Ultrasound-Mediated Kallidinogenase-Loaded Microbubble Targeted Therapy for Acute Cerebral Infarction

Yanling Liang, MD, PhD,*,[1] Jia Chen, MM,*,[1] Xuan Zheng, MM,* Zhiyi Chen, MD, PhD,† Yuming Liu, MM,* Siying Li, MM,* and Xiaobo Fang, MM*

Background: The neuroprotective effects of kallidinogenase against acute cerebral infarction have been demonstrated, and the use of microbubbles has been suggested as a therapeutic mechanism for drug delivery. This study was designed to investigate the optimal parameters for preparing kallidinogenase-loaded microbubbles (KLMs) and to evaluate the effects of KLM-targeted therapy on neurogenesis and angiogenesis following experimental acute cerebral infarction in rats. *Materials and Methods:* KLMs were prepared by mechanical shaking. Male Wistar rats were randomly divided into an ultrasound-mediated KLM-treated group and 4 control groups. Treatments were administered via daily tail vein injection on 6 consecutive days, starting at 24 hours after middle cerebral artery occlusion (MCAO). The ultrasound-treated groups were subjected to a 2-MHz pulse of ultrasonic irradiation on the lateral skull of the ischemic side for 10 minutes during injection. Cell proliferation was examined using a 5-bromo-2-deoxyuridine assay. Infarct volume and neurological function were evaluated on days 3 and 7 after MCAO. *Results:* The ultrasound-mediated KLM and kallidinogenase treatments significantly increased the numbers of doublecortin-immunoreactive cells in the subventricular zone (SVZ) and laminin$^+$ cells in the peri-infarction region on day 7 after MCAO, compared with the other 3 groups (all $P < .05$). The neurological function scores of the ultrasound-mediated KLM-treated group were significantly better than those of rats treated with kallidinogenase alone or with the other treatments (all $P < .05$). *Conclusions:* Treatment with the ultrasound-mediated KLMs promoted the proliferation of SVZ neuroblasts and vascular regeneration, which contributed to functional improvement after stroke. These findings provide a novel therapy for ischemic stroke. **Key Words:** Ultrasound—kallidinogenase—microbubbles—neurogenesis—angiogenesis—cerebral infarction.

Introduction

Stroke is a leading cause of morbidity and mortality worldwide.[1] The outcome of the acute stage of stroke has been improved by thrombolytic therapy using recombinant tissue plasminogen activator.[2] However, because the effective window is narrow, only a small portion of stroke patients can benefit from such treatment. In addition, recombinant tissue plasminogen activator therapy carries a high risk of secondary hemorrhage and is often associated with high medical costs.[3] Therefore, it is necessary to develop a novel, effective, and affordable therapy for stroke patients.

From the *Department of Neurology; and †Department of Ultrasound Medicine, The Third Affiliated Hospital of Guangzhou Medical University, Guangzhou, China.

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[1] These authors contributed equally to this work.

Address correspondence to Yanling Liang, MD, PhD, Department of Neurology, The Third Affiliated Hospital of Guangzhou Medical University, Guangzhou 510150, China. E-mail: elaine.liang@163.com.

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Recently, ultrasound contrast and noninvasive targeted-therapy technologies have been developed.[4] For instance, ultrasound-mediated microbubble contrast agents and drug- and gene-targeting therapies are applied in the clinical treatment of various diseases. With the advantages of high efficiency and robust targeting,[5] these technologies have the potential to reduce treatment costs and to eliminate side effects without reducing drug activity. The mechanisms of action underlying these technologies have not been fully elucidated but are mainly related to the "cavitation effect" of ultrasound, which refers to the compression and expansion of microbubbles (cavitation nuclei) in liquid under ultrasound irradiation.[6] Microbubbles can be destroyed by ultrasound, resulting in a bioeffect that could be used for local drug delivery, to induce angiogenesis and vascular remodeling, or for tumor destruction.[7]

Under low sound pressure, the compression and expansion of microbubbles is symmetrical, and the diameter of the microbubbles remains relatively constant without the bubble bursting, a phenomenon termed steady-state cavitation. In contrast, under high sound pressure, compression and expansion of the microbubbles are asymmetrical, finally leading to implosion, a process termed transient cavitation. At this stage, the microbubble contrast agent can emit a shock wave and a microjet at the same time, reversibly or irreversibly perforating cell membranes in the surrounding tissues. This process will increase the permeability of the cell membrane and the capillary walls.[8] Simultaneously, drugs or genes are released from the broken microbubbles and efficiently enter the targeted organs and tissues with the driving force of the shock wave or microjet. The use of microbubble contrast agents can increase cell phagocytosis, remarkably improving the cellular uptake of the drug or gene under the effect of ultrasound.[9] In a rat xenograft study, when gene-silencing plasmids were mixed with a microbubble agent, the anticancer activity of the plasmids was significantly improved following ultrasound wave irradiation.[10] In addition, microbubble contrast agents exhibit poor solubility and dispersion in blood but good stability. Therefore, the use of drug-loaded microbubbles can alleviate rapid drug degradation in the circulation, thereby reducing the required dosage of the drug and related side effects and treatment costs. Several clinical studies have demonstrated that the use of a microbubble contrast agent can reduce the required intensity of ultrasound therapy, further improving the efficiency of the ultrasound and lowering the incidence of hemorrhage and other side effects. In addition, several studies have demonstrated the safety of microbubble contrast agents in humans.[11,12] Therefore, we proposed to apply this technology in the treatment of acute cerebral infarction. Microbubble drug loading refers to combining therapeutic drugs with microbubbles via adhesion, embedding, or noncovalent bonding. This approach is used to reduce the effects of a drug of interest on other drugs in circulation, to prevent degradation and side effects of the drug, to improve therapeutic efficacy, and to extend the therapeutic window.

Human urinary kallidinogenase can promote healing after acute cerebral infarction by 2 mechanisms of action: establishing collateral circulation and promoting neural restoration.[13,14] A previous study indicated that human urinary kallidinogenase promoted angiogenesis and improved neurological function in a rat middle cerebral artery occlusion (MCAO) model.[15] Therefore, the present study was designed to investigate the effects of ultrasound-mediated kallidinogenase-loaded microbubbles (KLMs) on neurogenesis and angiogenesis in an ischemic stroke model involving MCAO in rats.

Materials and Methods

Preparation of KLMs

Sono Vue microbubbles (Bracco Suisse SA, Bern, Switzerland) were dissolved in normal saline. The concentration of microbubbles was 8 μL (equivalent to 45 μg) of SF_6/mL of microbubble suspension. One bottle of kallidinogenase (Techpool Bio-Pharma Co., Ltd., Guangzhou, China) (.15 U of peptide nucleic acid (PNA), i.e., urinary kallidinogenase dissolved in 1 μmol/L of Val-Leu-Arg-PNA, pH = 8.0) was dissolved in 20 mL normal saline and divided into 4 samples, according to the volume ratio of microbubble to kallidinogenase (2:1, 1:1, 1:2, and 1:4). The suspensions were kept in 4-mL pharmaceutical glass vials and protected from light until use. The mechanical oscillation was refitted from the capsule-type mixer with horizontal and reciprocating vibrations. The working frequency was 4500 times per minute or more, and the vibration amplitude was 15 ± 1 mm. The portioned kallidinogenase and the lipid microbubble mixed liquid were subjected to mechanical oscillation for various times (15, 30, 45, 60, or 90 seconds). After vibration, traces of the mixers were dropped on glass slides, and a fluorescence-resistant quenching agent was used as a sealant. Experiments at each volume ratio and oscillation time were repeated 3 times. The study included 3 sample groups, 3 control groups, and 3 replication groups.

Testing Procedure

We conducted 3 parallel tests using the procedure outlined in Table 1. PNA was calculated as follows: PNA U/mL = 173.6 × A405 nm × T/1000, where the reaction constant is 173.6, and T is the dilution factor (the factor of 1000 corrects the dilution factor for the difference between U/L and U/mL). The PNA unit per milliliter value should be between 90% and 115%. This title is made by Techpool Bio-Pharma Co., Ltd.

Characterization of KLMs

To facilitate observation, some kallidinogenase was labeled with fluorescein isothiocyanate. Kallidinogenase