ORIGINAL ARTICLE

Kallikrein Gene Transfer Induces Angiogenesis and Further Improves Regional Cerebral Blood Flow in the Early Period After Cerebral Ischemia/Reperfusion in Rats

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Keywords

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SUMMARY

Aims: The aims of this study were to find out whether kallikrein could induce angiogenesis and affect the cerebral blood flow (rCBF) in the early period after cerebral ischemia/reperfusion (CI/R). **Methods:** The adenovirus carried human tissue kallikrein (HTK) gene was administrated into the periinfarction region after CI/R. At 12, 24, and 72 h after treatments, neurological deficits were evaluated; expression of HTK and vascular endothelial growth factor (VEGF) were detected by immunohistochemistry staining; the infarction volume was measured; and rCBF was examined by ¹⁴C-iodoantipyrine microtracing technique. **Results:** The expression of VEGF was enhanced significantly in pAdCMV-HTK group than controls over all time points ($P < 0.05$). Furthermore, the rCBF in pAdCMV-HTK group increased markedly than controls at 24 and 72 h after treatment ($P < 0.05$), and the improved neurological deficit was accompanied by reduced infarction volume in pAdCMV-HTK group 24 and 72 h posttreatment. **Conclusion:** In the early period after CI/R, kallikrein could induce the angiogenesis and improve rCBF in periinfarction region, and further reduce the infarction volume and improve the neurological deficits.

Introduction

The kallikrein–kinin system (KKS) is an important inflammatory modification system *in vivo* [1]. It plays an essential role in physiological and pathological mechanisms by modulating secretion of many inflammatory factors, such as interleukin, cytokine, and arachidonic acid [2,3].

A recent study has shown that kallikrein was also involved in the pathological course of cerebral ischemia/reperfusion (CI/R) [4,5]. Tissue kallikrein gene transfer protected against CI by promoting glial cell survival, migration and inhibiting apoptosis [6]. Moreover, exogenous kallikrein enhanced neurogenesis and angiogenesis in the subventricular zone and the periinfarction region and improved neurological function after focal cortical infarction *in vivo* [7]. Furthermore, it has been demonstrated that kallikrein can improve regional microcirculation in the is-

chemic myocardium, thereby attenuating ischemia/reperfusion injury [8,9]. However, it is unclear whether kallikrein improves neurological function after CI/R through its effects on regional cerebral blood flow (rCBF) as well, as it is known that the changes of rCBF in periinfarction region in the early period after CI/R play an important role in the recovery of neurological deficits [10].

Therefore, in this study, we tried to find out whether the angiogenesis induced by kallikrein could further improve the rCBF after CI/R.

Materials and Methods

Animals and Materials

Ninety clean, healthy male Sprague Dawley (SD) rats weighing 200–300 g were enrolled in the study and were provided by the

Guangdong Province experimental animal center. All experimental procedures were approved by the local Institutional Animal Case and Use Committee and in accordance with the guidelines for animal use [8]. The utmost effort was made to minimize the animals' suffering during the experiment.

The pUC19-human tissue kallikrein (HTK) had been constructed and stored in our lab. The Lipofectamine FuGENE was from the Roche Company (Nutley, NJ, USA); HindIII, SalI, and PacI were from the Sigma Company (St. Louis, MO, USA); and the fetal buffalo serum (FBS) was purchased from the Invitrogen Company (Grand Island, NY, USA). The mouse anti-human kallikrein 1 monoclonal antibody was from the R&D Company (Wiesbaden-Nordenstadt, Germany), whereas the rabbit anti-rat vascular endothelial growth factor (VEGF) polyclonal antibody and the horseradish peroxidase conjugated rabbit anti-mouse IgG and the horseradish peroxidase conjugated mouse anti-rabbit IgG were from the Santa Cruz Company (Santa Cruz, CA, USA). The S-P immunohistochemistry kit and DAB reaction kit was purchased from the Maxine Company (Fuzhou City, China).

Construction and Titer of pAdCMV-HTK

The construction of pAdCMV-HTK was completed following the protocol reported previously [11]. In brief, the sequence of pUC19-HTK was detected for mutation or loss of bases. And then HindIII and SalI cut pUC19-HTK, and the small fragments of HTK gene were collected. Meanwhile, pTrack-CMV was cut by the same restriction endonucleases. The HTK gene, the cut Track-CMV, and the T4DNA ligation were mixed and incubated overnight at 4°C, following which the product was transfected to *E. coli* BJ5138. The monoclones were picked up and identified by PCR and electrophoresis. The pAdeasy and pTrack-CMV-HTK were then cotransfected to the *E. coli* BJ5138 and then to the DH5 α . Monoclones were picked up and amplified. The plasmids were recollected and analyzed again by PCR and electrophoresis.

The renal 293 cells were cultured and regenerated in 1640 medium (contained equal volumes of 10% FBS), and it was ready for virus infection when cells covered 70% of the plate. Meanwhile, PacI was used to cut the reconstructed pAdeasy-CMV-HTK. Its larger fragments were collected and infected to the renal 293 cells with Lipofectamine. Ten days after infection, cells were destroyed and centrifuged. The supernatant was collected and infected the other cells. The third generation of virus was chosen and collected for absorbance value detection. The titer of virus was evaluated in 96-well plates.

CI/R Model and Treatments

Rats were subjected to produce focal cortical ischemia/reperfusion using a previously described model of middle cerebral artery occlusion model (MCAO) [12]. In brief, 10% chloral hydrate (0.32 mL/100 g) was administered into the rat's abdomen for anesthesia. The right cerebral middle artery was then terminated, and the perfusion was recovered after 90 min. The animals which were operated upon were then assigned into the following groups ($n = 30$) randomly at 12 h after operation: group A, blank control group; group B, vehicle group; group C, pAdCMV-HTK group.

In groups A and B, 5- μ L vehicle and pAdCMV-HTK (MOI = 20) [11] were administered into the periinfarction region, respectively, whereas only the needle puncture was performed without any injection in the blank control group. Finally, rats were sacrificed for the following detection at 12, 24, and 72 h ($n = 10$) after treatments.

Evaluation of the Neurological Deficits

The Neurological Severity Scores (NSS) was performed as described previously [13] at 12, 24, and 72 h after treatment to evaluate the neurological function of rats. A score of 0 indicated no neurological deficits, a score of 1 correlated with mild focal neurological deficits, and rats with a score of 18 suffered from the most severe neurological deficits, that is, higher points indicated more severe neurological deficits.

Infarction Volume Measurement

Brain samples were dipped into the ice-cold 0.9% sodium chloride solution for 10 min, and then they were cut in coronary (2 mm per section) on a cryostat vibratome (VT 2800 N; Leica, Heidelberg, Germany). Sections were then incubated in 2% TTC solution at 37°C for 30 min, followed by fixed in 4% methanol buffer. Photos were taken by DC240 digital camera (Kodak Company, USA) after 24 h, and all data were stored in the computer and later were analyzed by Adobe Photoshop 7.0 image software to calculate the infarction area (the normal tissue was in pink, whereas the infarction region was in white). To exclude the influence of edema, the calculation was modified: the calculated infarction volume (mm^3) = (area of the contralateral brain area of the normal tissue in the infarction brain) $\times$ 2 mm.

Immunohistochemistry Staining

At 12, 24, and 72 h after treatment, blocks containing tissue around the infarct lesion (5 mm anterior to bregma) were collected and fixed in 4% paraformaldehyde for immunohistochemical staining. In brief, sections were dehydrated and then dipped into the acetic acid buffer for 15 min. The endogenous peroxidase was inhibited by 1% H_2O_2 methanol solution for 15 min. This is followed by the addition of the mouse anti-human Kallikrein 1 monoclonal antibody (1:250 in PBS); incubated at 37°C for 1–2 h; and then washed in PBS for 5 min thrice. Thereafter, the horseradish peroxidase conjugated rabbit anti-mouse IgG (1:400 in PBS) was incubated with the sections for 1.5 h at room temperature. Sections were washed again in PBS for 5 min thrice and then treated by DAB substrate and the hematoxylin. The positive cells were finally counted (cells/field of view, 400 $\times$) for 10 fields of view per section.

^{14}C -Iodoantipyrine Microtracing Detection

At 60 min before harvesting, ^{14}C -iodoantipyrine was administered intravenously (50 μCi) into the rats, and the animals were subsequently harvested. Brains were frozen in liquid nitrogen and

then they were cut in 5 mm on a cryostat vibratome (VT 2800 N; Leica, Heidelberg, Germany). Samples were collected from different regions (5 mm anterior to bregma) (including the cortex and white matter of infarction lesion, and the cortex and white matter of the contralateral regions). Samples were then weighted in prepared scintillation liquid bottles followed by adding the 0.3 mL solubilizer (Luma Solve, Lumac Inc., Netherlands) into the bottles, and digesting the samples in incubator at 50°C for 24–48 h. Thereafter, 10 mL scintillation liquid (Lipo Luma, Lumac Inc., Netherlands) was added and liquid scintillometer was used to detect the ^{14}C in samples. The regional cerebral blood flow was calculated by: dose% per 100 g = (^{14}C in samples/total ^{14}C) $\times$ ([1/weight of sample] $\times$ 100% $\times$ 100).

Statistical Analysis

Statistical analysis was completed in the department of statistic of Sun Yat-sen University (Guangzhou, China). All data were presented as average $\pm$ standard error (SE). The numerical data were analyzed with SPSS12.0 statistical software (SPSS Inc., Chicago, IL, USA). The comparisons of different groups were analyzed by the two-tailed Student's *t*-test or Mann–Whitney U test. A probability value of less than 0.05 ($P < 0.05$) was considered statistically significant.

Results

Quantitative Analysis of Experimental Animals

There were five rats who died within 72 h after operation, and the others survived till the end of the study. Therefore, more than five rats were operated and assigned into the study randomly. Ninety rat models were included in the final analysis.

Expression of HTK in Periinfarction Region

HTK was detected in the cytoplasm at 12 h after pAdCMV-HTK injection. HTK-positive cells were primarily neural cells, and a small number of glial cells (shown in Figure 1) in periinfarction region. Thereafter, HTK expression gradually increased and reached a peak at 24 h after injection. These results were significantly different from the blank control and vehicle groups ($P < 0.01$). At

Figure 1 HTK expression at 12 h after treatments around the cerebral infarction ($\times 400$). (A) In vehicle group, no HTK expression was found around the cerebral infarction lesion. (B) In pAdCMV-HTK group, HTK was expressed (stained in brown) in cytoplasm of neurons and some glia around infarction lesion. Scale bar 20 μm .

Table 1 The number of HTK positive cells in the periinfarction region ($n = 10$)

Groups	Time points after treatment		
	12 h	24 h	72 h
Blank control group	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
Vehicle group	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
pAdCMV-HTK group	14.4 $\pm$ 0.4 ^{a,b}	20.1 $\pm$ 0.5 ^{a,b}	13.2 $\pm$ 0.2 ^{a,b}

^aCompared with the blank control group, $P < 0.01$.

^bCompared with the vehicle group, $P < 0.01$.

72 h, HTK expression began to decrease, but remained higher than levels in the vehicle and blank control groups ($P < 0.01$) (shown in Table 1). The results suggested that the pAdCMV-HTK could effectively infect the neurons and could express the target gene successfully without any toxic effects (data not shown).

Kallikrein Enhanced the Expression of VEGF in Periinfarction Region

The VEGF positive stained cells were mainly diffused in the endothelium of regenerated vessels, which are located in the cortex and the white matter around the cerebral infarction lesions (shown in Figure 2). The expression of VEGF in the pAdCMV-HTK group was much higher than that in blank control and vehicle groups ($P < 0.05$); it was even higher at 72 h after treatment ($P < 0.01$). On the contrary, the expression of VEGF between the vehicle group and the blank control group showed no difference ($P > 0.05$, shown in Table 2). These data indicate that exogenous kallikrein enhanced angiogenesis in the early period after CI/R.

Kallikrein Gene Transfer Elevated rCBF after CI/R

The rCBF of the cortex and white matter in periinfarction region in all groups was slightly less than the contralateral region. At 12 h after pAdCMV-HTK treatment, the rCBF of the cortex and white matter around the infarction lesion elevated slightly ($P > 0.05$); however, it was much higher at 24 and 72 h after treatment ($P < 0.05$, shown in Table 3).

The Effects of Kallikrein Gene Transfer on the Neurological Function after CI/R

The NSS were evaluated at 12, 24, and 72 h after treatments in groups. Results were shown with a significant difference among the different time points ($P < 0.01$). At 24 h after treatment, the NSS in pAdCMV-HTK treated group was significantly lower than that in both vehicle and blank control groups ($P < 0.05$), whereas it was even lower at 72 h after treatment ($P < 0.01$, shown in Table 4). While comparing the NSS in vehicle group with that in blank control group, there was no significant difference ($P > 0.05$). These data indicated that the overexpression of kallikrein gene around the lesion might attenuate the neurological deficit of rats after CI/R.

Figure 2 The expression of VEGF at 72 h after treatments in groups ($\times 400$). (A) The blank control group; (B) the vehicle group; and (C) the pAdCMV-HTK group. Scale bar 100 μm .

Table 2 The number of VEGF expressed cells in periinfarction region in groups (cells/field of view) ($n = 10$)

Groups	Time points after treatment		
	12 h	24 h	72 h
Blank control group	36 $\pm$ 9	42 $\pm$ 11	51 $\pm$ 13
Vehicle group	41 $\pm$ 15	52 $\pm$ 12	56 $\pm$ 10
pAdCMV-HTK group	65 $\pm$ 14 ^{a,b}	77 $\pm$ 17 ^{a,b}	96 $\pm$ 13 ^{c,d}

^aCompared with the blank control group, $P < 0.05$.

^bCompared with the vehicle group, $P < 0.05$.

^cCompared with the blank control group, $P < 0.01$.

^dCompared with the vehicle group, $P < 0.01$.

Kallikrein Gene Transfer Reduced the Infarction Volume after CI/R

At 24 and 72 h after treatments, the cerebral infarction volumes of groups were: pAdCMV-HTK group ([67.05 $\pm$ 23.66] mm^3 , [45.44 $\pm$ 18.25] mm^3), vehicle group ([284.92 $\pm$ 68.95] mm^3 , [292.92 $\pm$ 56.23] mm^3), and the blank control group ([298.25 $\pm$ 70.56] mm^3 , [285.32 $\pm$ 62.13] mm^3). There was significant difference in groups ($P < 0.05$, shown in Figure 3), and the infarction volumes in pAdCMV-HTK group were much lesser than that in the control and vehicle group ($P < 0.05$); the difference was more significant at 72 h after treatment ($P < 0.01$). However, there was no statistical difference between the blank control and vehicle groups ($P > 0.05$).

Discussion

Cerebral infarction is a common cerebral vascular disease of elderly. Recent research have shown that the improvement of rCBF

Table 4 Neurological severity score in groups ($n = 10$)

Groups	Time points after treatment		
	12 h	24 h	72 h
Blank control group	7.96 $\pm$ 0.13	8.25 $\pm$ 0.22	7.91 $\pm$ 0.15
Vehicle group	7.61 $\pm$ 0.08	7.72 $\pm$ 0.17	6.82 $\pm$ 0.14
pAdCMV-HTK group	7.42 $\pm$ 0.10	5.10 $\pm$ 0.14 ^{a,b}	4.23 $\pm$ 0.21 ^{b,c}

^aCompared with the blank control group, $P < 0.05$.

^bCompared with the vehicle group, $P < 0.05$.

^cCompared with the blank control group, $P < 0.01$.

in periinfarction region plays an important role in the recovery of neurological function after CI/R [10].

As a novel angiogenic factor, kallikrein gene has been successfully applied in therapeutic angiogenesis in various experimental ischemic models, such as, hindlimb ischemia, cardiac infarction, and renal ischemia [14–18], and the angiogenesis effects of kallikrein gene transfer have been documented in an animal model of cerebral infarction [6]. However, it is still unknown whether there is a relationship between the newly born microvessels induced by kallikrein and the recovery of rCBF after CI/R, and further results in the attenuation of neurological deficits.

Consistent with previous reports [4], our results demonstrated that kallikrein gene transfer could induce the expression of VEGF in newly born microvessels around the infarction lesion. This change began at 12 h, and peaked at 72 h after pAdCMV-HTK treatment. It suggested that kallikrein gene transfer would induce the microvascular proliferation around the infarction in the early period after CI/R.

Table 3 rCBF in groups after cerebral ischemia/reperfusion (dose% per 100 g) ($n = 10$)

Time points after treatment	Groups	White matter around infarction lesion	Cortex around infarction lesion	White matter of contralateral brain	Cortex of contralateral brain
12 h	Blank control group	1.68 $\pm$ 0.078	2.04 $\pm$ 0.052	2.21 $\pm$ 0.056	1.98 $\pm$ 0.024
	Vehicle group	1.77 $\pm$ 0.065	2.03 $\pm$ 0.210	2.24 $\pm$ 0.067	2.04 $\pm$ 0.035
	pAdCMV-HTK group	1.85 $\pm$ 0.052	2.16 $\pm$ 0.123	2.31 $\pm$ 0.075	1.97 $\pm$ 0.067
24 h	Blank control group	1.75 $\pm$ 0.014	2.09 $\pm$ 0.078	2.31 $\pm$ 0.062	2.09 $\pm$ 0.036
	Vehicle group	2.07 $\pm$ 0.016	2.15 $\pm$ 0.195	2.52 $\pm$ 0.085	1.86 $\pm$ 0.052
	pAdCMV-HTK group	2.99 $\pm$ 0.056 ^a	3.12 $\pm$ 0.096 ^a	2.46 $\pm$ 0.061	2.42 $\pm$ 0.078
72 h	Blank control group	1.98 $\pm$ 0.124	2.12 $\pm$ 0.122	1.96 $\pm$ 0.075	2.01 $\pm$ 0.042
	Vehicle group	2.42 $\pm$ 0.098	2.28 $\pm$ 0.178	2.32 $\pm$ 0.053	2.13 $\pm$ 0.075
	pAdCMV-HTK group	3.24 $\pm$ 0.062 ^{a,b}	3.45 $\pm$ 0.098 ^{a,b}	2.43 $\pm$ 0.086	2.57 $\pm$ 0.089

^aCompared with the blank control group, $P < 0.05$.

^bCompared with the vehicle group, $P < 0.05$.

Figure 3 Cerebral infarction volumes after treatments in groups (TTC stained, continuous coronal sections). (A) At 24 h after treatment in the blank control group; (B) 24 h after pAdCMV-HTK treatment; (C) 72 h after pAdCMV-HTK treatment; (D) 24 h after vehicle treatment. The infarction region was in white, whereas the normal region was in red.

To confirm the improvement of microcirculation in periinfarction region after kallikrein gene transfer, a further measurement of rCBF was performed. Results indicated that after the ischemia/reperfusion, rCBF of the cortex and the white matter around the infarction lesions were markedly reduced. Interestingly, the overexpression of kallikrein elevated the rCBF significantly while compared with the controls. The change was time-dependent and it was more notable at 72 h after treatment. Interestingly, the changes of rCBF were parallel with the elevation of VEGF expression. As expected, these results demonstrated that kallikrein gene transfer could further improve the rCBF, besides inducing the regeneration of microvessels.

Furthermore, we found that treatment with pAdCMV-HTK reduced infarction volume after CI/R. The rats were treated early

after reperfusion and the reduction was significantly over 24–72 h after treatment. These data indicated that kallikrein gene transfer in the early period after CI/R could improve the rCBF and further minimize the infarction area.

Function is the key principle for determining whether kallikrein gene transfer improves the outcome of CI/R. We investigated the neurological deficits by performing NSS. We found that at 24 h after treatment, the NSS in pAdCMV-HTK-treated group was significantly lower than that in vehicle and blank control groups ($P < 0.05$), and it was even lower at 72 h after treatment ($P < 0.01$). This result is consistent with previous results [19, 20].

In summary, our findings suggest that in the early period of CI/R, kallikrein gene transfer might enhance the microvascular regeneration, resulting in the attenuation of the infarction volume and recovery of neurological function. Furthermore, our findings indicated that kallikrein improves neurological function after CI/R by improving rCBF. This study provides novel evidences that human tissue kallikrein gene transfer may have a potential therapeutic perspective on CI/R by its effects on both angiogenesis and elevation of rCBF.

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Conflicts of Interest

The authors have no conflicts of interest to declare.

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