GP6 promotes the development of cerebral ischemic stroke induced by atherosclerosis via the *FYN-PKA-pPTK2*/FAK1 signaling pathway

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Abstract

Background. Cerebrovascular disease is currently a serious threat to human health and life, commonly including cerebral infarction, cerebral hemorrhage and transient cerebral ischemia, among others.

Objectives. To explore the role and e molecular mechanism of *GP6* in the development of cerebral ischemic stroke (CIS) induced by atherosclerosis (AS).

Materials and methods. Forty-five male New Zealand white rabbits were randomly divided into 3 groups: the control, CIS model and anti-*GP6* group. Carotid artery tissues and blood of the white rabbits were collected for analysis. Hematoxylin and eosin (H&E) staining was used to analyze the pathological characteristics of vascular endothelial injury. Flow cytometry (FCM) was performed to analyze the content of *Th1* and *Th17* in blood. Immunohistochemistry was used to analyze the distribution and relative expression of *FCER1G*, *ITGA2* and *GP6* proteins in the carotid artery and cerebrovascular tissues. Western blot was applied to determine the protein expression of *GP6*, *FYN*, *PKA*, *pPTK2*, and *pFAK1* in carotid artery tissues of the rabbits.

Results. In the CIS model group, there was lymphocyte infiltration, fibrous tissue formation, and the formation of thrombus and lipid plaques. In the anti-*GP6* group, scattered thin plaques were observed, and no obvious foam cell deposition was observed. The *Th1* and *Th17* content was significantly decreased in the CIS model group compared to the control and anti-*GP6* group. The relative expression of *FCER1G*, *ITGA2* and *GP6* in the CIS model group was significantly higher compared to those in the control group and anti-*GP6* group. The protein expression of *GP6*, *FYN*, *PKA*, *pPTK2*, and *pFAK1* in the CIS model group were markedly higher compared to those in the control group and anti-*GP6* group.

Conclusions. *GP6* can promote the development of CIS by activating the *FYN-PKA-pPTK2*/FAK1 signaling pathway.

Key words: CIS, AS, *GP6*, thrombus, vascular endothelial cells

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Background

Cerebrovascular disease is currently a serious threat to human health and life, the most common being cerebral infarction, cerebral hemorrhage and transient cerebral ischemia, among others.[1] It mainly occurs in middle-aged and elderly people, but the age of onset has shown a trend of decreasing, often occurring in people 30–50 years old.[2] The disease often leads to cognitive disorders or disorders affecting the physical activity of patients. Many patients are unable to take care of themselves after onset and quality of life is seriously reduced, resulting in them becoming a heavy burden to their families and society.[3] At present, there is no radically effective cure for the disease, and the prognosis is often closely related to the initial severity of the disease.[4] Even if patients survive, most of them are accompanied by lifelong sequelae, and cerebrovascular diseases can occur repeatedly, causing far more pain to the patients and their families than malignant tumors and cardiovascular diseases. Therefore, how to prevent and treat cerebrovascular disease more effectively is the most serious problem that neurology faces presently.[5] Cerebral ischemic stroke (CIS) is ischemic necrosis or softening of localized brain tissue due to cerebral blood circulation disorder, ischemia or hypoxia. It is the most common type of cerebrovascular disease, accounting for about 70% of all acute cerebrovascular diseases. About 30–40% of CIS is caused by carotid atherosclerotic stenosis.[6]

Extracellular matrix (ECM) contact exposed at the site of platelet and vascular injury is the first line of defense for repairing damaged tissue and stopping bleeding. Collagen, one of the macromolecular components of ECM, can not only adhere to platelets through direct and indirect channels but also cause platelet aggregation and expression of pro-coagulant activity. The interaction between platelets and collagen in arteries or damaged vessels with high shear stress is particularly important. Current research suggests that platelets have 2 types of collagen receptors on their surfaces, α2β1 and glycoprotein 6 (*GP6*). It is a glycoprotein receptor that acts on collagen and is located on the long arm of chromosome 19 (19q13.4). It contains 8 exons and has a molecular weight of 62 kDa. In humans, *GP6* is encoded by the *GP6* gene. *GP6* mediates the initial adhesion of platelets to collagen, generates signal transduction, improves the binding affinity of integrin receptors, and causes platelet aggregation, platelet release and thrombosis.[7] When serum *GP6* concentration is increased, it can promote the initial adhesion of collagen and platelets, improving the affinity of the integrin receptor, which induces platelet aggregation and thrombosis.[8] Kubisz et al. found that a variation of the *GP6* gene was related to platelet aggregation.[9] Sokol et al. found that a *GP6* gene polymorphism may be related to the heightened aggregation ability of platelets.[10] Despite the complex composition of the ECM, the adhesion of platelets and collagen plays an important role in initiating hemostasis and thrombus in the body. Inhibition of *GP6* function can significantly inhibit collagen-induced platelet adhesion, aggregation and platelet thrombosis under high shear stress in vitro.[11] Therefore, we believe that atherosclerotic CIS is closely related to the structural and functional integrity of *GP6*, but the mechanism of action of *GP6* in atherosclerosis (AS) has rarely been studied.

Objectives

In this paper, we wanted to investigate the mechanism of action of *GP6* in AS. Helper T (Th) cell content, *Th1* and *Th17*, was significantly decreased in the CIS model group compared to the control group, while having no marked differences compared to the anti-*GP6* group. Immunohistochemistry showed that the proteins *FCER1G*, *ITGA2* and *GP6* were all distributed in the cell membrane. *GP6* can promote the formation of cerebral ischemic stroke via upregulating the expression of *FCER1G*, *ITGA2*, *FYN*, *PKA*, *pPTK2*, and *pFAK1*. This may be a new target for treating cerebral ischemic stroke.

Materials and methods

Model establishment and grouping

Forty-five male New Zealand white rabbits (~16–18 weeks old, weight: ~1.5–2.0 kg) were obtained from the Naval Medical Institute Animal Center (Shanghai, China). They were randomly divided into 3 groups (n = 15 in each group): the control group, CIS group and anti-*GP6* group. In the CIS group and the anti-*GP6* group, rabbits were first fed with a specific high-fat diet for 8 weeks, and then 3% hydrogen peroxide solution was applied to perfuse the bilateral common carotid arteries of the rabbits, causing vascular endothelial oxidative stress injury. In the anti-*GP6* group, the rabbits were given anti-*GP6* antibody via the tail vein. Carotid artery tissues of the white rabbits were collected and rapidly stored in –80°C liquid nitrogen for further use.

In this study, atherosclerotic stenosis injury rabbit models were constructed and separated into 3 groups: the control group, CIS group and anti-*GP6* group. Hematoxylin and eosin (H&E) staining was used to analyze the pathological features of vascular endothelial injury. Flow cytometry (FCM) was used to determine the concentration of *Th1* and *Th17* cells in blood samples. The expressions of *GP6*, *FYN*, *PKA*, *pPTK2*, and FAK1 were also assessed.

The animal experiments were approved by the Ethics Committee of Xinhua Hospital affiliated with Shanghai Jiaotong University School of Medicine (Shanghai, China).

Hematoxylin and eosin staining

After the rabbits were sacrificed by the cervical dislocation method, hippocampal tissue was taken from their brains. The tissues were fixed with 4% formaldehyde for 24 h, embedded in paraffin and sectioned into 5 μm-thick sections. Histopathological changes were observed under a light microscope (Olympus Corp., Tokyo, Japan) after H&E staining.

Flow cytometry

Cells were digested with trypsin, washed twice with phosphate-buffered saline (PBS) and collected into a centrifuge tube. Binding buffer was added to prepare cell suspensions with a final concentration of 1×10^6/mL. Annexin V-FITC kit (BioVision, Milpitas, USA) instructions were used for labeling, which was performed according to the instructions of Annexin V-FITC kit. Annexin V was added for staining at room temperature in the dark for 15 min.

Western blot

Cells were lysed with cell lysis buffer on ice for 30 min and then centrifuged at 12,000 rpm for 25 min. Supernatant was collected and the Bradford method was used for protein quantification. Protein in the amount of 25 μg was loaded for sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane and sealed with 5% skim milk at room temperature for 1 h; then, primary antibody was added and the membrane was incubated overnight at 4°C. The next day, the secondary antibody was added and incubated at room temperature for 1 h, and then, electrochemiluminescence (ECL) hypersensitive luminescence solution was added for developing.

Immunohistochemistry

Tissues were dewaxed and endogenous oxidase was inactivated with 3% hydrogen peroxide. After washing in PBS 3 times, the antigen was retrieved by microwave heating. The slices were placed in sodium citrate buffer with pH 6.0, heated to boiling in a microwave and then cooled. The slices were again microwaved in sodium citrate buffer with pH 6.0 and then washed with PBS. After cleaning, sections were removed and sealed for 10 min. Then, mouse anti-human *FCER1G*, *ITGA2* and *GP6* monoclonal antibodies (1:150) were added and incubated at 4°C for 12 h. After washing, biotin-labeled secondary antibodies were added and incubated at 4°C for 20 min each. After incubation and washing, the samples were washed again and immersed in DAB color developing solution for 5 min. Then, the samples were washed, re-dyed, dehydrated, sealed and observed under an optical microscope.

Statistical analyses

IBM SPSS v. 19.0 software (IBM Corp., Armonk, USA) was used for statistical analysis. The results were expressed as mean values ± standard deviation (SD). Differences between groups were compared using one-way analysis of variance (ANOVA) tests followed by Bonferroni post hoc test. The assumption for ANOVA was verified with the Brown–Forsythe test. A value of $p < 0.05$ was considered statistically significant.

Results

Anti-*GP6* inhibits vascular endothelial injury

After the successful establishment of the evaluation models, the rabbits were anesthetized with isopentane and sacrificed. Carotid artery tissue and cerebrovascular tissues were collected from the white rabbits, and H&E staining was used to analyze the pathological characteristics of the vascular endothelial injury. As shown in Fig. 1, in the control group, the smooth muscle cells were arranged neatly, tightly and orderly, the endothelial layer was continuous and there was no lipid deposition in the subcutaneous tissue. The structure of each layer was clear. In the CIS group, the endothelium was discontinuous, the intimal hyperplasia was serious, the tube wall was full of plaques, a large number of foam cells could be seen, the membranes in the middle and outside were irregularly thickened, and foam cell infiltration could be seen. In the anti-*GP6* group, endothelial cells were continuous, lipid deposition was observed under the intima, scattered thin plaques were observed, and no obvious foam cell deposition was observed. The thickness of the membrane was the same as that of the control group membrane, which was thinner than the CIS model group membrane.

Content of *Th1* and *Th17*

The content of *Th1* and *Th17* in blood was analyzed with FCM and the results are shown in Fig. 2. The *Th1* ($p < 0.01$, degrees of freedom (df) = 2, $F = 337.7$) and *Th17* ($p < 0.01$, df = 2, $F = 150.6$) content was significantly decreased in the CIS model group compared to the control group, while there was no marked difference compared to the anti-*GP6* group.

Results of immunohistochemistry

Immunohistochemistry was used to analyze the distribution and relative expression of *FCER1G*, *ITGA2* and *GP6* proteins in the carotid artery and cerebrovascular tissues of white rabbits. The proteins *FCER1G*, *ITGA2* and *GP6* were all distributed in the cell membrane

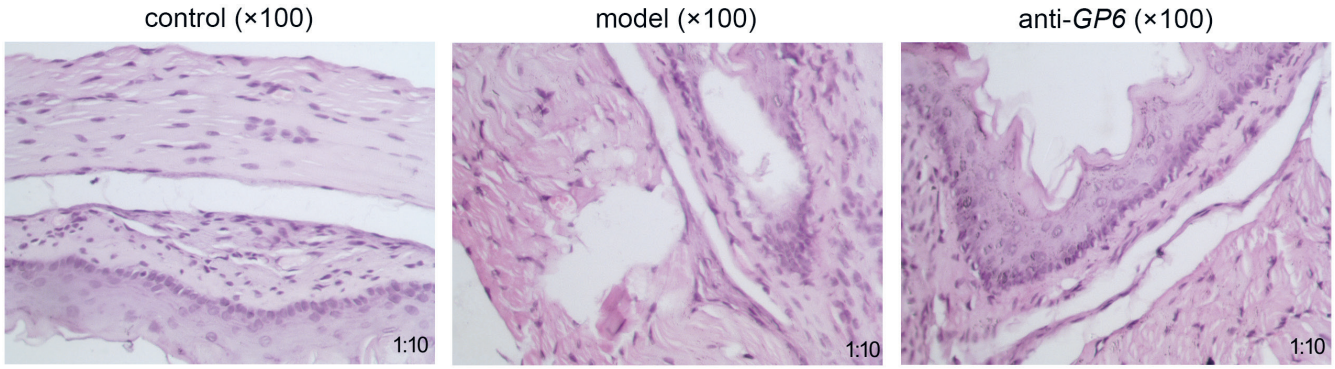

Fig. 1. The pathological features of vascular endothelial injury in the control, cerebral ischemic stroke (CIS) model and anti-*GP6* groups analyzed using H&E staining. In the control group, the smooth muscle cells were arranged neatly, tightly and orderly, the endothelial layer was continuous and there was no lipid deposition in the subcutaneous tissue; in the CIS model group, the endothelium was discontinuous, the intimal hyperplasia was serious, the tube wall was full of plaques, and a large number of foam cells could be seen; and in the anti-*GP6* group, scattered thin plaques were observed and no obvious foam cell deposition was observed

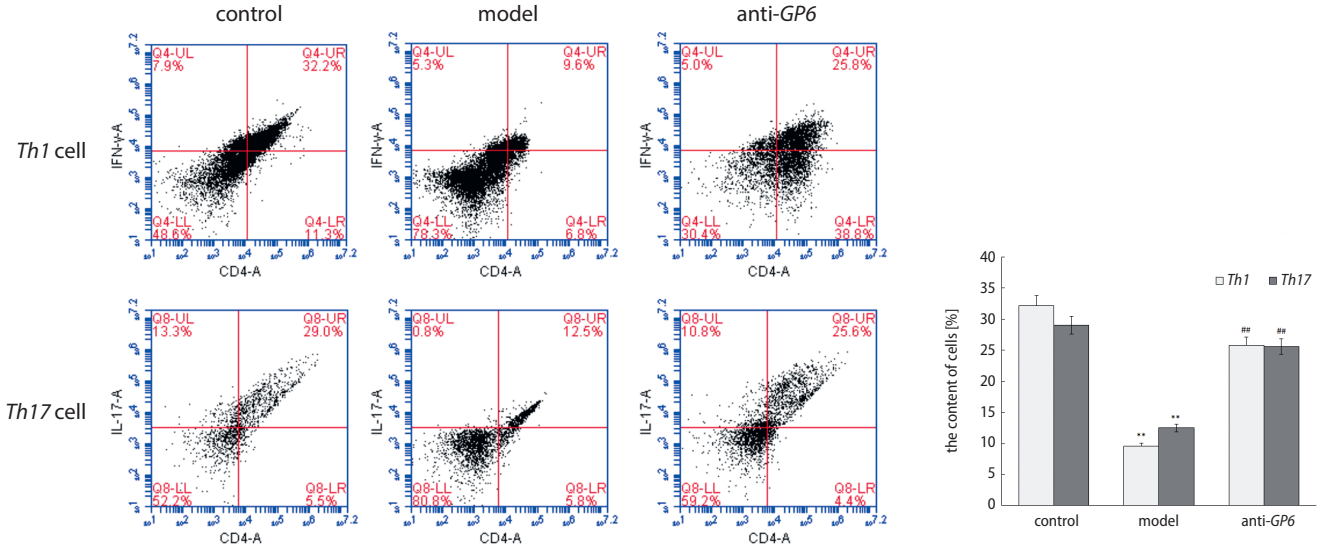

Fig. 2. The content of *Th1* and *Th17* in blood samples of rabbits from the control, cerebral ischemic stroke (CIS) model and anti-*GP6* groups determined with flow cytometry (FCM). The *Th1* and *Th17* content was significantly decreased in the CIS model compared to the control group and anti-*GP6* group

*$p < 0.05$ or $^{\#\#}p < 0.01$, indicating the significant difference using one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test.

(Fig. 3). The relative expression of *FCER1G*, *ITGA2* and *GP6* in the CIS model group was significantly higher compared to the control group and anti-*GP6* group. The relative expression of *FCER1G*, *ITGA2* and *GP6* did not significantly differ between the control group and anti-*GP6* group.

Expression of *GP6*, *FYN*, *PKA*, *pPTK2*, and *pFAK1*

Western blot was applied to determine the protein expression of *GP6*, *FYN*, *PKA*, *pPTK2*, and *pFAK1* in carotid artery tissues of rabbits from the control, CIS model and anti-*GP6* group. The protein expression of *GP6* ($p < 0.001$, $df = 2$, $F = 392.9$), *FYN* ($p < 0.001$, $df = 2$, $F = 380.3$), *PKA* ($p < 0.001$, $df = 2$, $F = 304.3$), *pPTK2* ($p < 0.001$, $df = 2$, $F = 369.9$), and *pFAK1* ($p < 0.001$, $df = 2$, $F = 562.8$) in the CIS model group were markedly higher compared to those in the control group and anti-*GP6* group (Fig. 4). Moreover, the expression of *GP6*, *FYN*, *PKA*, *pPTK2*, and *pFAK1* were significantly upregulated in the anti-*GP6* group compared with the control group.

Discussion

With a remarkable rise in people's living standards and an accelerated pace of life, the onset of CIS is observed earlier than before and the incidence rate increases year after year. In particular, progressive CIS has a poor prognosis and high morbidity and mortality, which poses a serious threat to people's health and life safety, and burdens patients and families.[12,13] Carotid AS is an important risk factor for CIS and is closely related to the occurrence, development and recurrence of cerebral infarction.[14] To date, surgery

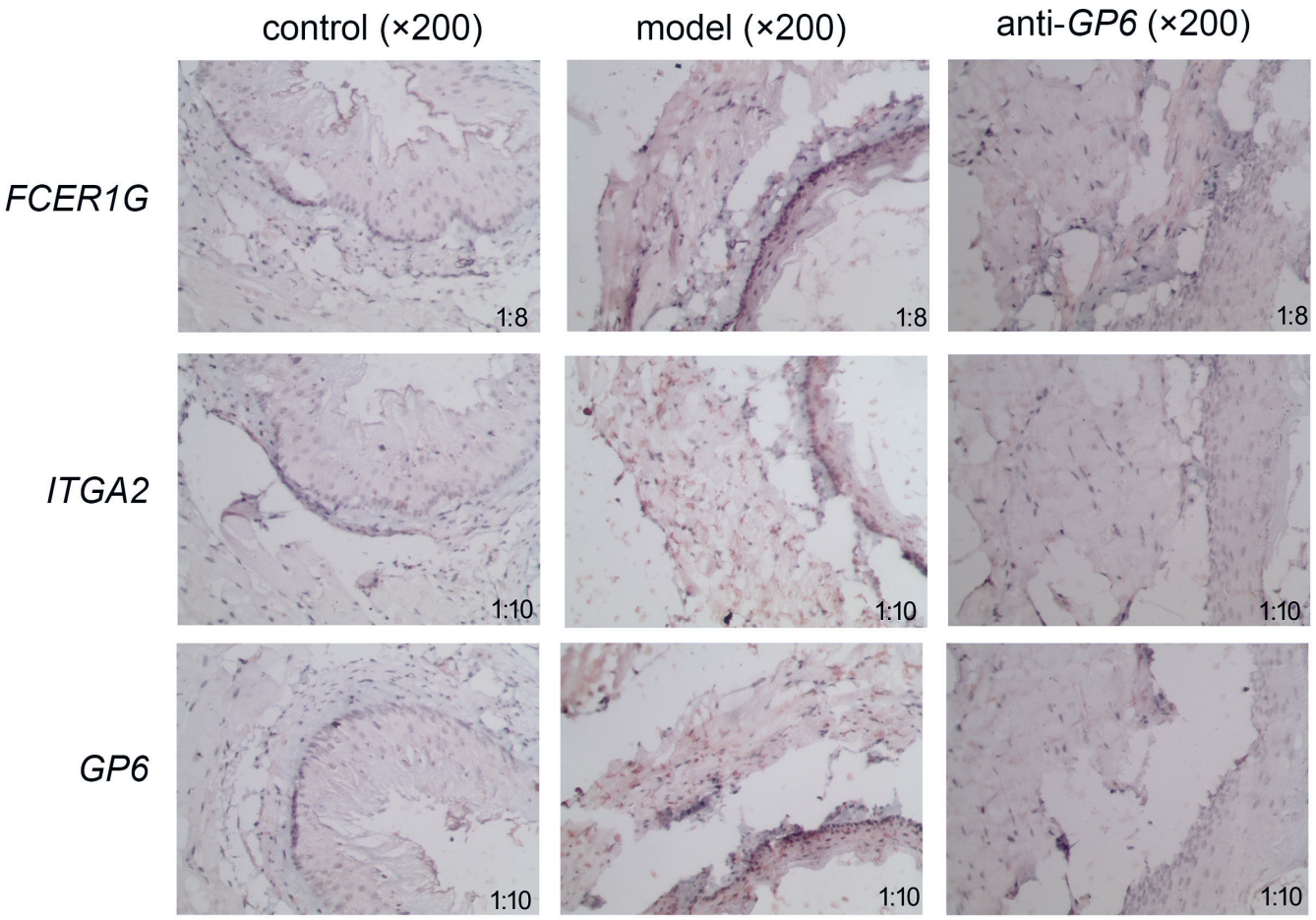

Fig. 3. Immunofluorescent staining of *FCER1G*, *ITGA2* and *GP6* proteins in the carotid artery and cerebrovascular tissues of white rabbits in the control, cerebral ischemic stroke (CIS) model and anti-*GP6* groups. The proteins *FCER1G*, *ITGA2* and *GP6* were all distributed in the cell membrane. The relative expression of *FCER1G*, *ITGA2* and *GP6* in the CIS model group was significantly higher compared to the control group and anti-*GP6* group

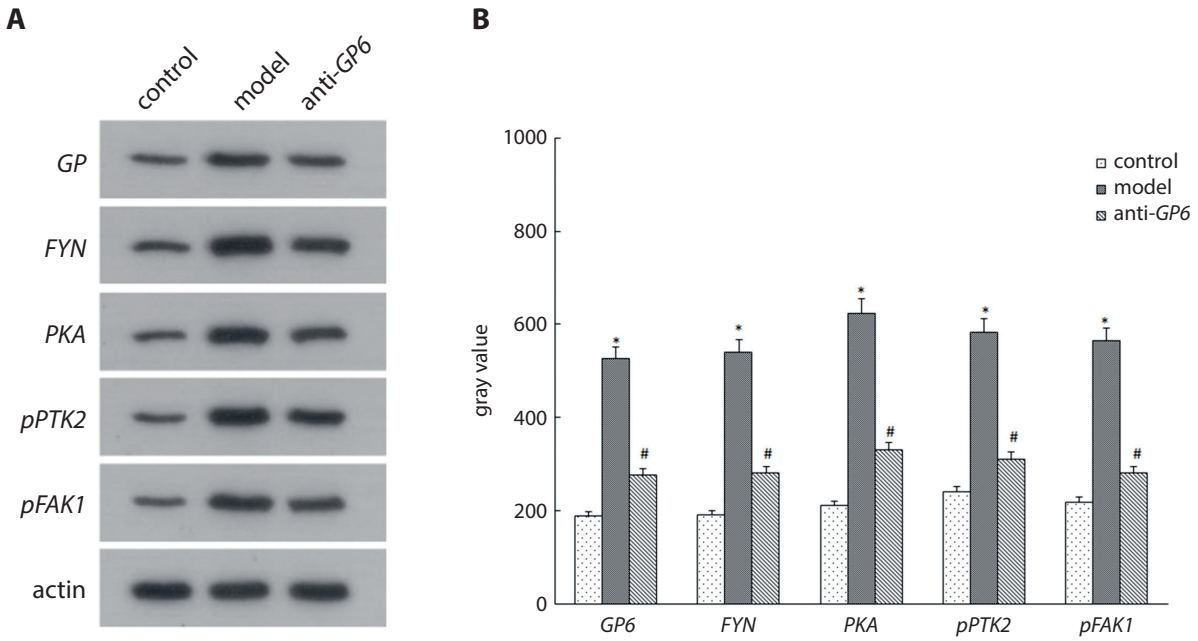

Fig. 4. The protein expression of *GP6*, *FYN*, *PKA*, *pPTK2*, and *pFAK1* in carotid artery tissues of rabbits in the control, cerebral ischemic stroke (CIS) model and anti-*GP6* groups measured with western blot assay. The protein expression of *GP6*, *FYN*, *PKA*, *pPTK2*, and *pFAK1* in the CIS model group was markedly higher compared to the control group and anti-*GP6* group

*$p < 0.001$ or #$p < 0.01$, indicating the significant difference using one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test.

or endovascular treatment for patients with recent ischemic diseases has been dependent on the degree of narrowing of the responsible vessel. For many years, the extent of artery stenosis has been regarded as the most reliable index to predict the risk of stroke in patients with AS. Thromboembolism derived from atherosclerotic plaque is considered to be the main pathogenesis of CIS in most atherosclerotic patients. Plaque rupture can lead to platelet aggregation, local thrombosis or thromboembolism. Plaque itself can also cause thromboembolism.[15] Protein function analysis revealed that *GP6* is a platelet glycoprotein, which plays an important role in platelet coagulation, thrombosis and arterial embolization.[16] The *GP6* signaling pathway mainly activates *FYN* and *PKA*, phosphorylates PTK2/FAK1 and promotes platelet adhesion to damaged vascular endothelium.

Vascular endothelial cells have anticoagulant and fibrinolytic properties and can influence the chemotaxis and adhesion properties of cell membranes.[17] Under normal circumstances, vascular endothelial cells cover the inner surface of the entire vascular system, providing an anticoagulant interface that prevents platelets and other blood cells from adhering to the subcutaneous tissue and preventing activation of the clotting response. Endocrine dysfunction caused by endothelial cell injury plays a key role in the pathophysiological mechanism of the early formation and development of AS.[18] In this study, male New Zealand white rabbits were randomly divided into 3 groups: control, CIS model and anti-*GP6* group. Results of H&E staining analysis of the vascular endothelial injury showed that in the CIS model group, the endothelium was discontinuous, the intimal hyperplasia was serious, the tube wall was full of plaques, a large number of foam cells could be seen, the membranes in the middle and outside were irregularly thickened, and foam cell infiltration could be seen. However, the injury degree was reduced in the anti-*GP6* group compared to the CIS model group. Results of FCM showed that *Th1* and *Th17* content was significantly decreased in the CIS model group compared to the control group, while it did not markedly differ from the anti-*GP6* group. Atherosclerosis occurs for various reasons, including the involvement of macrophages, T lymphocytes, endothelial cells, smooth muscle cells, mast cells, and other cells. After entering the vascular wall, T lymphocytes are activated under antigen stimulation to produce inflammatory cytokines, which can aggravate the progression of AS by amplifying the inflammatory response. Different T cell subsets in the vascular wall are not only involved in the early plaque formation of AS but also promote the progression of AS, which is closely related to the pathological process of AS.[19] The $CD4^+$T cells play a crucial role in the development of AS. According to biological characteristics and different cytokines produced, $CD4^+$T cells are mainly divided into *Th1*, *Th2* and *Th17*. The *Th1* cells are differentiated from initial T cells in response to interleukin (IL)-12 and produce interferon gamma (IFN-γ), which acts as a defense against microorganisms in the cell. The role of *Th17* cells in disease is mainly to promote defense against organ-specific autoimmune diseases and chronic infections. Results of animal experiments carried out by Davenport et al. showed that *Th1* and *Th2* immune responses were involved in the formation of atherosclerotic lesions.[20] Kim et al. assessed 124 patients with chest pain who underwent coronary angiography and found the expression of *Th1* and *Th17* cells in patients with stable angina pectoris was significantly increased, which indicated that *Th1* and *Th17* cells are associated with the development of AS.[21] Interleukin 17 (IL-17) is a major effector of *Th17* cells and an early promoter of T-cell-induced inflammatory response, which can amplify the inflammatory response. Immunohistochemistry results in our study showed the proteins *FCER1G*, *ITGA2* and *GP6* were all distributed in the cell membrane. The relative expression of *FCER1G*, *ITGA2* and *GP6* in the CIS model group was significantly higher compared to the control group and anti-*GP6* group. The *FCER1G* gene is located on chromosome 1q23 (1:161185024-161190489) 24 and encodes an 86-amino acid protein.[22] *ITGA2* is a protein-coding gene. Diseases associated with *ITGA2* include anus disease and thrombocytopenia.[23] Results from western blotting indicated that the protein expression of *GP6*, *FYN*, *PKA*, *pPTK2*, and *pFAK1* in the CIS model group was markedly higher compared to the control group and anti-*GP6* group. Protein kinases are enzymes that catalyze protein phosphorylation, which include an important protein kinase, protein kinase A (*PKA*).

Limitations

At present, a large number of studies have been carried out on the *GP6* gene in China and abroad. The consistent view is that *GP6* gene polymorphisms are related to platelet aggregation ability, but whether they lead to an increase or decrease in platelet aggregation, is still in question. If a polymorphism leads to an increase in platelet aggregation, then it is likely to be associated with thrombotic diseases. However, some scholars found that the ti3254c gene polymorphism of *GP6* is not significantly associated with the occurrence of coronary heart disease. Scholars generally agree that the expression of *GP6* is enhanced in patients with acute ischemic diseases such as stroke and myocardial infarction. However, regarding whether *GP6* gene polymorphisms are related to AS-based diseases (such as coronary heart disease and cerebral infarction), current research results are mostly negative and more in-depth studies are needed for verification. The safety of long-term drug use and the role of drug immunity and cardiovascular events need to be further explored. Chronic inflammation, which is repeated over a long time, can also lead to an inability to cure the disease. The presence of other pathways in the mechanism of *GP6* in the treatment of AS requires further study.

Conclusions

GP6 can promote the formation of cerebral ischemic stroke by upregulating the expression of *FCER1G*, *ITGA2*, *FYN*, *PKA*, *pPTK2*, and *pFAK1*. It may be a new treatment target for cerebral ischemic stroke.

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