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ORIGINAL ARTICLE

A synthetic peptide derived from staphylokinase enhances plasminogen activation by tissue-type plasminogen activator

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Summary. *Background:* A synthetic nonadecapeptide (SP; GPYLMVNVTGVDGKGNELL) previously enhanced the activation of plasminogen by the SAK/plasmin complex. *Objectives:* To identify the binding site for SP on plasminogen and elucidate the effects of SP on plasminogen activation by the tissue-type plasminogen activator (t-PA). *Methods:* The effects of SP on plasminogen activation were estimated using a chromogenic substrate and from the cleavage of plasmin on SDS-PAGE under reduced conditions. The binding to SP of various peptides derived from the amino acid sequence of plasminogen was analyzed with an IAsys biosensor. The SP-mediated structural change to plasminogen was analyzed by circular dichroism (CD) spectroscopy. The thrombolytic effects of SP were examined using a mouse model of thrombosis. *Results:* SP enhanced the activation of plasminogen by t-PA. The catalytic efficiency (k_{cat}/K_m) of Glu-plasminogen activation by t-PA was 11.4-fold higher in the presence than absence of SP. The binding of SP to plasminogen was greatly inhibited by a synthetic peptide, FEKDKYILQGVTSWGLG, located close to the C-terminal of the plasminogen B region. Near-ultraviolet CD spectra of the complex between SP and Glu-plasminogen significantly differed from those of Glu-plasminogen. When SP was administered in a mouse model of thrombosis, early recanalization was observed in a dose-dependent manner. However, SP did not cause recanalization in t-PA gene-deficient mice. *Conclusions:* SP bound to the B region and promoted the activation of plasminogen by t-PA, and then induced effective thrombolysis.

Keywords: endothelial cell, plasminogen, plasminogen activation, synthetic peptide, thrombolysis, tissue-type plasminogen activator.

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Introduction

The fibrinolytic process involves two protease/protease inhibitor systems; the plasminogen activator (PA)/PA inhibitor (PAI) and plasmin/α_2-antiplasmin (α_2-AP) systems. Plasmin degrades fibrin and induces thrombolysis in blood vessels [1]. Physiological PAs include urokinase-type PA (u-PA) and tissue-type PA (t-PA). Both types directly cleave peptide bonds in the plasminogen molecule, and convert plasminogen to plasmin [2]. However, streptokinase (SK) [3,4] and staphylokinase (SAK) [5] from bacteria do not directly convert plasminogen to plasmin by themselves, but form a stoichiometric complex with plasmin(ogen), and these complexes exhibit PA activity.

SAK is a profibrinolytic protein produced by *Staphylococcus aureus* [6]. It consists of 136 amino acids and has a molecular weight of approximately 15 500. SAK alone does not directly convert plasminogen to plasmin and has no fibrinolytic activity. When SAK forms a complex with plasmin, however, the active site in the complex is exposed and this complex activates other plasminogen molecules to form plasmin [7]. PA activity in the SAK/plasmin complex is inhibited by α_2-AP immediately [8]. However, in the presence of fibrin, the plasmin in the complex binds to fibrin via lysine-binding sites (LBS), and the PA activity of the complex is protected from inhibition by α_2-AP [9]. Moreover, plasminogen activation by the SAK/plasmin complex was increased by fibrin [10]. In order to improve the characteristics of SAK, several mutants were developed [11,12]. In an *in vitro* model [13], animal models [14] and clinical studies [15–17], SAK showed efficient thrombolytic activity without a systemic lytic state. These findings suggest that the SAK/plasmin complex possesses specific thrombolytic properties [18].

Previously, the interaction between SAK and plasminogen was investigated using peptides synthesized based on the amino acid sequence of SAK [19]. The nonadecapeptide, GPYLMVNVTGVDGKGNELL (SP), which corresponds to Gly22-Leu40 of SAK, bound to plasminogen and enhanced the activation of plasminogen by the SAK/plasmin complex. SP neither showed plasminogen activator activity nor influenced the binding between plasmin and SAK. The binding site of Glu-plasminogen for SP was different to that for SAK.

Receptors and binding sites for fibrinolytic factors are present on various cells, and the cell-bound fibrinolytic factors express proteolytic activity at the cell surface [20,21]. Also, plasminogen binds to endothelial cells [22]. We reported previously that the activation of plasminogen by the SAK/plasmin complex was enhanced in human endothelial cells [23]. Moreover, it was further enhanced by a fibrin clot in the presence of endothelial cells.

The present study shows that SP binds to a region near the active site of plasminogen and induces local structural changes. The binding enhances plasminogen activation by t-PA. SP binds to Glu-plasminogen already bound to endothelial cells and promotes the activation process. Intravenous administration of SP resulted in early recanalization by intrinsic t-PA in a model of carotid artery thrombosis, indicating SP to be a useful thrombolytic peptide.

Materials and methods

Reagents

The following materials were obtained from the commercial sources indicated: H-D-Valyl-L-leucyl-L-lysine-p-nitroanilide (S-2251; Chromogenix, Milano, Italy), human t-PA, human u-PA, human Glu-plasminogen, human Lys-plasminogen, human plasmin and mouse plasminogen (Cosmo Bio, Tokyo, Japan), CNBr-digested fibrinogen (Technoclone, Vienna, Austria), and ε-aminocaproic acid (EACA; Seikagaku Corporation, Tokyo, Japan). All other reagents and chemicals were of the highest grade available.

Synthetic peptide

A nonadecapeptide, GPYLMVNVTGVDGKGNELL (SP), which corresponds to Gly22-Leu40 of SAK, was synthesized using an automated solid-phase peptide synthesizer (PSSM-8, Shimadzu, Kyoto, Japan). As the ability of SP to bind plasminogen was inhibited by the anti-plasminogen B region IgG as reported [19], we synthesized various peptides derived from the plasminogen B region (Table 1) in order to identify the region of Glu-plasminogen acting on the SP-induced activation. The substitution of amino acids (Glu748, Lys749, Asp750 and Lys751) with Ala or other residues in B11 (B11 mutant peptides shown in Table 2) was performed as mentioned above. The newly synthesized peptides were purified by reversed phase chromatography using the SMART System (GE Healthcare UK Ltd, Little Chalfont, UK) and a C18 column (Nacalai Tesque, Tokyo, Japan).

Cell culture

The human endothelial cells used in this study were of a cloned established cell line (TKM-33) [24]. The antigens for thrombomodulin and von Willebrand factor were demonstrated in TKM-33 cells by immunohistochemical studies.

Assay of plasminogen activation with a chromogenic substrate

The effects of SP on the activation of plasminogen by PA were estimated using a chromogenic substrate [19] as follows. Glu-plasminogen or Lys-plasminogen (final concentration, 100 nM) and S-2251 (0.5 mM) were incubated with t-PA or u-PA (1 nM) in the absence or presence of SP (0.16–10 μM) in 50 mM phosphate buffer (pH 7.4) containing 150 mM NaCl and 0.01% Tween-80 (reaction buffer) in a 96-well microtiter plate at 37°C for 1 h. The level of plasmin activity was estimated based on the amidolysis of S-2251 with a microplate reader (Thermomax^{TM}, Molecular Devices, Sunnyvale, CA, USA).

The effect of SP (10 μM) on the activation of Glu-plasminogen (100 nM) by t-PA (1 nM) in the presence of CNBr-digested fibrinogen fragments (150 μg mL^{-1}) was examined using the same assay.

Table 1 Amino acid sequences of peptides

B1		B2			B3			B4			B5			B6			B7			B8			B9			B10			B11			B12		
566–586	C	588–602	C		604–623			624–643			644–664		C	666–678		C	680–708		C	710–724		C	726–735		C	737–745		C	747–763		C	765–790		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29					
B1 (566–586)	V	A	H	P	H	S	W	P	W	Q	V	S	L	R	T	R	F	G	M	H	F													
B2 (588–602)	G	G	T	L	I	S	P	E	W	V	L	T	A	A	H																			
B3 (604–623)	L	E	K	S	P	R	P	S	S	Y	K	V	I	L	G	A	H	Q	E	V														
B4 (624–643)	N	L	E	P	R	V	Q	E	I	E	V	S	R	L	F	L	E	P	T	R														
B5 (644–664)	K	D	I	A	L	L	K	L	S	S	P	A	V	I	T	D	K	V	I	P	A													
B6 (666–678)	L	P	S	P	N	Y	V	V	A	D	R	T	E																					
B7 (680–708)	F	I	T	G	W	G	E	T	Q	G	T	F	G	A	G	L	L	K	E	A	Q	L	P	V	I	E	N	K	V					
B8 (710–724)	N	R	Y	E	F	L	N	G	R	V	Q	S	T	E	L																			
B9 (726–735)	A	G	H	L	A	G	G	T	D	S																								
B10 (737–745)	Q	G	D	S	G	G	P	L	V																									
B11 (747–763)	F	E	K	D	K	Y	I	L	Q	G	V	T	S	W	G	L		G																
B12 (765–790)	A	R	P	N	K	P	G	V	Y	V	R	V	S	R	F	V	T	W	I	E	G	V	M	R	N	N								

The upper column shows the B region of plasminogen. The numbers in boxes correspond to the B region of plasminogen (566–790). C between boxes indicates cysteine. The peptides (B1–B12) whose amino acid sequence corresponds with the B region of plasminogen. Each amino acid is shown as a single letter.

Table 2 Amino acid sequences of B11 mutant peptides

	747	748	749	750	751	752	753	754	755	756	757	758	759	760	761	762	763
B11	F	E	K	D	K	Y	I	L	Q	G	V	T	S	W	G	L	G
E748A	F	***A***	K	D	K	Y	I	L	Q	G	V	T	S	W	G	L	G
E748K	F	***K***	K	D	K	Y	I	L	Q	G	V	T	S	W	G	L	G
K749A	F	E	***A***	D	K	Y	I	L	Q	G	V	T	S	W	G	L	G
K749D	F	E	***D***	D	K	Y	I	L	Q	G	V	T	S	W	G	L	G
D750A	F	E	K	***A***	K	Y	I	L	Q	G	V	T	S	W	G	L	G
D750K	F	E	K	***K***	K	Y	I	L	Q	G	V	T	S	W	G	L	G
ΔD750	F	E	K	–	K	Y	I	L	Q	G	V	T	S	W	G	L	G
K751A	F	E	K	D	***A***	Y	I	L	Q	G	V	T	S	W	G	L	G
K751D	F	E	K	D	***D***	Y	I	L	Q	G	V	T	S	W	G	L	G
E748A/D750A	F	***A***	K	***A***	K	Y	I	L	Q	G	V	T	S	W	G	L	G
K749A/K751A	F	E	***A***	D	***A***	Y	I	L	Q	G	V	T	S	W	G	L	G
E748K/K749E/D750K/K751D	F	***K***	***E***	***K***	***D***	Y	I	L	Q	G	V	T	S	W	G	L	G

The amino acid sequence of wild-type B11 corresponds with 747Phe to 763Gly in the B region of plasminogen. Each amino acid is shown as a single letter. Mutated amino acids are shown in boldfaced italics. The bar (–) shows the deletion of AN amino acid.

The inhibitory effects of peptides derived from the plasminogen B region (shown in Tables 1 and 2) for SP-mediated plasminogen activation were estimated using the chromogenic substrate assay. Briefly, Glu-plasminogen (100 nM) and S-2251 (0.5 mM) were incubated with t-PA (1 nM) in the presence of SP (5 μM) and peptides derived from the plasminogen B region (5 μM). Glu-plasminogen activation on the TKM-33 cells by t-PA or u-PA was estimated using the same conditions with or without SP as mentioned above.

The kinetic parameters for plasminogen activation by t-PA were calculated as follows [25,26]. The activation of Glu-plasminogen (20–100 μM) by t-PA (100 nM) was measured in the absence or presence of SP (10–500 μM). The amount of plasmin generated at different points in time was measured with S-2251 (0.5 mM) after a 20-fold dilution of the sample. The activation obeyed Michaelis–Menten kinetics, as revealed by a linear double-reciprocal plot of initial activation vs. the plasminogen concentration.

Assay of plasminogen activation by t-PA using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

The effects of SP on the activation of Glu-plasminogen by t-PA were estimated using SDS-PAGE. Briefly, Glu-plasminogen (2 μM) was reacted with t-PA (67 nM) in the absence or presence of SP (25 μM), and incubated at 37°C for 0–60 min. The degree of activation was estimated by determining the cleavage of plasmin into its heavy and light chains, as observed by SDS-PAGE under reduced conditions. Standard molecular weight proteins were used as markers. The protein was visualized with Coomassie brilliant blue and the gel was scanned. The activation rate for plasminogen was calculated from the intensity of the plasminogen band. The density of the band of untreated plasminogen (0 min) was taken as 0%.

Assay of plasmin activity with a chromogenic substrate

The effects of SP on plasmin activity were estimated using a chromogenic substrate as follows. Plasmin (100 nM) and S-2251 (1 mM) were incubated in the presence of SP (0–10 μM) in 50 mM phosphate buffer (pH 7.4) containing 150 mM NaCl, 10% glycerol and 0.01% Tween-80 in a 96-well microtiter plate at 37°C for 15 min. The level of plasmin activity was estimated based on the amidolysis of S-2251 with a microplate reader.

Stability of SP

SP was incubated at 4 or 37°C in 50 mM phosphate buffer (pH 7.4) containing 150 mM NaCl and 0.01% Tween-80 for 1, 3 and 7 days. The stability of SP in the buffer was estimated by using the chromogenic substrate assay. To investigate the effect of a protease such as plasmin or t-PA on SP activity, SP was incubated with plasmin-Sepharose or t-PA-Sepharose for 1 h at 37°C. Glu-plasminogen activation by t-PA was estimated using the chromogenic substrate assay with or without SP that had been treated with the protease.

Binding of plasminogen, plasminogen activators or synthetic peptides derived from the plasminogen B region to SP

The ability of plasminogen or plasminogen activators to bind SP was measured with an IAsys resonant mirror biosensor (Affinity Sensors, Cambridge, UK). Protein (1 μM) or SP (10 μM) in 10 mM sodium acetate (pH 5.0) was covalently coupled to the activated carboxymethyl dextran-coated biosensor cuvette [6]. The binding of SP (0.5–10 μM) to human or murine Glu-plasminogen, human t-PA or human u-PA was followed in real time for at least 5 min in 50 μL of 25 mM phosphate buffer (pH 7.4).

The binding of synthetic peptides derived from the plasminogen B region to SP was measured with the IAsys biosensor (Affinity Sensors, Cambridge, UK). The binding of B11 peptide (0.5–12.5 μM) or B11 mutant peptide (12.5 μM) was followed in real time for at least 5 min in 50 μL of 25 mM phosphate buffer (pH 7.4). Binding parameters were calculated from the association and dissociation phases of the binding

reactions using the FastFit software (Affinity Sensors) provided with the instrument.

The inhibitory effects of B11 on the binding of SP to plasminogen were estimated using the IAsys biosensor. SP (10 μM) was pre-incubated with B11 (10 μM) and then its ability to bind immobilized-plasminogen was analyzed.

The binding of SP to plasminogen on the TKM-33 cells was estimated as follows. First, the binding of Glu-plasminogen (25, 50, or 100 nM) to immobilized-SP was analyzed; then the binding of TKM-33 cells (1×10^4) to the Glu-plasminogen bound with immobilized-SP was analyzed.

Spectropolarimetry

The circular dichroism (CD) spectra were recorded using a JASCO J-720 automatic spectropolarimeter (Nihon Bunko, Tokyo, Japan) at 25°C. CD spectra of Glu-plasminogen (10 μM) or Lys-plasminogen (10 μM) in the absence or presence of SP (10 μM) or EACA (10 mM) in 0.1 M sodium phosphate buffer, pH 7.4, were recorded at wavelengths of 250–310 nm. CD spectra of Glu-plasminogen (1 μM) or Lys-plasminogen (1 μM) in the absence or presence of SP (1 μM) or EACA (10 mM) in 0.1 M sodium phosphate buffer, pH 7.4 were recorded at 200–250 nm.

Photo-irradiation-induced carotid artery thrombosis model

Experiments were performed on 2–3-month-old male t-PA gene-deficient (t-PAKO), PAI-1 gene-deficient (PAI-1KO) and wild-type (WT) mice [27,28]. The Institutional Animal Care and Use Committee of Kinki University School of Medicine approved the experimental protocol.

The procedure used to produce a thrombus through endothelial injury was described previously elsewhere [29]. In brief, mice (n = 4–6) were anesthetized with an intraperitoneal injection of 44 mg kg^{-1} of sodium pentobarbital. The right common carotid artery, the left jugular vein and the right femoral artery were exposed, and then catheters were inserted into the left jugular vein for the injection of the photosensitizer dye, rose bengal (30 mg kg^{-1}). Blood flow in the carotid artery was continuously monitored using a Doppler flow probe (Model PDV-20; Crystal Biotech, Northborough, MA, USA). Green light irradiation (540 nm) was continued for 15 min after the injection of rose bengal, resulting in the destruction of endothelial cells in the irradiated area. In this animal model, saline was used as the vehicle. SP was dissolved in saline, and then 62.5 or 125 or 250 μg kg^{-1} of SP (10% administered intravenously [i.v.] as a bolus and 90% by i.v. infusion for 30 min) was administered 30 min after the interruption of blood flow by occlusion thrombi. To investigate the effect of SP or thrombolytic potential of t-PA infusion, t-PA was dissolved in saline, and then 0.3 or 0.6 mg kg^{-1} of t-PA (10% administered by i.v. bolus and 90% by i.v. infusion for 30 min) with or without SP (62.5, or 250 μg kg^{-1}) was administered. Blood flow was continuously monitored during experiments.

Results

Effects of SP on the activation of Glu-plasminogen

SP enhanced the activation of Glu-plasminogen by t-PA in a concentration-dependent manner. This reaction was examined kinetically. Figure 1 shows the double-reciprocal plots of initial activations (1/v) vs. plasminogen concentrations (1/[plasminogen]) in the absence or presence of SP. The K_m value was lower in the presence than the absence of SP (8.5 ± 1.0 μM vs. 105 ± 7.0 μM). The k_{cat} value was 0.105 ± 0.012 s^{-1} and 0.115 ± 0.021 s^{-1}, respectively. Thus, the catalytic efficiency (k_{cat}/K_m) of Glu-plasminogen activation by t-PA was 11.4-fold higher in the presence than the absence of SP (0.0124 ± 0.0035 μM^{-1} s^{-1} vs. 0.00109 ± 0.00045 μM^{-1} s^{-1}). Also, SP promoted Glu-plasminogen activation by u-PA (data not shown). However, SP did not enhance the activation of Lys-plasminogen by t-PA or u-PA (data not shown).

Analyses using SDS-PAGE showed that t-PA converted Glu-plasminogen to plasmin in a time-dependent manner (data not shown). Plasmin was clearly observed at 3 min when SP was absent and at 1 min when it was present (data not shown). The time required for 50% maximum plasmin production was 20 min in the absence of SP and 5 min in the presence of SP.

The effects of CNBr-digested fibrinogen fragments on the activation of Glu-plasminogen by t-PA were examined. SP enhanced the activation by t-PA with the fragments. The CNBr-digested fibrinogen fragments had an additive effect on the enhancement by SP (data not shown).

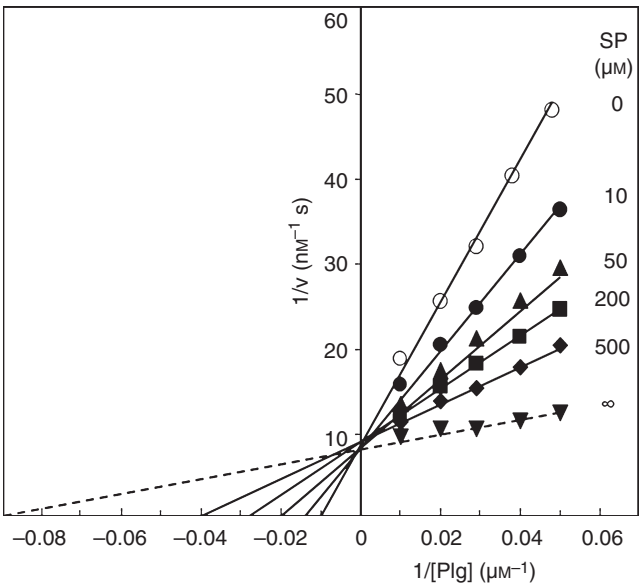

Fig. 1. Double-reciprocal plots of initial activations (1/v) vs. plasminogen concentrations (1/[plasminogen]) in the absence or presence of nonadecapeptide, GPYLMVNVTGVDGKGNELL (SP). The activation of Glu-plasminogen (20–100 mM) by tissue-type PA (t-PA) (100 nM) was measured in the absence (○, 0 μM) or presence (●, 10 μM; ▲, 50 μM; ■, 200 μM; ♦, 500 μM) of SP. The dotted line (▼) represents 1/v at infinite plasminogen concentration. The initial activation rates were estimated based on the amidolysis of S-2251 (0.5 mM) spectrophotometrically with a microplate reader. The data shown represent the average for three experiments.

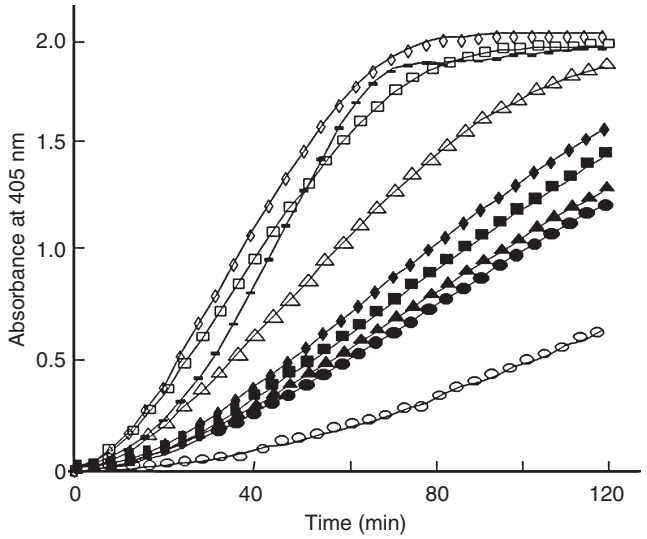

Fig. 2. Effects of nonadecapeptide, GPYLMVNVTGVDGKGNELL (SP) on the activation of Glu-plasminogen by tissue-type PA (t-PA) on TKM-cells. Activation of Glu-plasminogen (100 nM) by t-PA (1 nM) in the presence of SP (○,; 0 μM; ▲, 0.16 μM; ■, 0.31 μM; ♦, 0.63 μM; Δ, 1.25 μM; –, 2.5 μM; □, 5 μM; ◇, 10 μM) on TKM-33 cells. The level of plasmin activity was estimated based on the amidolysis of S-2251 spectrophotometrically with a microplate reader. The data shown represent the average for three experiments.

The effects of SP on the activation of Glu-plasminogen by t-PA on endothelial cells were investigated using the chromogenic substrate assay (Fig. 2). Glu-plasminogen activation by t-PA increased. Moreover, SP had a concentration-dependent effect.

The effect of SP on plasmin activity was estimated using chromogenic substrate. SP exhibited no influence of plasmin activity (data not shown).

Binding of plasminogen to SP

SP bound to immobilized-human Glu-plasminogen or murine Glu-plasminogen in a concentration-dependent manner (data not shown). SP did not bind to immobilized-human t-PA or u-PA.

Furthermore, the binding of Glu-plasminogen to immobilized-SP increased in a concentration-dependent manner (Fig. 3). The binding of TKM-33 cells to Glu-plasminogen also increased in a concentration-dependent manner (Fig. 3B). Namely, the binding depended on Glu-plasminogen, which had already bound to SP. However, TKM-33 cells did not directly bind to immobilized-SP (data not shown).

Stability of SP

More than 80% of SP activity was maintained when SP was incubated in buffer at 37°C for 7 days (data not shown).

Furthermore, SP activity was completely maintained when SP was incubated with plasmin-Sepharose or t-PA-Sepharose at 37°C for 1 h (data not shown).

Effects of plasminogen B region peptides on the SP-induced activation of Glu-plasminogen

Among the 12 peptides listed in Table 1, the B7 peptide (Phe680–Val708), B9 peptide (A726–S735) and B11 peptide (Phe747–Gly763) suppressed the SP-mediated activation of Glu-plasminogen by t-PA (Fig. 4A). As its inhibitory effect was the most powerful, B11 was mainly used in subsequent experiments. The B11 peptide had a concentration-dependent effect on the SP-mediated activation by t-PA (Fig. 4B). However, it did not directly inhibit Glu-plasminogen activation by t-PA (data not shown).

To identify the amino acids important to the inhibitory effect of B11, we synthesized various mutant forms of the peptide (Table 2). The substitution of Glu748 and Asp750 with Ala (E748A/D750A) and of Asp750 with Ala (D750A) or Lys (D750K) significantly decreased the inhibitory effect on the SP-mediated activation of Glu-plasminogen (Table 3). Also, the substitution of Glu748/Lys749/Asp750/Lys751 with Lys748/Glu749/Lys750/Asn751 (E748K/K749E/D750K/K751D)

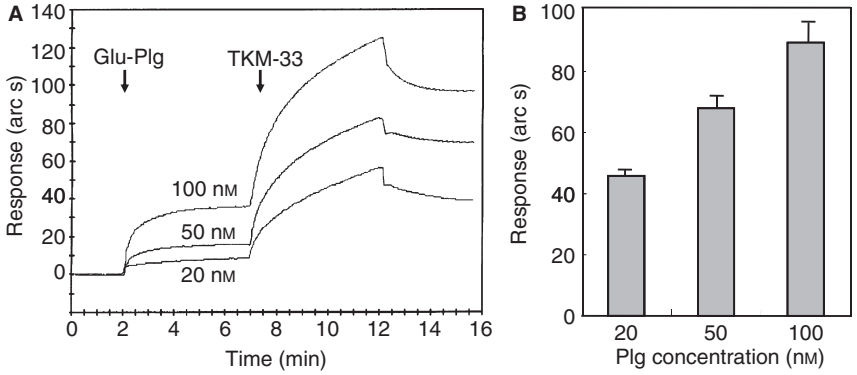

Fig. 3. The binding of nonadecapeptide, GPYLMVNVTGVDGKGNELL (SP) to Glu-plasminogen on endothelial cells. (A) The binding of Glu-plasminogen (20, 50 and 100 nM) to immobilized-SP and subsequent binding of endothelial cells (1×10^4) to Glu-plasminogen were assayed using an IAsys resonant mirror biosensor. The ordinate shows the strength of binding. The abscission shows the time-course of the binding reaction. (B) The binding of endothelial cells to Glu-plasminogen bound to immobilized-SP shown as the response (arc seconds) at 12 min in (A). Each value is represented as the mean ± SD ($n = 3$).

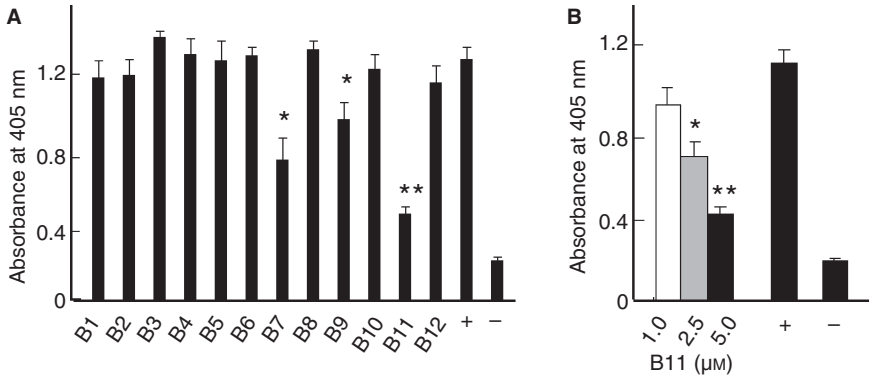

Fig. 4. Effects of peptides derived from the B-chain of plasminogen (B1–B12) on the nonadecapeptide, GPYLMVNVTGVDGKGNELL (SP)-mediated activation of Glu-plasminogen by t-PA. Amino acid sequences of B1 to B12 are listed in Table 1. The level of plasmin activity was estimated based on the amidolysis of S-2251 spectrophotometrically with a microplate reader. The ordinate shows the absorbance at 405 nm after 30 min. (A) The plus (+) mark indicates the activation of Glu-plasminogen (100 nM) by tissue-type PA (t-PA) (1 nM) in the presence of 5 μM SP. The minus (−) mark indicates the activation of Glu-plasminogen (100 nM) by t-PA (1 nM) in the absence of SP. Each value indicates the SP-mediated activation of Glu-plasminogen by t-PA in the presence of peptides derived from the B-chain of plasminogen (B1–B12, each 5 μM) and is represented as the mean ± SD (n = 3). Statistically significant differences vs. SP-mediated Glu-plasminogen activation by t-PA in the absence of peptides derived from the B-chain of plasminogen (+) are represented by asterisks, $*P < 0.05$ or $**P < 0.01$. (B) The meaning of the plus (+) and minus (−) marks is mentioned above. Each value indicates SP-mediated activation of Glu-plasminogen by t-PA in the presence of B11 (1.0, 2.5 or 5 μM). Statistically significant differences are represented as mentioned above.

Table 3 The relative inhibitory activity of B11 mutant peptides on SP-mediated activation of Glu-plasminogen

Synthetic peptides	Relative inhibitory activity (%)
B11	100
E748A	88.9 ± 10.7
E748K	95.7 ± 3.3
K749A	85.4 ± 12.6
K749D	90.9 ± 8.1
D750A	10.8 ± 6.3**
D750K	15.3 ± 7.8**
K751A	83.9 ± 15.3
K751D	87.6 ± 11.0
E748A/D750A	10.1 ± 4.7**
K749A/K751A	82.9 ± 16.8
E748K/K749E/D750K/K751D	25.7 ± 11.6*
ΔD750	8.3 ± 5.6**

Glu-plasminogen is activated by t-PA and the amount of plasmin generated was estimated as described in Materials and methods. The inhibitory activity of B11 for SP-mediated activation of Glu-plasminogen is set at 100%. Each value is represented as the mean ± SD (n = 3). Statistically significant differences vs. relative inhibitory activity by B11 are represented as asterisks, $*P < 0.05$ or $**P < 0.01$.

significantly reduced the inhibitory effect. Furthermore, the deletion of Asp750 (ΔD750) significantly weakened the inhibitory effect. On the other hand, the substitution of Glu748, Lys749 or Lys751 with Ala (E748A, K749A, K751A) had no effect. The substitution of both Lys749 and Lys751 with Ala (K749A/K751A) did not affect the SP-mediated actvation of Glu-plasminogen either. In addition, the substitution of Lys749 or Lys751 with Asp (K749D, K751D) had no effect.

Binding of peptides derived from the plasminogen B region to SP

The IAsys assay showed that B11 bound to SP in a concentration-dependent manner (Fig. 5A). Among the mutant peptides, E748A (Fig. 5B-3), K749A (Fig. 5B-2), K751A (Fig. 5B-1) and K749A/K751A (Fig. 5B-4) bound to SP, but D750A (Fig. 5B-5) and E748A/D750A (Fig. 5B-6) did not. D750K, E748K/K749E/D750K/K751D and ΔD750 did not bind to SP either (data not shown).

Binding parameters for SP and B11 were as follows: 2.2×10^4 M^{-1} s^{-1} for the association rate constant (ka), 6.3×10^{-2} s^{-1} for the dissociation rate constant (kd), and 3.49×10^5 M^{-1} for the affinity constant (Ka). However, the binding of SP to plasminogen was inhibited by the B11 peptide (data not shown).

Effect of SP on CD spectra of Glu-plasminogen or Lys-plasminogen

The far-ultraviolet (200–250 nm) CD spectra of Glu-plasminogen as well as Lys-plasminogen in the absence and presence of SP are very similar (data not shown). At 208 nm, the ellipticity of Glu-plasminogen and Lys-plasminogen in the absence or presence of SP was about −4200 degrees cm^2 dmol^{-1}. Figure 6(A,B) shows the near-ultraviolet (250–310 nm) CD spectra of Glu-plasminogen and Lys-plasminogen. Each spectrum has positive ellipticity at around 280 nm. The spectrum for the complex between SP and Glu-plasminogen was shifted down from that of Glu-plasminogen alone. The CD spectrum of the complex between SP and Glu-plasminogen differed from that of Glu-plasminogen in the presence of EACA (Fig. 6A). Furthermore, the spectrum of the complex between SP and Lys-plasminogen was shifted more than that of the complex between SP and Glu-plasminogen (Fig. 6). The spectrum of Lys-plasminogen in the presence of EACA was similar to that of Lys-plasminogen alone. However, the CD spectrum of the complex between SP and Lys-plasminogen differed from that of Lys-plasminogen (Fig. 6B).

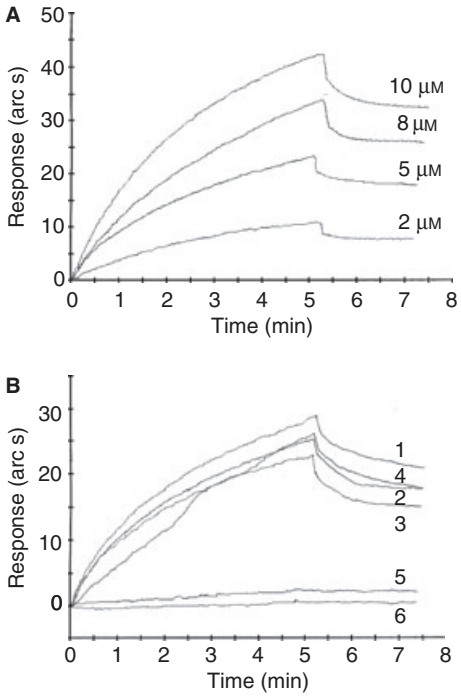

Fig. 5. The binding of peptides derived from the B-chain of plasminogen (B11) or B11 mutant peptides to nonadecapeptide, GPYLMVNVTGV-DGKGNELL (SP). (A) The abilitiy of B11 (2–10 μM) to bind immobilized SP assayed using an IAsys biosensor. The ordinate shows the strength of binding between SP and B11. (B) The ability of B11 mutant peptides (10 μM) to bind immobilized SP. The abscission reveals the time-course of the binding reaction. 1, K751A; 2, K749A; 3, E748A; 4, K749A/K751A; 5, D750A; 6, E748A/D750A.

Effect of SP on thrombolysis in vivo

After the interruption of blood flow, various concentrations of SP and vehicle were delivered intravenously. The time profiles of vascular patency in each group of mice are schematically illustrated in Fig. 7. The closed columns show vascular occlusion. In mice treated with SP (Fig. 7B–D), recanalization was observed earlier than in vehicle-treated controls (Fig. 7A). Moreover, total recanalizing time was longer for mice treated with SP (2.38 ± 1.11 min for 62.5 μg kg^{-1} of SP, 9.25 ± 2.12 min for 125 μg kg^{-1} of SP, 13.45 ± 6.14 min for 250 μg kg^{-1} vs. 0.67 ± 0.55 min for vehicle). The recanalization was dependent on the concentration of SP. However, SP did not induce the degradation of fibrinogen in plasma in this model.

As the recanalization was considered to be due to intrinsic t-PA, the effect of SP on thrombolysis was investigated using t-PA-deficient mice (t-PAKO mice). In t-PAKO mice treated with vehicle or SP, a reduction in cyclic flow and persistent patency were not observed at all and only persistent occlusion was evident (Fig. 7E,F).

Next, because PAI-1 plays an important role as an inhibitor for t-PA, the effect of SP on thrombolysis was investigated by using PAI-1-deficient mice (PAI-1KO mice). Recanalization occurred earlier in the mice treated with SP than in those given vehicle only (Fig. 7G,H).

Total recanalizing time was longer for the mice treated with t-PA (0.3 mg kg^{-1}, i.v.) and SP (250 μg kg^{-1}, i.v.) than for mice treated with t-PA (0.3 mg kg^{-1}, i.v.) alone (30.7 min vs. 3 min). Total recanalizing time for the administration of t-PA (0.3 mg kg^{-1}, i.v.) with SP (250 μg kg^{-1}, i.v.) was comparable to that on the administration of t-PA (0.6 mg kg^{-1}, i.v.) alone (30.7 min vs. 39.0 min).

Discussion

Thromboembolic diseases such as myocardial infarction, cerebral infarction and pulmonary embolism can be lethal.

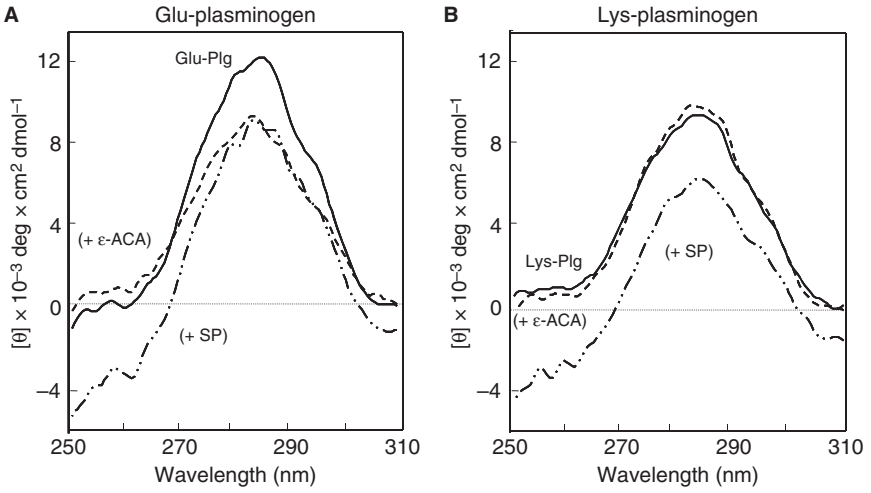

Fig. 6. Effects of nonadecapeptide, GPYLMVNVTGVDGKGNELL (SP) on circular dichroism (CD) spectra in the near-ultraviolet region of Glu-plasminogen or Lys-plasminogen. (A) CD spectra of Glu-plasminogen (10 μM; — —) alone or with 10 μM SP (-¨—) or 10 mM ε-aminocaproic acid (EACA) (——) measured using a JASCO J-720 automatic spectropolarimeter. (B) CD spectra of Lys-plasminogen (10 μM; —;—;) alone or with 10 μM SP (-¨—) or 10 mM EACA (——).

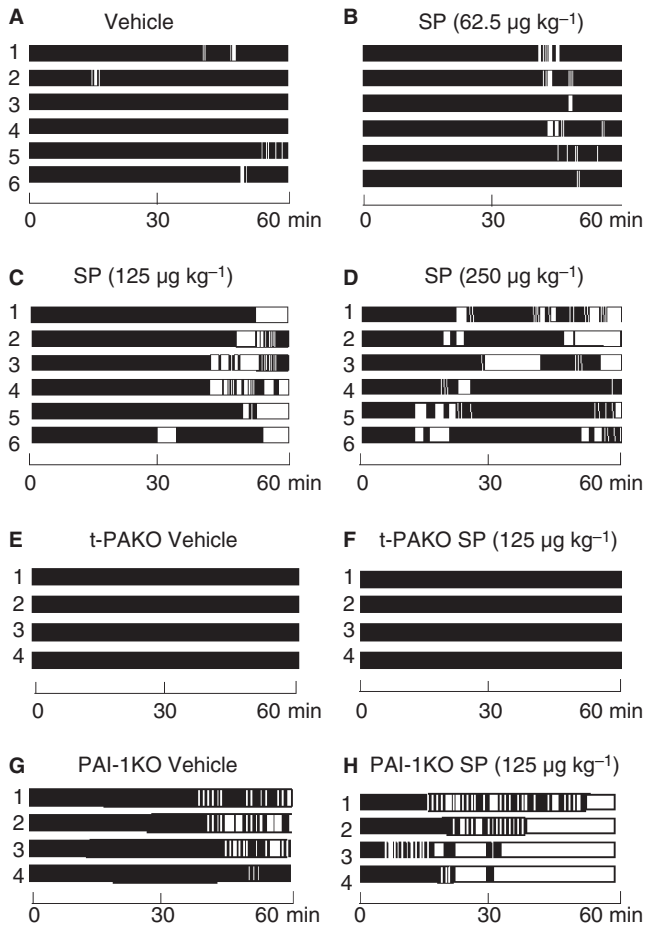

Fig. 7. Time profile of vascular patency in mice treated with nonadecapeptide, GPYLMVNVTGVDGKGNELL (SP) or vehicle alone. The thrombolytic effect of SP was examined in the photo-irradiation-induced model of carotid artery thrombosis as described in 'Materials and methods'. The effect of the intravenous administration of SP ([A], vehicle; [B], 62.5 μg kg^{-1}; [C], 125 μg kg^{-1}; [D], 250 μg kg^{-1}) in wild-type mice on vascular patency was examined. The effect of the intravenous administration of SP ([E] and [G]; vehicle, [F] and [H]; 125 μg kg^{-1}) in t-PAKO ([E] and [F]) or PAI-1KO ([G] and [H]) on vascular patency was also examined. The closed column shows vascular occlusion, and the open column, recanalization.

As these diseases are based on atherothrombosis, they may soon become a leading cause of death worldwide [30]. To dissolve a thrombus and induce recanalization of an occluded vessel, fibrinolytic agents such as t-PA and u-PA are widely used [31]. As t-PA has affinity for fibrin [32,33] and expresses fibrin-specific PA activity [34], it is a more efficient fibrinolytic agent than u-PA [35]. However, it is very expensive. It is therefore necessary to develop a new fibrinolytic agent to replace t-PA.

Having previously reported that SP enhanced the activation of plasminogen by the SAK/plasmin complex [19], here we investigated in detail whether SP enhances the activation of plasmin by t-PA. SP enhanced the activation of plasminogen by t-PA in a concentration-dependent manner. The mechanism of SP's effect was further investigated by conducting a kinetic analysis (Fig. 1). SP increased by 11.4-fold the catalytic efficiency of Glu-plasminogen activation by t-PA, due to a reduction in the K_m value (8.5 ± 1.0 μM in the presence of SP vs. 105 ± 7.0 μM in the absence of SP). SP seemed to increase the affinity between Glu-plasminogen and t-PA.

We previously demonstrated that a lysine residue at the 14th position from the N-terminal of SP was critical for binding with Glu-plasminogen [19]. However, the amino acid or region in Glu-plasminogen vital for binding with SP was not elucidated. As it has been confirmed that SP bound to the B region (residues 562–791) of Glu-plasminogen, peptides in this region sandwiched between cysteine residues were synthesized (B1–B12 in Table 1). Among these peptides, B11 exhibited the greatest competition for binding between Glu-plasminogen and SP, indicating that the amino acid region in Glu-plasminogen, which corresponded to B11, was the most important region for binding to SP. In order to define the amino acid in Glu-plasminogen necessary for binding with SP and SP-mediated Glu-plasminogen activation by t-PA, B11 mutant peptides (Table 2) were synthesized. The substitution of Asp750 with Ala abolished the binding with SP (Fig. 5B). The deletion of Asp750 or substitution of Asp750 with Lys also abolished the binding (data not shown). These findings suggested that Asp750 in Glu-plasminogen was critical for binding with SP. Furthermore, these mutations in B11 decreased the relative inhibitory effect on the SP-mediated activation of Glu-plasminogen by t-PA (Table 3), suggesting that Asp750 was critical to the activation. The substitution of Asp750 with Ala or Lys as well as the deletion of Asp750 in B11 abolished the binding of the peptide to SP. Furthermore, it was previously reported that substitution of Lys14 in SP with Ala abolished the enhancement of Glu-plasminogen activation by the plasmin-SAK complex. As Asp750 and Lys14 have opposite electric charges, electrostatic force may be involved in the binding of SP to Glu-plasminogen.

We confirmed that SP bound to murine Glu-plasminogen using the IAsys system. It was previously reported that SAK did not bind to murine and bovine plasminogen/plasmin. Parry *et al.* [36] found that the major amino acids in human μ-plasmin responsible for the affinity for SAK are Arg719 and Arg767 (Arg175 and Arg221 according to chymotrypsinogen numbering) and the minor amino acids are Asn169, Tyr171, Asn173, Glu180, Leu217 and Lys224. Tyr171 in human plasminogen, thought to be a minor amino acid for binding to SAK, is substituted by Val in murine plasminogen, or by Asn in bovine plasminogen. This substitution causes no-binding of SAK to murine or bovine plasminogen. In contrast, the amino acid sequence of the SP-binding site in human plasminogen was preserved in murine and bovine plasminogen. It was thought that this preservation causes the binding of SP to murine plasminogen. Thus, the binding of SP to plasminogen has a completely different mechanism to that of SAK to plasminogen.

Because SP bound to Glu-plasminogen and enhanced its activation by t-PA, it was speculated to cause a structural change to Glu-plasminogen. The far-ultraviolet (200–250 nm) CD spectra of Glu-plasminogen and Lys-plasminogen in the

absence or presence of SP are very similar (data not shown). At 208 nm, the ellipticity of Glu-plasminogen or Lys-plasminogen with or without SP was about -4200 degrees $\times$ cm^2 $\times$ dmol^{-1}, which means that the contribution of the α-helical structure in these proteins must be negligible (3.2–3.5%) and SP has no influence on this contribution. Because Glu-plasminogen has a closed tightly formed structure and Lys-plasminogen has an opened relaxed form [37], the near-ultraviolet CD spectrum of Glu-plasminogen was quite different to that of Lys-plasminogen (Fig. 6A,B). The activation of Lys-plasminogen by t-PA is greater than that of Glu-plasminogen [32], due to its relaxed form [38]. As described previously [39], the CD spectrum of Glu-plasminogen with EACA (lysine derivative) was similar to that of Lys-plasminogen in the absence or presence of EACA (Fig. 6A,B). EACA binds to LBS in the Kringle domain in the plasminogen A region and induces the relaxed conformation that causes enhancement of activation. On the other hand, the CD spectra of Glu-plasminogen or Lys-plasminogen with SP significantly differed from that with EACA (Fig. 6A,B). Thus, SP binds to Glu- or Lys-plasminogen, and induces local structural changes. However, the CD spectra of Glu-plasminogen with SP were quite different to those with EACA. These results show that SP induces the change in the CD spectra of Glu-plasminogen, which is different to the relaxed form produced by EACA, and subsequently enhances the activation of Glu-plasminogen by t-PA. However, to confirm the results obtained with the CD spectra, more detailed experiments will be needed. SP binds to the B-region of Glu-plasminogen, and induces local structural change as shown in Fig. 6. Because the K_m value dropped in the presence of SP, it is thought that SP induces structural change by which affinity for t-PA is augmented. As the binding site for SP in plasminogen is different to that for SAK and the molecular weight of SP is much smaller than that of SAK, the structural change of Glu-plasminogen induced by binding with SP is thought to be quite different to that caused by binding with SAK. The mechanism of SP is therefore considered different to that of SAK.

Since binding sites for plasminogen or t-PA exist on human endothelial cells [20], plasminogen and t-PA are accumulated on endothelial cells. In fact, the activation of plasminogen by t-PA was greater in the presence (Fig. 2, ●) than the absence of endothelial cells (Fig. 2, ○). Furthermore, SP enhanced the activation of plasminogen by t-PA on human endothelial cells (Fig. 2). It was also revealed that SP bound to Glu-plasminogen that had already bound to endothelial cells (Fig. 3). However, SP did not bind to endothelial cells directly (data not shown). These findings suggest that the region of the plasminogen molecule for binding endothelial cells is different to that for binding SP. It was demonstrated that plasminogen binds to endothelial cells via LBS in plasminogen [20] and to SP independently of LBS [19].

In a photo-irradiation-induced carotid artery thrombosis model [29,40,41], treatment of the mice with SP resulted in an earlier and longer recanalization (Fig. 7A–D). However, it was considered that SP did not induce the degradation of fibrinogen due to the inhibition of plasmin generated in plasma by α_2-AP. In t-PAKO mice treated with vehicle or SP, recanalization was not observed at all and only persistent occlusion was shown (Fig. 7E,F). As a reason for these phenomena, it was thought that the absence of intrinsic t-PA could not induce the spontaneous thrombolysis observed in wild-type mice. On the other hand, in PAI-1KO mice treated with SP (Fig. 7H), recanalization was observed earlier than in wild-type mice treated with SP (Fig. 7C). Thus, it is suggested that SP enhances thrombolysis *in vivo*. Because SP alone induced thrombolysis, it may be used to replace t-PA. Furthermore, SP enhanced the thrombolytic potential of t-PA. Therefore, SP may be given in combination with t-PA or SAK. In this case, SP may be able to reduce the amount of t-PA or SAK needed.

SP binds to Asp750 in the B-chain of plasminogen and enhances plasminogen activation by t-PA, resulting in more effective thrombolysis. Therefore, SP can be used not only alone but also in combination with a thrombolytic agent such as t-PA.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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