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Brief Report

Intervention of Death-Associated Protein Kinase 1–p53 Interaction Exerts the Therapeutic Effects Against Stroke

Xiaoxi Wang, MD*; Lei Pei, PhD*; Honglin Yan, MD*; Zhongping Wang, PhD; Na Wei, MD; Shan Wang, MD; Xin Yang, MD; Qing Tian, MD; Youming Lu, PhD

Background and Purpose—Death-associated protein kinase 1 (DAPK1) interacts with the tumor suppressor gene p53 via a direct binding of a death domain of DAPK1 to a DNA-binding motif (DM) of p53 (p53DM) and converges multiple cell death pathways in stroke. The goals of this study are to determine whether disruption of DAPK1–p53 interaction is therapeutically effective against stroke.

Methods—We synthesized a membrane-permeable p53DM peptide (Tat-p53DM) and tested the therapeutic effects of Tat-p53DM in a mouse model with stroke.

Results—We showed that Tat-p53DM blocked DAPK1–p53 interaction in brain cells in vivo. When administered 6 hours after stroke onset in adult male mice, Tat-p53DM was still therapeutically effective against brain damages and improved neurological functions.

Conclusions—DAPK1–p53 interaction is a preferred target for therapeutic intervention of stroke. **(*Stroke*. 2014;45:00-00.)**

Key Words: death-associated protein kinases ■ stroke

Death-associated protein kinase 1 (DAPK1) is a Ca^{2+}/calmodulin-dependent kinase[1] and plays roles in several modes of cell death in ischemic stroke.[2,3] To define a promising target that allows a delayed intervention being clinically effective against stroke, we searched for cell death signals downstream of DAPK1. We identified a direct binding of DAPK1 to a DNA-binding motif (DM) of the tumor suppressor protein p53 (p53DM), which is known as a transcriptional regulator of genes that control cell necrosis and apoptosis.[4–7] We thus synthesized a membrane-permeable p53DM peptide consisting of amino acid 271 to 282 (Tat-p53DM) that specifically blocked DAPK1–p53 interaction.[3] Activation of DAPK1 phosphorylates p53 at serine-23 (pS^{23}) in the cultured cortical neurons.[3] In the nucleus, the pS^{23} induces expression of the proapoptotic genes such as *Bax*, whereas in the mitochondrial matrix, the pS^{23} interacts with a necrotic factor of cyclophilin D. Application of Tat-p53DM effectively blocks these dual pathways of the pS^{23} actions in the cortical cultures in vitro,[3] suggesting that Tat-p53DM could be clinically effective against stroke. To test this possibility, here we validated the therapeutic effects of Tat-p53DM in vivo in a mouse model with stroke.

Methods

Focal Cerebral Ischemia

Focal cerebral ischemia was induced by intraluminal middle cerebral artery occlusion (MCAO), as we described previously.[1] Briefly, a 7/0 surgical nylon monofilament with rounded tip was introduced into the left internal carotid through the external carotid stump and advanced 10 to 13 mm past the carotid bifurcation. The filament was left in place for 60 minutes and then withdrawn. Sham-operated animals were treated identically, except that the middle cerebral artery was not occluded after the neck incision.

Peptides

Tat-p53DM (Tat-RVCACPGRDRRT) or Tat-s-p53DM (Tat-CCPGECVRTRRR) peptides with 99% purity were synthesized by Bioyeargene Biosciences (Wuhan, China). The peptides were numbered, and the experimenters were unaware of which one was applied in all experiments.

Peptides Dose–Effect Experiments

Sixty mice underwent a 60-minute MCAO and were injected (intravenously) with vehicle (control) or with Tat-p53DM or Tat-s-p53DM at a single dose of 0.1, 0.5, 1, or 2 mg/kg body weight. And 1 mg/kg was identified as the optimal dose. Twelve hours later, all mice were euthanized and the cortical extracts were prepared. The proteins were

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From the Department of Physiology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China (X.W., L.P., H.Y., N.W., S.W., X.Y., Q.T., Y.L.); Department of Physiology and Pathophysiology, Jiujiang University, Jiujiang, China (Z.W.); Institute for Brain Research, Huazhong University of Science and Technology, Wuhan, China (X.W., L.P., H.Y., Z.W., N.W., S.W., X.Y., Y.L.).

*Drs X. Wang, Pei, and Yan contributed equally.

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Correspondence to Qing Tian, MD, Institute for Brain Research, Huazhong University of Science and Technology, Wuhan 430030, China. E-mail tianq@hust.edu.cn or Youming Lu, PhD, Institute for Brain Research, Huazhong University of Science and Technology, Wuhan 430030, China. E-mail lym@hust.edu.cn

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precipitated with anti-DAPK1 and blotted with anti-p53 and anti-DAPK1 antibodies, respectively. The ratio of the precipitated p53 in each treatment versus control was expressed as 100%.

Cell Death Analysis

For peptide injection and cell death measurement, 63 mice were divided into 7 groups at random. One group received sham operation. The other 6 groups received MCAO operation. Three or 6 hours after MCAO, mice were injected with vehicle or Tat-p53DM or Tat-s-p53DM (1 mg/kg body weight, IV). Seven days after the administration, mice were euthanized and the brain sections were stained with Fluoro-Jade C (FJ). The number of FJ-positive (FJ^+) cells in each group was counted.

Behavioral Analysis

After MCAO and peptide administration, the neurological deficits were assessed throughout 4-week period of observations 24 hours after the MCAO. Neurological performance was scored daily using modified 7-point neurological scales. An accelerating rotarod was used to test the motor coordination. A foot fault task was performed to assess motor impairments of limb functioning. Behavioral analysis was always assessed by the blinded independent investigators who were unaware of the experimental conditions and treatments.

Statistical Analysis

All variance values in the text and figure legends are represented as mean±SEM. Parametric tests including *t* test and ANOVA were used where assumptions of normality and equal variance (*F* test) were met. In some studies, Mann–Whitney *U* was used. Main effects and interactions for all ANOVAs or Mann–Whitney *U* tests are described in the figure legends. All *t* tests were 2 tailed using a *P* value of 0.05.

Please see the online-only Data Supplement for expanded Methods section.

Results

Tat-p53DM Intercepts DAPK1–p53 Interaction

We recently reported that a death domain (DD) of DAPK1 (DAPK1DD) binds to a p53 DM consisting of amino acid 241 to 282 (p53DM).[3] We subsequently generated a membrane-permeable p53DM peptide by fusing p53DM to the transduction domain of the HIV Tat protein (Tat-p53DM). Our data revealed that Tat-p53DM at a dose of 1 mg/kg (IV) effectively intercepted an association between DAPK1 and p53 in brain cells in vivo (Figure 1A and 1B). F1

Tat-p53DM Protects Against Stroke

We next determined whether selectively blocking DAPK1–p53 binding by Tat-p53DM produced the therapeutic effects in ischemic stroke. We operated adult male mice with MCAO for 60 minutes and then reperfusion, which were confirmed in all animals via monitoring cerebral blood flow. Three hours after the MCAO operation, animals were intravenously administered with a single dose of 0.1, 0.5, 1.0, or 2.0 mg/kg Tat-p53DM or a scrambled Tat-s-p53DM (Figure 1C). We observed that Tat-p53DM at a dose of 1 mg/kg sufficiently uncoupled p53 protein from DAPK1 complex in the cortical neurons of MCAO mice.

To examine the therapeutic effects of Tat-p53DM, we operated adult male mice with MCAO. Six hours after the operation, mice were administered with Tat-p53DM or Tat-s-p53DM at a single dose of 1, 2, 3, 4, or 5 mg/kg. The brain infarct was measured 72 hours later with ~~TTC~~ staining (Figure 2A). We found that Tat-p53DM at a single dose AQ8 F2

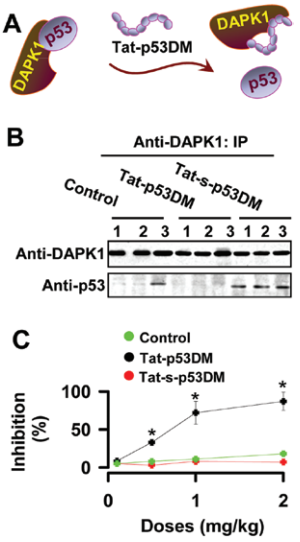

Figure 1. Tat-p53 intercepts death-associated protein kinase 1 (DAPK1)–p53 binding in vivo. **A**, DNA-binding motif of p53 (p53DM) peptide inhibition of DAPK1–p53 binding, **B**, Representative blots show the time course of Tat-p53DM action in an inhibition of DAPK1–p53 association in the cortical neurons of adult mice, **C**, The dose–response curves of Tat-p53DM in an inhibition of DAPK1–p53 association in the cortical cells in vivo. Data are mean±SEM (ANOVA, **P*<0.05; n=5). AQ7

of 1 mg/kg) sufficiently decreased the cerebral infarction to 18.6±3.7 mm^3 from 31.9±5.8 mm^3 in controls, in which mice were administered with Tat-s-p53DM (Figure 2B).

To extend our analysis of neuronal protection, we also examined cell death in mice 7 days after operation with MCAO by staining the brain sections with FJ (Figure 2C).

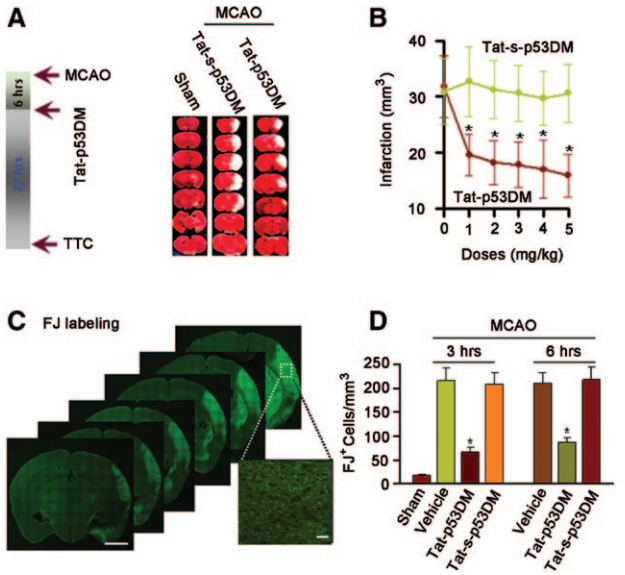

Figure 2. Administration of Tat-p53DM protects against stroke damages. **A**, Experimental schedule, **B**, A graph shows the sizes of cerebral infract. Data are mean±SEM (ANOVA, **P*<0.05; n=11). **C**, Representative images show the Fluoro-Jade C (FJ)–labeled cells in the cortex of mice. Scale bar in serial pictures is 2.5 mm, and scale bar in magnified picture of the box area is 100 μm. **D**, Bar graph summarizes the numbers of the FJ-labeled cells. Data are mean±SEM (ANOVA, **P*<0.05; n=9). MCAO indicates middle cerebral artery occlusion. AQ9

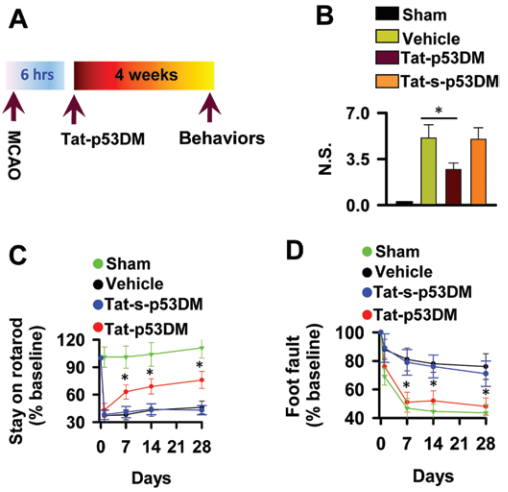

Figure 3. Tat-p53DM improves overall neurological functions. **A**, Adult mice were administered (intravenously) with a single dose (1 mg/kg) of vehicle, Tat-s-p53DM, or Tat-p53DM 6 hours after operation with sham or middle cerebral artery occlusion (MCAO). (**B** to **D**, Neurological behaviors including overall neurological scores (N.S., **B**). The performance on rotarod (**C**) and foot fault (**D**) was analyzed throughout the 4-week period after operation. Data are mean±SEM (~~Mann–Whitney *U*40=3.00~~, **P*<0.05; n = 11). AQ10

In this study, mice were treated with Tat-p53DM 3 or 6 hours after MCAO operation. We found that in both conditions, Tat-p53DM profoundly reduced the number of degenerated cells in the vulnerable brain regions including the striatum and the ~~hippocampus~~. The reduction of FJ labeling was of a similar magnitude to the reduction of the brain infarction (n=9; ANOVA, *P*<0.05; Figure 2D). Thus, a delayed administration of Tat-p53DM has the capability to protect against stroke damages.

Tat-p53DM Is Therapeutically Effective Against Stroke

We next performed behavioral analysis throughout the 4-week observation period (Figure 3A) after MCAO operation using a modified neurological scoring (7 points) system that is analogous to the National Institutes of Health Stroke Rating Method used in clinic stroke trials.[2] A score of 7 points represents severe bilateral neurological deficits, and 0 is normal. Animals treated with 1 mg/kg body weight of Tat-p53DM showed a better neurological score throughout the 4-week observation period from 24 hours to 4 weeks after the Tat-p53DM treatment (n=11; ~~2-way~~ ANOVA, *P*<0.05; Figure 3B) and revealed a complete recovery of motor coordination (Figure 3C and 3D). Thus, a delayed administration improves overall neurological functions. F3

Discussion

In the present study, we have demonstrated that Tat-p53DM specifically blocks a delayed cellular signaling downstream of DAPK1. These include a translocation of p53 protein into the nucleus and expression of p53 proapoptotic genes such as *Bax* and *Puma*, as well as accumulation of a cleaved version of caspase-3.[3] We have validated the therapeutic effects of Tat-p53DM in vivo in mouse models and shown that Tat-p53DM had an extended efficacy with a remarkable long time window (>6 hours) for the treatments of stroke. Thus, Tat-p53DM can be considered a promising therapeutics for stroke.

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Disclosures

None.

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