Targeted Delivery of Extracellular Matrix Protected against Neurologic Defects after Focal Ischemia Reperfusion in Rats

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Ischemic stroke is one of the leading causes of morbidity and mortality worldwide and characterized by defective angiogenesis. The functional sequences (RGDs, GRGDSPASSPISC) derived from fibronectin have been confirmed to augment angiogenesis in vivo and in vitro. However, delivery of peptides into the brain parenchyma has been hampered by the presence of the blood–brain barrier (BBB). We fused RGDs with penetratin (Antp) derived from Drosophila antennapedia homeodomain protein to improve the penetration of peptides through BBB into ischemic hemisphere. We found Antp-RGDs successfully not only penetrate the SH-SY5Y cells but also penetrated through BBB into ischemic hemisphere by intraperitoneal injection. In addition, application of Antp-RGDs to the focal cerebral ischemic reperfusion injury in rats resulted in the reduction of cerebral ischemic volume and the improvement of neurologic score according to the 21-point score. We further demonstrated that activation of phosphorylation-extracellular-signal related kinase 1/2 (p-ERK 1/2) and upregulation of gene *VEGF* resulted from post-treatment with Antp-RGDs 2 hours after reperfusion onset might at least partly contribute to the benefic changes after focal cerebral ischemic reperfusion injury in rats. Our data suggested that Antp-RGDs may serve as an attractive therapeutic intervention for treating ischemic stroke. **Key Words:** Ischemia reperfusion injury—penetratin—fibronectin—neuroprotection—angiogenesis.

Introduction

Stroke is the leading cause of disability in the developed and developing world, and ischemic stroke is the most prevalent type.[1,2] Cerebral ischemic stroke is considered to be an acute onset clinical syndrome characterized by a sudden decrease in blood flow to brain tissue.[1] To date, recombinant tissue plasminogen, targeting at vessel recanalization, is the only approved drug for treating ischemic stroke by the US Food and Drug Administration.[3] However, only 5% of patients are eligible to receive recombinant tissue plasminogen treatment because of safety concerns and narrow therapy window (<4.5 hours).[1] Although they successfully prevented cerebral ischemic injury in rodents, all these neuroprotective agents tested in patients failed in clinical trials because of deleterious side effects and/or low efficacy.[4] Therefore, it is still urgent to develop new strategies for preventing cerebral ischemic reperfusion injury.

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Angiogenesis plays an important role in various physiologic processes and is considered to be a hallmark of various ischemic diseases. In the case of ischemic diseases, an angiogenesis stimulator can be used to induce therapeutic angiogenesis due to deficient angiogenesis.[5] Thus, to find the new chemicals for inducing, angiogenesis maybe still an attractive process to treat focal cerebral ischemia injury.

Extracellular matrix (ECM) takes part in the process of angiogenesis of ischemic vascular disease. ECM plays an essential role in supporting key signaling events involved in regulating endothelial cells (ECs) migration, invasion, proliferation, and survival during angiogenesis proceeding.[6] In addition, the establishment of a continuous basement membrane is necessary for the maturation of vessels.[7] It is well known that fibronectin is one of the ECM. More importantly, a functional group from fibronectin, which has the capabilities of enhancing ECs migrate and proliferate during the process of angiogenesis, has been confirmed.[8] The functional sequences (RGDs, GRGDSPASSPISC) have been demonstrated to exert the capabilities of improving the angiogenesis in vivo and in vitro.[8,9] These data suggest that functional sequences from fibronectin maybe a promising candidate for treating cerebral ischemic stroke. However, to our limited acknowledgment, there is little research to study the biological effect of functional sequences from fibronectin on focal cerebral ischemic reperfusion injury.

In recent years, biomaterials made from self-assembling short peptides and peptide derivatives are extensively investigated in regeneration medicine because of their biocompatibility and biodegradability.[10] The metabolite of biomaterials can serve as nutrition for cell growth in vivo. Implantation of different biomaterials as scaffold has been confirmed to enhance regeneration from loss in the brain tissue.[11] However, little effort is made to investigate the biomaterials' effect on focal cerebral ischemic reperfusion injury. In addition, stereotaxic implantation is invasive strategy and hard for clinical trial although it eliminates the problem that drugs crossed the blood–brain barrier (BBB) into brain tissue. To date, penetration of drugs or therapeutic proteins through BBB into cells is still the urgent problem to be solved for us. It is well known that protein transduction domains (PTD) are small peptide molecules with the capabilities of delivering small particles, proteins, peptides, and nucleic acids into cells and host cells without significant side effects.[12,13] In addition, it is well acknowledged that short peptide derived from PTD can be internalized in most cell types, as well as allowing the cellular delivery of conjugated biomolecules, which include antigenic peptides, peptide nucleic acids, antisense oligonucleotides, full-length proteins, or even nanoparticles and liposomes.[14] Penetratin (Antp) derived from Drosophila antennapedia homeodomain protein and transactivator of transcription derived from human immunodeficiency virus are the most common PTDs. It has been demonstrated that transactivator of transcription successfully delivered exogenous neurogenin-2 fused with laminin binding domain through the BBB into cerebral ischemic zone. Thus, neurogenin-2 successfully exerts a reduction of the infarct volume and improvement of neurologic functional outcomes after reperfusion.[15] In addition, fusion peptides with PTD peptide Antp (16 peptides: RQIKIWFQNRRMKWKK) could also successfully deliver large molecules into cells or through BBB in vivo.[16]

Therefore, in this present study, we generated new peptides fusing RGDs with Antp and aimed to investigate whether Antp-RGD peptides cross the BBB into ischemic zone. In addition, we tried to study their biological effects and potential mechanisms on focal ischemic reperfusion injury in rats.

Materials and Methods

Preparation of Peptides Solution

The peptides used in the research were synthesized by the Shanghai Biotech Bioscience and Technology Corporation (Shanghai, China). The sequences of peptides (Antp-RGDs) were RQIKIWFQNRRMKWKK (Antp)-GRGDSPASSPISC-OH (Fibronectin, RGDs). An N-terminal tag of biotin or FITC was added to the peptides. Biotin-labeled or fluorescein isothiocyanate (FITC)-labeled peptides were dissolved in Ringer solution (7.5 g/L NaCl, .35 g/L KCl, 0.2 g/L $CaCl_2$; pH, 7.6-7.8).

Assessment of Peptides Cell Penetration

SH-SY5Y cells were cultured in the complete medium containing DMEM/F12 supplement with 10% fetal bone serum, 1% L-glutamine, and 1% Penicillin–streptomycin (all from Invitrogen, Grand Island, NY). When the cells get to the 90% confluence, the complete medium was discharged and FITC-labeled peptides (10 μM) in serum-free medium were added to SH-SY5Y cells grown in 5-cm tissue-culture dishes. After 30 minutes in a 37°C incubator with 5% (v/v) CO_2, the cells were washed twice with phosphate buffer saline (PBS) and then trypsinized and resuspended in flow buffer (PBS containing 2% fetal bovine serum) as a single-cell suspension. The cells at a concentration of 10^6 cells per 200 μL were for the analysis using flow cytometry by LSRFortessa cell analyzer (BD, Franklin Lakes, NJ). Data were analyzed using Cytomation Summit 5.2 software (Cytomation Inc, Fort Collins, CO). As controls, cells were incubated with peptide- and serum-free medium.

Animals

Male Sprague–Dawley rats (n = 40) weighing 250-300 g were purchased from Animal Research Center of Shandong Traditional Medicine University (Jinan, China). The animals were maintained in temperature- and