The Temporal Profile of OPG Expression and Regulatory Role on Ischemic Brain Injury in Rats After MCAo

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ABSTRACT

Objective To investigate the temporal profile of osteoprotegerin (OPG) in middle cerebral artery occlusion (MCAo) rats and the serum level of RANKL and ALP in OPG deficient and wild type rats.

Methods Rats was anesthetized and subjected to MCAo by transient occlusion of middle cerebral artery occlusion. The serum level of OPG, RANKL and ALP in rats after MCAo was examined by ELISA assay. The protein expression of OPG in ischemic brain was determined by Western blot analysis.

Results The level of OPG in the rat serum was significantly increased from 6 h after MCAo and peaked at 12-24 and 72-168 h. The protein expression of OPG was upregulated from 12 h after MCAo and peaked at 72 h. The level of RANKL and ALP was significantly decreased in OPG deficient rats after MCAo.

Conclusion OPG/RANKL signaling is associated with brain injury after MCAo, indicating a potential therapeutic target for ischemic stroke.

Keywords cerebral ischemia; osteoprotegerin; receptor activator of nuclear factor- κ B ligand

INTRODUCTION

Stroke is one of the main cause of death and disability worldwide following cancer and cardiovascular diseases, rising with the increase of age^[1]. The burden of ischemic stroke increased significantly and led to brain injury, edema, hemorrhage and neuronal death^[2]. However, the pathogenesis is not fully understand. Hundreds of targeting chemicals were developed, but there are few effective drugs against stroke.

Osteoprotegerin (OPG) is a soluble protein which binds to a receptor activator of nuclear factor- κ B ligand (RANKL) [3]. It is reported that elevated level of OPG in the serum was closely related to the severity and poor functional outcome of ischemic stroke^[4-6]. OPG/RANKL signaling has been known to work on vascular calcification and immune system^[7, 8]. In the present study, we examined the temporal expression of OPG in ischemic brain and the regulation on RANKL and ALP in a transient middle cerebral artery occlusion model using OPG^{-/-} rats.

MATERIALS AND METHODS

ANIMAL PROCEDURE

All animal procedures were undertaken in accordance with the guidelines for animal care and welfare and were approved by the Ethical Committees of Weifang Medical University. All animals were kept at about 23 $\pm$ 2°C and a 12 h light/12h dark cycle with free access to tap water and diet.

TRANSIENT MIDDLE CEREBRAL ARTERY OCCLUSION (MCAo)

Rats were randomly divided into sham operated group and MCAo group. Rats were anesthetized with intraperitoneal injection of chloral hydrate and subjected to MCAo as previously reported. In short, a silicon coated 4-0 monofilament nylon suture with a rounded tip was inserted from the right common carotid artery and gently advanced until it occlude the middle cerebral artery. The monofilament was gently removed after reperfusion. In sham-operated animals, all procedures were performed except occlusion of the middle cerebral artery. The animals received humane care and kept under 37°C.

ELISA

The blood was obtained at the end of the experiment and centrifuged at 3000 rpm for 15 min to collect serum. The level of osteoprotegerin (OPG), receptor activator for nuclear factor- κ B ligand (RANKL) and alkaline phosphatase (ALP) in the plasma was determined using commercially available ELISA kit according to the manufacture's instructions.

WESTERN BLOT ANALYSIS

The brain tissues were homogenized in lysis buffer containing containing pepstatin 1, leupeptin 2, phenylmethyl -sulfonyl fluoride 1. The lysates were centrifuged at 12000 rpm for 30 min and the concentration was detected using coomassie brilliant blue staining method. 60 μ g of protein in each sample were separated using SDS-PAGE. The protein samples were transferred into polyvinylidene fluoride (PVDF) membranes for immunoblotting. The PVDF membranes were blocked using 7.5% defatted milk. Then the primary antibody against OPG and GAPDH were used to incubate the membranes at 4°C overnight. After incubated with anti-rabbit IRDye700DX®-conjugated second antibody or anti-mouse IRDye800DX®-conjugated antibody (1:5000, Rockland, USA), the bands were detected by an Odyssey infrared imaging system. The intensity of the bands were quantitatively evaluated using Quantity One® analysis software.

STATISTICAL ANALYSIS

All data were presented as mean $\pm$ SD. The significance of differences among groups was assessed using one-way ANOVA followed by Bonferroni's post hoc test using the software Prism 5.0 (GraphPad Software).

$P < 0.05$ was considered statistically significant.

RESULTS

Temporal profile of OPG content and expression.

To investigate the regulatory role of OPG after ischemia, we examined the level of OPG in the rat serum after MCAo. As shown in Fig.1, the level of OPG in the serum was increased at 6 h after MCAo and peaked at 12-24 h and 72-168 h after MCAo. In rats, OPG protein expression in ischemic brain was increased and peaked at 12 h and 72 h after MCAo (Fig.2). These data indicate that OPG primarily acts in the acute stage of ischemic brain.

Fig.1 The level of OPG in rat serum after MCAo. The plasma OPG was examined using ELSIA assay at 3, 6, 12, 24, 48, 72, 168 h after MCAo in rats. $n=4$ in each group. * $P < 0.05$, ** $P < 0.01$ vs sham-operated rats.

Fig.2 The protein expression of OPG in rats after MCAo. The expression of OPG protein was analyzed by Western blot. $n=3$ in each time point. * $P < 0.05$, ** $P < 0.01$ vs sham-operated rats.

Changement of RANKL and ALP in the plasma of rats after MCAo.

To elucidate the role of OPG/RANKL signaling, we examined the changes of RANKL and ALP in the plasma using ELISA detection assay. 24 h after MCAo, we examined the content of RANKL and ALP in the plasma in OPG^{-/-} and WT rats. As shown in Fig.3A and 3B, the level of RANKL and ALP in the plasma was

significantly increased 24 h after MCAo compared with OPG^{-/-} sham-operated rats. The level of RANKL and ALP in OPG^{-/-} rats was significantly decreased compared with that in WT rats after MCAo. These results indicated that the OPG/RANKL signaling played important roles in MCAo rats.

Fig.3 The content of RANKL and ALP in the serum of rats. Serum RANKL and ALP was examined using ELISA assay at 24 h after MCAo in OPG^{-/-} rats. n=3 in each time point. ** $P < 0.05$ vs OPG^{-/-} sham operated rats, ## $P < 0.01$ vs WT rats subjected to MCAo.

DISCUSSION

Stroke is the leading cause of mortality and disability in adults since millions of Chinese people died from ischemic stroke^[9-11]. Therefore, it is important to develop more effective treatment of ischemic stroke. In the present study, we investigate the temporal expression of OPG in the plasma and ischemic brain in the middle cerebral artery occlusion for different points of reperfusion.

It has been reported that OPG/RANKL/RANK signaling was involved in brain ischemia^[12, 13]. Increased level of OPG in the serum has been observed in patients with ischemic stroke, which is related with the severity of stroke. The regulatory role of OPG in ischemic brain injury is not yet known. Here we demonstrated that the level of OPG protein in the infarct region was increased from 6 to 24 h after middle cerebral artery occlusion. The protein expression of OPG peaked again 72h after MCAo. Moreover, in OPG deficient rat, the level of RANKL and ALP in the plasma was decreased significantly after ischemia. Further studies are necessary to clarify the mechanisms that how OPG deletion regulated the expression of RANKL.

The present study demonstrated that OPG/RANKL signaling could be associated with cerebral and other diseases in central nervous system. Further studies on the OPG/RANKL system in the brain might shed light on molecular mechanisms involved in brain injury and be a potential therapeutic target for ischemic stroke.

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