA) (closed squares) served as control. After incubation and washing, Glu-plasminogen (10 µg/ml) was added and bound Glu-plasminogen was determined using kringle 1-3 specific mouse monoclonal anti-plasminogen antibody. (B) For the inhibition ELISA kringle 1-3 of plasminogen (open circles) or mini-plasminogen (closed circles) served as inhibitors. Glu-plasminogen bound to immobilized β2GPI was detected using kringle 4 specific mouse monoclonal anti-plasminogen antibody. Assays were run in triplicate. (C) Competitive ELISA using EACA, a lysine homologue. Binding of nicked β2GPI (0.2 µM) to
immobilized Glu-plasminogen was tested by ELISA using Cof-22 antibody in the presence of various concentrations of EACA (0-0.20 µg/ml). (D) Soluble fibrin monomer (5µg/ml) was coated on the surface of microtiter plate and blocked. Biotinylated Glu-plasminogen (5µg/ml) was pre-incubated with intact or nicked β2GPI and added to the wells. After incubation and washing, ALP-conjugated streptavidin was used for detection. Assays were run triplicate. Error bars indicate standard deviations. β2GPI: β2-glycoprotein I, BSA: bovine serum albumin, K: kringle, mini-plg: mini-plasminogen, EACA: ε-aminocaproic acid.

Figure 6. Inhibitory effect of nicked β2GPI on plasmin generation.

(A) Plasmin generation was measured by parabolic rate assay using synthetic substrate S-2251 in the presence of tPA, Glu-plasminogen and fibrin monomer. Nicked β2GPI (closed circles), intact β2GPI (open circles), β2GPI domain I-IV mutant (closed squares), or BSA (open squares) was added to the reaction in the indicated concentrations. After 12 hours of incubation, absorbance at 405 nm was measured and expressed as tPA activity (Unit/ml) using tPA as standard. (B) Fibrinolytic activity was measured using fibrin plate assay. Solution reaction containing tPA, Glu-plasminogen, and nicked (closed circles) or intact β2GPI (open circles) were placed onto fibrin plates. After 36 hours of incubation, the ring area of lysis was measured. Assays were performed in triplicate. Error bars indicate standard deviations. β2GPI; β2-glycoprotein I, BSA; bovine serum albumin, D; domain, tPA; tissue plasminogen activator.
Figure 1

![Figure 1: Scatter plot showing the comparison between healthy subjects and stroke patients. The plot indicates a statistically significant difference between the two groups.](image)

- Healthy subjects:
  - No Lacunar Infarct: 676 (8%)
  - Lacunar Infarct: 1452 (27%)

- Stroke patients:
  - No Lacunar Infarct: 3952 (63%)
  - Lacunar Infarct: 784 (12%)

Statistical significance:
- p < 0.0001
- p = 0.0002
- p = 0.042
Figure 2
Figure 3

![Graph showing a scatter plot with age on the x-axis and Nkhd 2Q12 ratio on the y-axis. The plot includes data points and a trend line with correlation R = 0.483, P < 0.0001.]

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Figure 4
Figure 5

(A)  

(B)
Figure 6
Nicked beta2-glycoprotein I: A marker of cerebral infarct and a novel role in the negative feedback pathway of extrinsic fibrinolysis

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