

GPVI-Fc-PEG improves cerebral infarct volume and cerebral thrombosis in mouse model with cerebral thrombosis

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Abstract. Cerebral thrombosis is one of the most common causes of cerebral infarction, and anticoagulation therapy is a routine treatment in patients with hemorrhagic cerebral venous thrombosis. The hemostatic function of platelets is important for the anticoagulation therapy of thrombosis. Glycoprotein VI (GPVI) is reported as the major signaling receptor for collagen and is exclusively expressed on platelets and megakaryocytes, initiating platelet recruitment at sites of vascular injury and demonstrating numerous beneficial effects for patients with cerebral thrombosis. In the present study, thrombus formation and platelet adhesion following endothelial injury was monitored in the jugular vein by intra-vital fluorescence microscopy. The morphological and clinical observations of cerebral thrombosis were investigated and analyzed in a mouse model with cerebral thrombosis. In addition, the present study investigated the effect of fusion protein GPVI modified with Fc and PEG, which is specifically linked to the extracellular domain of GPVI (GPVI-Fc-PEG), on thrombus formation following vessel wall injury and on experimental mice with cerebral thrombosis. The maximum tolerated dose (MTD) was identified as 0.18 mg. GPVI-Fc-PEG competitively bound to and prevented von Willebrand Factor-collagen interactions. The results of the present study demonstrated that cerebral thrombosis was greatly relieved and improved functional outcomes treatment with an MTD of GPVI-Fc-PEG following endothelial injury, compared with GPVI-Fc-treated mice. In addition, cerebral edema and infarct size was improved compared with GPVI-Fc-treated mice with ischemic stroke immediately prior to reperfusion. Furthermore, treatment of GPVI-Fc-PEG led to increased reperfusion and improved survival following cerebral thrombosis compared with treatment with either

single agent alone. Taken together, GPVI-Fc-PEG relieved cerebral thrombosis following ischemic stroke and improved prognostic preclinical outcomes without intracranial bleeding, which suggested that GPVI-Fc-PEG may be a potential candidate for cerebral thrombosis therapy.

Introduction

Cerebral infarction, known as ischemic stroke, is caused by various causes of local blood supply obstacles in the brain tissue, which lead to cerebral ischemic necrosis, anoxic lesions and even corresponding clinical nerve function loss (1-3). Currently, the incidence rate of cerebral infarction presents a rising trend with the growth in living standards throughout the world (4,5). Cerebral thrombosis has been identified as one of the most common cardiovascular diseases and the most frequent disabling disease that leads to mortality at the age of >60 years (6). Therefore, cerebral infarction severely affects the lives of patients. The underlying cause is rupture of atherosclerotic plaques following platelet adhesion and thrombus formation or embolization in cerebral thrombosis (7,8).

Activation of platelets is essential for normal hemostasis at sites of endothelial injury, however a congealing clot in the blood can cause stoppage of flow leading to a heart attack, aneurysm or stroke, depending on the location of the blocked vessel (9-11). A clot in the blood is a major pathomechanism underlying acute ischemic disease states including stroke, atherosclerosis, myocardial infarction and cerebral hemorrhage, which may lead to severe disability. They cause the majority of mortalities in clinical emergencies all over the world (12,13). Glycoprotein VI (GPVI) has been identified as the major signaling receptor for collagen and is exclusively expressed on platelets and megakaryocytes, initiating platelet recruitment at sites of vascular injury and demonstrating numerous beneficial effects for patients with cerebral thrombosis (14). Platelet GPVI is upregulated in patients with acute stroke, coronary syndrome and is associated with acute cerebral infarction (15). In addition, GPVI may be a potential target and helpful to control infarct volume in patients with myocardial necrosis and acute vascular syndromes (16,17).

The activation of platelets mediated by GPVI and subsequent shedding of GPVI serves as a decisive factor in the blood of patients with acute vascular syndromes (14). A

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previous study (18) reported that GPVI-Fc combined with von Willebrand Factor (vWF) and inhibited platelet adhesion, serving an essential role in vascular syndromes therapy and eliminating thrombus formation. Therefore, the efficacy of GPVI-Fc maybe a potential candidate target for the pharmacological inhibition of pathological thrombus formation in patients with vascular syndromes (19,20). The importance of GPVI-mediated signals pathway has been investigated in a recent clinical study (21).

Recently, polyethylene glycol (PEG) has been reported as a small molecule, which can modify various protein drugs to formed nanoparticles, leading to improved pharmacodynamics in clinical outcomes (22,23). In addition, the effects of this modification of pharmaceuticals by different PEG-containing block-copolymers on the preparation of ovalbumin-loaded PLGA nanoparticles has been studied and applied in clinical settings and has demonstrated improved efficacy for patients (24). Therefore, protein modification by PEG may be conducted to improve the therapeutic effects of protein drugs.

In the present study, PEG-modified GPVI was tested for the treatment of cerebral thrombosis and cerebral damage. The preclinical outcomes demonstrated that experimental cerebral thrombosis was relieved following treatment with PEG (2000) modified GPVI-Fc (GPVI-Fc-PEG) in a cerebral thrombosis animal model, suggesting that GPVI-Fc-PEG may be a potential candidate for cerebral thrombosis therapy.

Materials and methods

Ethics statement. The present study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, MD, USA). The protocol was approved by Chinese Association for Laboratory Animal Sciences, Animal Health Products and the committee on the Ethics of Animal Experiments Defense Research. All surgery and euthanasia were performed under sodium pentobarbital (30 mg/kg; Jiangsu Lianshui Pharmaceutical Co., Ltd., Lianshui, China) anesthesia followed by cervical dislocation, and all efforts were made to minimize suffering.

Enzyme-linked immunosorbent assay (ELISA). In order to assess the capacity binding of GPVI-Fc-PEG (cat. no. ab133065; Abcam, Cambridge, UK) or vWF (cat. no. ab108918; Abcam) to collagen, commercially available ELISA kits were used. The ELISA assays were performed according to the manufacturer's instructions (25). The result was measured at 450 nm in an ELISA reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and finally converted to the affinity of GPVI-Fc-PEG for bovine and mouse collagen. Competitive affinity analysis of GPVI-Fc-PEG to collagen with vWF was also determined by competitive ELISA.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from cerebroarterial cells using an RNAeasy Mini kit (Qiagen Sciences, Inc., Gaithersburg, MD, USA) in experimental and control mice. Total RNA (1 µg) was reverse transcribed into cDNA using a reverse transcription kit (Qiagen Sciences, Inc.) at 37°C for 30 min and the quality was confirmed by 30% SDS-PAGE.

Table I. Sequences of primers were used for reverse transcription-quantitative polymerase chain reaction in the present study.

Gene	Sequence
IgG	F: 5'-CTCCAGCAGTCTTCATGTTCCCCC-3' R: 5'-AAGCTTGATGGTCTTCTGCGTGTGGT-3'
TGF-β	F: 5'-GCTTTGGATGCCGCCTATTGC-3' R: 5'-GCTGCATTTGCAAGACTTTAC-3'
PDGF	F: 5'-AAGACCATGAGCCTGGGTACC-3' R: 5'-CTCGGTCACAGGCCGTGCTGC-3'
β-actin	F: 5'-AGAAAATCTGGCACCACACC-3' R: 5'-TAGCACAGCCTGGATAGCAA-3'

F, forward; R, reverse; Ig, immunoglobulin; TGF, transforming growth factor; PDGF, platelet-derived growth factor.

The cDNA (10 ng) was subjected to qPCR with the SYBR Green Master Mix system (Bio-Rad Laboratories, Inc.). Thermocycling conditions were as follows: Initial denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 20 sec, at 58°C for 20 sec and at 72°C for 20 sec, with a final extension at 72°C for 5 min. All the forward and reverse primers were synthesized by Invitrogen; Thermo Fisher Scientific, Inc. (Waltham, MA, USA) and are presented in Table I. Relative mRNA expression changes were calculated by the $2^{-\Delta\Delta C_q}$ method (26). The results are expressed as the n-fold way vs. control.

Animal studies in vivo. A total of 6 eight-week-old female C57BL/6 mice (weight, 30-35 g) were purchased (Bioray Laboratories, Inc., Shanghai, China) and housed in specific pathogen-free conditions. All animals were housed in a temperature-controlled facility at 23±1°C with a relative humidity of 50±5%, under a 12-h light/dark cycle with free access to food and water. A lesion of the endothelium, induced by a transient ligation of the left common carotid artery, was used to test the antithrombotic effect of GPVI-Fc-PEG on an injured arterial wall. To visualize platelet adhesion to the injured vessel wall under *in vivo* conditions, platelets were fluorescently labeled and injected intravenously and monitored *in situ* with an intravital microscope over 45 min following the endothelial damage. The MTD of GPVI-Fc-PEG was conducted as previously described (27). Administration of GPVI-Fc-PEG or GPVI-Fc (0.18 mg) once daily was performed immediately prior to inducing the endothelial lesion in the common carotid artery.

GPVI-Fc-PEG in vivo functional outcome in mice with cerebral thrombosis. The influence of GPVI-Fc-PEG on arterial thrombosis induced by deeper lesions of the arterial wall was investigated in a mouse model of wire-induced different degrees of vascular injury. Following preparation of the carotid artery, a coronary guiding wire was introduced via the external carotid artery and rubbed over the endothelium of the mouse common carotid artery. GPVI-Fc-PEG or GPVI-Fc was injected intravenously prior to the intervention as in a previous study (18). Thrombus size was quantified

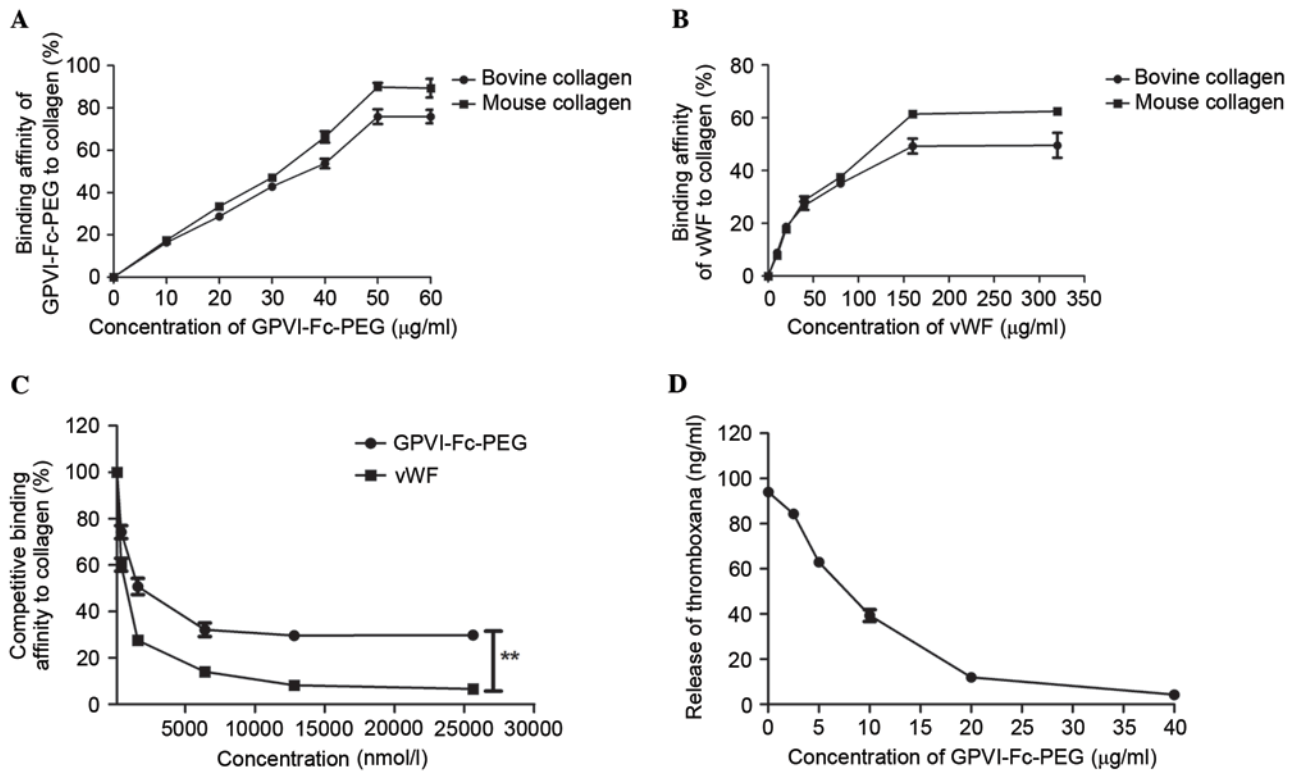


Figure 1. Characteristics of GPVI-Fc-PEG and competitive binding to collagen with vWF. The affinity of (A) GPVI-Fc-PEG and (B) vWF to binding with mouse or bovine collagen by ELISA. (C) Competitive ELISA of GPVI-Fc-PEG and vWF for collagen binding. (D) Inhibition of GPVI-Fc-PEG for collagen-related peptide-stimulated thromboxane release in human platelets in a dose-dependent manner. Data are presented as mean \pm standard deviation (n=3). Significance (* $P<0.05$ and ** $P<0.01$) was analyzed using one-way analysis of variance. GPVI-Fc-PEG, Fc and PEG modified glycoprotein VI; ELISA, enzyme-linked immunosorbent assay; vWF, von Willebrand Factor.

following digital imaging and quantification using Image-Pro Plus software version 6.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Determination of GPVI-Fc-PEG for platelet function *in vivo*.

An optical microscope was equipped with a video camera recorder to analyze the role of GPVI-Fc-PEG in platelet function *in vivo*. For determining the vessel diameter, a video was captured at x100 magnification. For the determination of transiently adherent platelets, video sequences of 30 sec were captured at x200 magnification 5, 10, 15, 20 and 30 min following endothelial damage. Transiently adherent platelets were counted in slow motion during 30 sec video sequences within a $150 \times 100 \mu\text{m}^2$ window, which was placed on the video screen directly over the endothelial lesion. At 30 and 60 min following endothelial damage, the platelet thrombus area was determined. For the determination of the thrombus area, three screen shots were captured and the area of mean total platelet thrombi were added up for an overall thrombus area and evaluated using Image-Pro Plus software version 6.0 (Media Cybernetics, Inc.).

Statistical analysis. All data are presented as the mean \pm standard deviation of triplicate experiments. Unpaired data was assessed by Student's t-test and comparisons of data between multiple groups were analyzed by one-way analysis of variance followed by a post hoc Dunnett's test for multiple comparisons. Kaplan-Meier was used to estimate the risk of relapse and

re-treatment during the 30 day treatment. $P<0.05$ and $P<0.01$ were considered to indicate a statistically significant difference.

Results

GPVI-Fc-PEG demonstrated completely binding to collagen with vWF. GPVI demonstrated a high affinity with collagen in a previous study (28) and in order to test the affinity of GPVI-Fc-PEG with collagen, ELISA was performed in the present study. The result, presented in Fig. 1A, revealed that GPVI-Fc-PEG demonstrated a specific affinity to bovine and mouse collagen in a linear dose-dependent manner. vWF specific affinity for bovine and mouse collagen was demonstrated, with the maximum bindings of 241 and 76 ng/ml, respectively (Fig. 1B). A competitive ELISA experiment was conducted to investigate the capacity of GPVI-Fc-PEG and vWF for completely binding to collagen. As presented in Fig. 1C, GPVI-Fc-PEG presented competition for the binding of vWF to collagen at increasing doses, while Fc and PEG did not exhibit competitive effects. Additionally, the results demonstrated that GPVI-Fc-PEG inhibited collagen-related peptide (CRP)-stimulated thromboxane release from human platelets in a dose-dependent manner (Fig. 1D).

Effect of GPVI-Fc-PEG suppressed thrombus formation on platelet-endothelial cell interactions following endothelial lesion in mice *in vivo*. A previous study (18) demonstrated that

Table II. Treatment-related adverse events of GPVI-Fc-PEG with an overall incidence $\geq 10\%$.

Adverse event	Total (n=36)	GPVI-Fc-PEG (0.04-0.12 mg) (n=12)	GPVI-Fc-PEG (0.18-0.32 mg) (n=12)	GPVI-Fc-PEG (0.40 mg) (n=12)
Hypertension	6	1	2	3
Proteinuria	7	2	2	3
Diarrhea	7	2	2	3
Constipation	4	1	1	2
Lethargy	10	2	3	5
Diarrhea	10	2	3	5
Vomiting	4	1	1	2

GPVI-Fc-PEG, Fc and PEG modified glycoprotein VI.

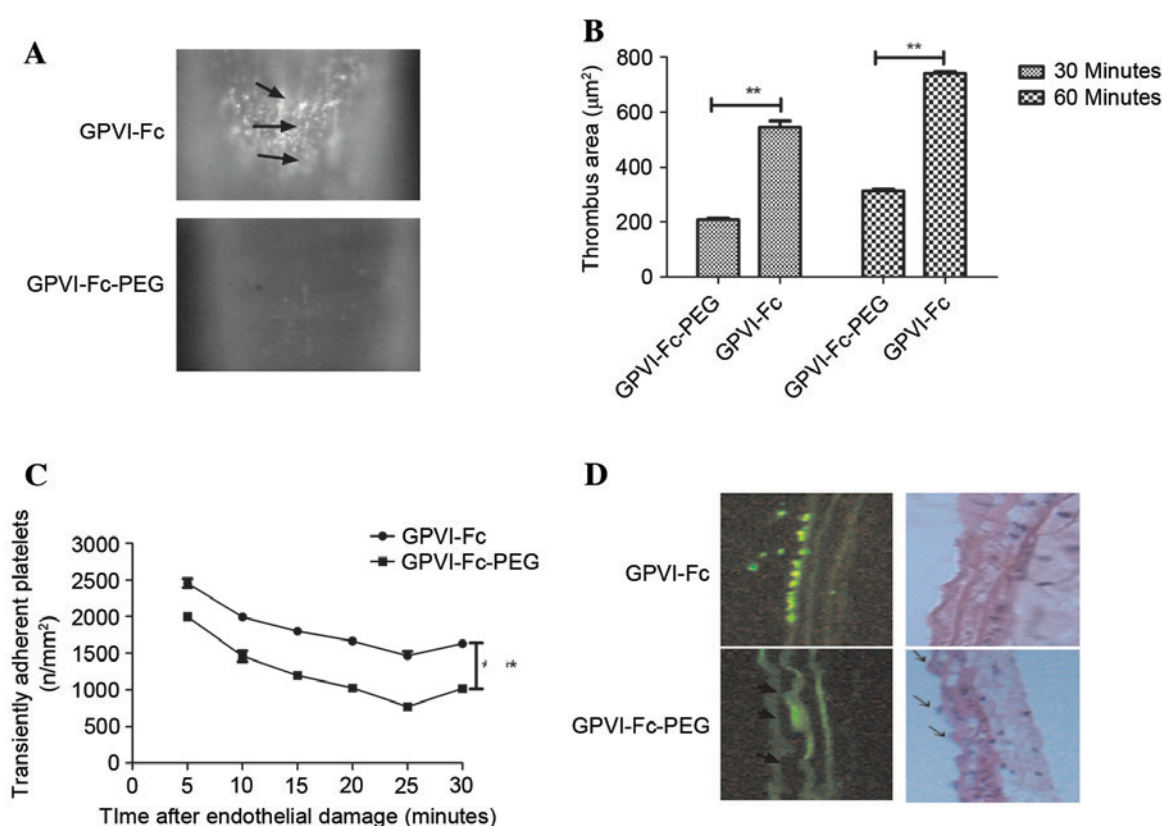


Figure 2. Inhibition effects of GPVI-Fc-PEG on cerebral thrombus formation. (A) Immunohistochemical analysis of inhibition effects of GPVI-Fc-PEG on cerebral thrombus formation by spectrophotometric assay. (B) Effects of MTD of GPVI-Fc-PEG (0.18 mg) on thrombus area 45 min following vascular injury. (C) Effects of MTD of GPVI-Fc-PEG (0.18 mg) on transient platelet adhesion. (D) Analysis of inhibitory effects of GPVI-Fc-PEG on endothelial lesion in mice *in vivo*. Data are presented as mean \pm standard deviation ($n=3$). $^{**}P<0.01$. GPVI-Fc-PEG, Fc and PEG modified glycoprotein VI; MTD, maximum tolerated dose.

GPVI-Fc inhibited thrombus formation on platelet-endothelial cell interactions following endothelial lesion in mice *in vivo*. In the present study, GPVI-Fc-PEG was used to analyze its inhibition effects on platelet-endothelial cell interactions and therapeutic effects in mice model of cerebral thrombosis. The MTD of GPVI-Fc-PEG in C57BL/6 mice was studied first and the median overall duration of treatment was 7 days. The dosing cohort of GPVI-Fc-PEG was 0.08, 0.16, 0.32, 0.64 and 0.80 mg/animal. In the results, 0.18 mg of GPVI-Fc-PEG once daily was identified as the MTD. The most common treatment-related adverse events were hypertension,

diarrhea, vomiting, lethargy, constipation, proteinuria and vomiting (Table II).

Subsequently, mice with cerebral thrombosis were treated with GPVI-Fc or GPVI-Fc-PEG or with PBS as a control. Endothelial erosion led to vascular injury with consecutive thrombus formation and was verified by histological analysis (Fig. 2A). As hypothesized, GPVI-Fc-PEG resulted in a significant reduction of cerebral thrombosis measured by platelet thrombus size following endothelial damage in the right common carotid artery compared with other drug-treated and control groups (Fig. 2B). In addition, the ability of platelets

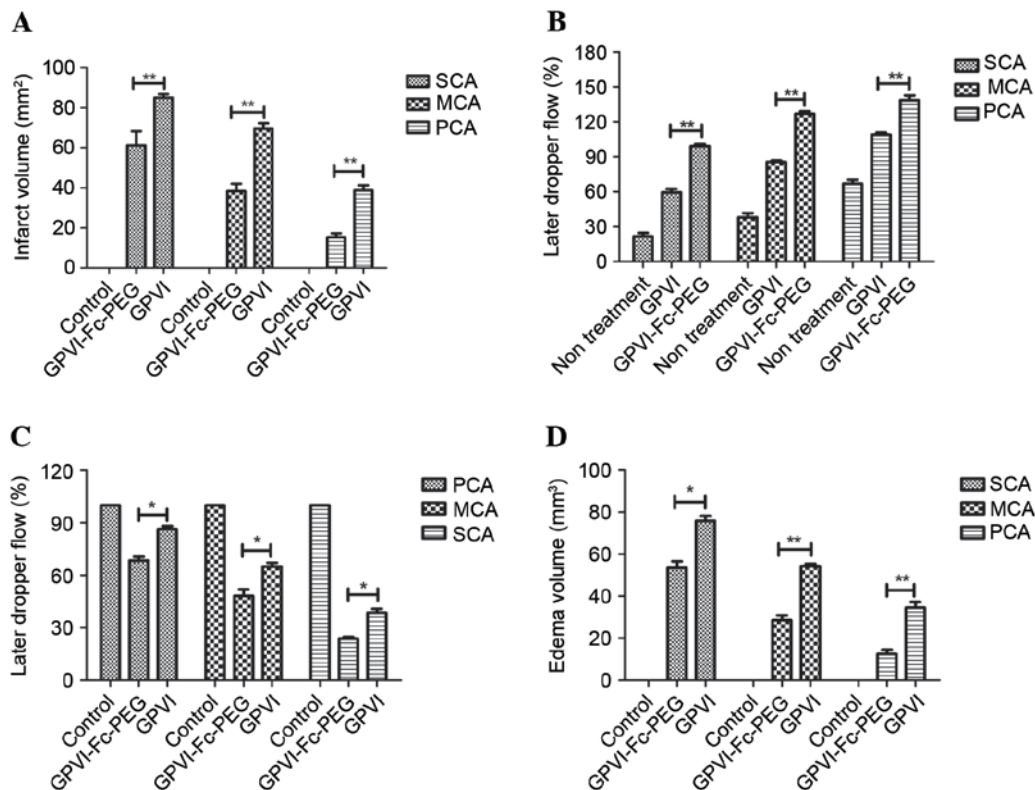


Figure 3. Efficacy of GPVI-Fc-PEG for thrombosis induced by vascular injury. (A) Infarct volume of brain slices in PCA, MCA and SCA following treatment with GPVI-Fc-PEG compared with GPVI-Fc. (B) Blood flow in SCA, MCA and PCA following treatment with GPVI-Fc-PEG compared with the GPVI-Fc group during the occlusion time. (C) Therapeutic efficacy of GPVI-Fc-PEG on reperfusion time SCA, MCA and PCA following treatment with GPVI-Fc-PEG. (D) Mean edema volumes in GPVI-Fc-PEG- and GPVI-Fc-treated mice with control, as detected by a flexible laser Doppler flow probe attached to the temporal skull. Significance (* $P < 0.05$ and ** $P < 0.01$) was determined by Student's t-test. GPVI-Fc-PEG, Fc and PEG modified glycoprotein VI; SCA, severe cerebral artery lesion (CA); MCA, moderate CA; PCA, primary CA.

to adhere to the endothelium was significantly decreased in the GPVI-Fc-PEG-treated group from 10 min after treatment following endothelial injury compared with the other groups (Fig. 2C). Histological analysis in Fig. 2D further confirmed the efficacy of GPVI-Fc-PEG in the treatment of cerebral thrombosis *in vivo*.

GPVI-Fc-PEG demonstrated efficacy for thrombosis induced by vascular injury. The efficacy of GPVI-Fc-PEG on differing degrees of vascular injury was investigated. Different degrees of vascular injury could be induced by wire that led to exposure of severe, moderate and primary layers of the vascular wall to the blood. GPVI-Fc-PEG inhibited arterial thrombosis increase following 24 h at MTD dose in wire-induced severe (S), moderate (M) and primary (P) cerebral artery (CA) vascular lesion in mice compared with the GPVI-Fc group (Fig. 3A). Blood flow in the SCA, MCA and PCA was recorded following 30 min occlusion and 30 min reperfusion. Fig. 3B demonstrates that the blood flow was increased by ~34, 40 and 30% in SCA, MCA and PCA, respectively compared with the GPVI-Fc group during the occlusion time (45 min). In addition, the reperfusion time was decreased ~15, 16 and 13% in SCA, MCA and PCA, respectively compared with the GPVI-Fc group (Fig. 3C). Additionally, morphological effects of GPVI-Fc-PEG on ischemic cerebral stroke by SCA, MCA and PCA occlusion were observed (data not shown). The results (Fig. 3D)

demonstrated that the edema volume of mice was significantly reduced following treatment with GPVI-Fc-PEG compared with the GPVI-Fc group.

Effect of GPVI-Fc-PEG on cellular inflammatory infiltration, reperfusion damage, functional outcome and survival rate in mice following stroke induced by different degree of occlusion. The therapeutic effects of GPVI-Fc-PEG on SCA, MCA and PCA were evaluated at 6 and 18 h following the onset of reperfusion. As presented in Fig. 4A grip strength was significantly increased in GPVI-Fc-PEG-treated mice compared with the GPVI-Fc and control groups following reperfusion. GPVI-Fc-PEG demonstrated beneficial outcomes although with a trend to less positive motor activity compared with GPVI-Fc-PEG. Changes in neurological function were noted following GPVI-Fc-PEG-treatment. The results (Fig. 4B) demonstrated significant differences between GPVI-Fc-PEG-treated and GPVI-Fc-treated mice. Improvement of neurological function was observed following 24 h in the GPVI-Fc-PEG-treated mice with GPVI-Fc as control. In addition, the results (Fig. 4C) indicated that the survival rate was prolonged following treatment with GPVI-Fc-PEG in mice with different degrees of cerebral artery lesion at 24, 48 and 72 h following reperfusion. Several factors that indicate inflammatory response to injury were assessed by RT-qPCR in brain sections of mice with SCM. A significant reduction of

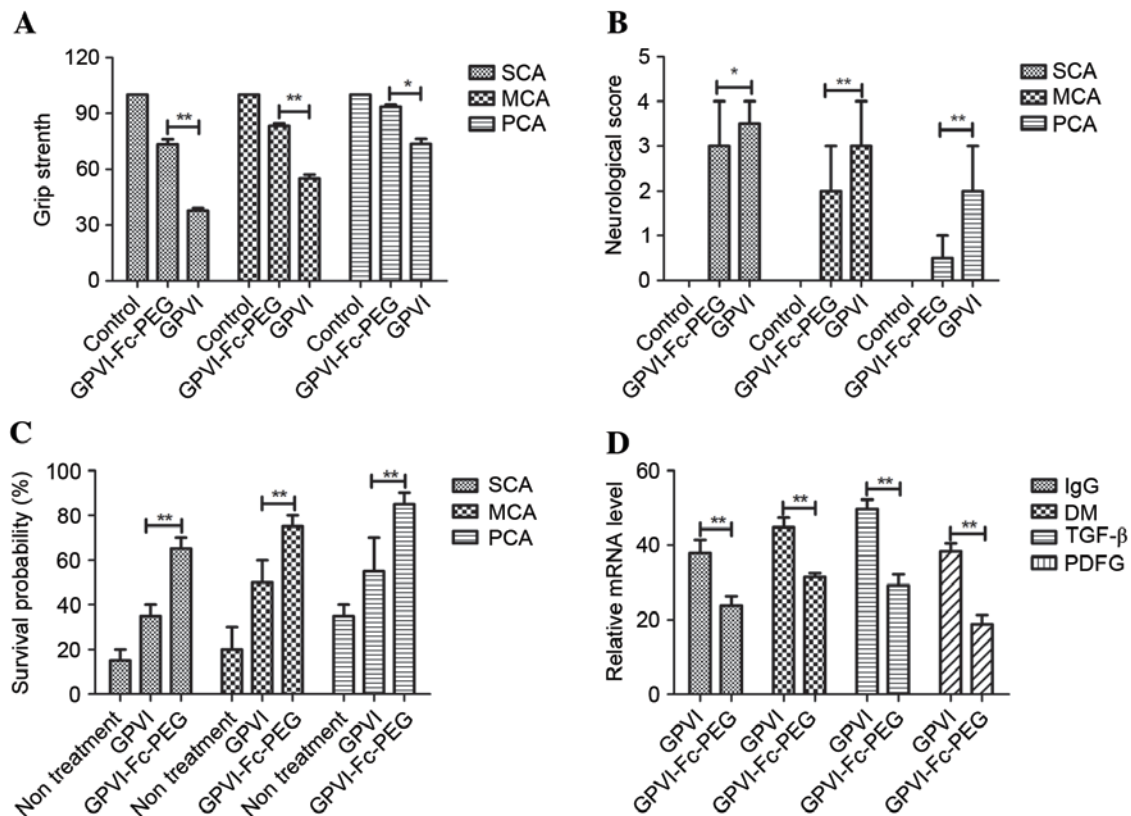


Figure 4. Detection of parameters of inflammatory factors in brain tissue by *in situ* immunohistochemistry in mice. (A) Relaxing effects of GPVI-Fc-PEG on the grip strength in mice with SCA, MCA and PCA. (B) Neurological Bederson score (0, no deficit; 1, forelimb flexion; 2, decreased resistance to lateral push without circling; 3, circling and 4, no spontaneous movement) of mice treated with GPVI-Fc-PEG; n=6 in each group and *P<0.05 was considered to indicate a statistically significant difference. (C) Survival rate of mice with SCA, MCA and PCA following treatment with GPVI-Fc-PEG in a 30-day observation. Kaplan-Meier was used to estimate the risk of relapse and re-treatment during 30-day treatment. *P<0.05 and **P<0.01 were considered statistically significant. (D) Decrease of inflammatory factors IgG, TGF-β, DM and PDGF in brains from GPVI-Fc-PEG- and GPVI-Fc-treated mice with SCA, MCA and PCA; n=6 in each group and *P<0.05 was considered to indicate a statistically significant difference. GPVI-Fc-PEG, Fc and PEG modified glycoprotein VI; SCA, severe cerebral artery lesion (CA); MCA, moderate CA; PCA, primary CA; IgG, immunoglobulin G; DM, density of macrophages; TGF-β, transforming growth factor-β; PDGF, platelet derived growth factor.

immunoglobulin G, density of macrophages, transforming growth factor (TGF)-β and platelet-derived growth factor was observed in GPVI-Fc-PEG-treated mice with cerebral thrombosis (Fig. 4D).

Discussion

Platelet activation is not only indispensable for initiation, formation and stabilization of cerebral thrombus, but also enhances the progression of vascular damage, increases inflammatory factor expression and even occludes reperfusion of the arteries (29). Platelet activation is indispensable for initiation although vWF or GPVI bind to platelet receptor glycoprotein Ib, leading to integrin αIIbβ3 activation and platelet aggregation in the platelet receptor (30). Subsequently, pathological thrombus formation is observed in the local blood supply causing obstacles in brain tissue area and it has been suggested that platelet activation is important in pathological thrombus formation, however its exact *in vivo* function has long remained undefined (31,32).

In the present study, the function of GPVI-Fc-PEG in cerebral thrombosis was investigated in different degrees of cerebral thrombosis (SCA, MCA and PCA). The findings demonstrated that treatment with GPVI-Fc-PEG by

intravenous injection led to an evolutionary relegation of thrombus formation and inflammatory response to injury following endothelial damage and a significant improvement of neurological function and prognostic outcome in addition to reduction of cerebral infarction area in mice with cerebral thrombosis or ischemic stroke. In addition, the data presented an improved anti-ischemic effect and greatly avoided the risk of cerebral hemorrhage. Thus, GPVI-Fc-PEG markedly enhanced the preclinical outcome of cerebral thrombosis without increasing the risk of cerebral hemorrhage, achieved by nanoparticles modified by PEG. According to the results of the present study, GPVI-Fc-PEG competitively inhibited the binding capacity of vWF to collagen and contributed to the improved therapeutic effects of GPVI-Fc-PEG for cerebral thrombosis.

Previous studies (33-35) have reported that the GPVI pathway is a potential treatment target for cerebral thrombus by the administration of GPVI antibody, which not only resulted in a decrease of GPVI protein level, but also demonstrated suppressive effects on other platelet signal pathways, including thrombin-dependent activation. In addition, a previous review (36) considered the complex signal pathway of GPVI and described the function, structure, post-translational, binding partners and modifications presently

known in cerebral thrombus. Furthermore, Walsh *et al* (37) demonstrated that Nox1 and Nox2 served an essential role in GPVI-dependent platelet activation and thrombus formation, and their results demonstrated that Nox1 is the key Nox homolog regulating GPVI-dependent reactive oxygen species production, essential for CRP-dependent thromboxane (Tx)A₂ production, and was mediated in part through p38 mitogen-activated protein kinase signaling. Coincidentally, Goebel *et al* (18) examined the effect of GPVI-Fc on cerebral thrombus following vessel wall injury in a mouse model of cerebral thrombus. However, the results for GPVI-Fc did not present an ideal efficacy for the pharmacodynamics of macromolecular particles.

In the present study, the preclinical efficacy of GPVI-Fc-PEG was synthesized and therapeutic outcomes of GPVI-Fc-PEG was explored in cerebral thrombus mouse model. The results demonstrated that the therapeutic outcomes of GPVI-Fc-PEG surpassed GPVI-Fc in cellular inflammatory infiltration, reperfusion damage, functional outcome and survival rate in mice following stroke induced by different degree of occlusion. In addition, the findings suggest that GPVI-Fc-PEG was a complete inhibitor with vWF in platelet activation via binding to collagen exposed at vascular injury.

In conclusion, the present study confirmed that GPVI-Fc-PEG could efficiently block the GPVI-mediated and bind competitively with vWF-mediated activation of platelets compared with GPVI-Fc, and block thrombus formation by decreasing the level of collagen following vascular injury. These improved efficacies were also identified in the injured brain ischemic tissue during cerebral thrombus and reperfusion, which presented less vascular damage in SCA, MCA and PCA compared with a previous study (38). However, more studies are required to further elucidate the mechanisms of the beneficial role of GPVI-Fc-PEG during cerebral thrombus.

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