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Nanoparticles accumulate in ischemic core and penumbra region even when cerebral perfusion is reduced

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ABSTRACT

The use of a liposomal drug delivery system is a promising strategy for avoiding side effects and enhancing drug efficiency by changing the distribution of the intact drug. We have previously shown that liposomal agents quickly accumulated in an ischemia–reperfusion region and ameliorated cerebral ischemia–reperfusion injury when they were injected after reperfusion in transient middle cerebral artery occlusion (t-MCAO) rats. In the present study, we hypothesized that liposomes also act effectively as a drug carrier in the ischemic state, since the integrity of the blood brain barrier is disrupted at an early stage after an ischemic event. To test this hypothesis, the cerebral distribution of fluorescence-labeled liposomes was observed in permanent MCAO (p-MCAO) rats. The liposomes accumulated in the ischemic core and the penumbra region when injected at 1 or 2 h after occlusion. The accumulation in the ischemic core region was clearly greater than that in the penumbra region, despite the cerebral blood perfusion of the core region being substantially reduced. This result suggests that drug delivery to an ischemic region using liposomes is possible even when cerebral blood circulation has not recovered. Because liposomal drug delivery systems have the potential to effectively employ a number of agents that have failed in clinical trials, they may offer an effective strategy for achieving neuroprotection in stroke patients.

1. Introduction

Ischemic stroke is the second most common cause of death and the leading cause of adult disability worldwide [1]. The reduction in cerebral blood flow that occurs with an ischemic event results in cerebral cell death unless rapidly reversed. Thrombolytic therapy by the injection of tissue plasminogen activator (t-PA) has been approved for the neuroprotection of the cerebral ischemic region, but the therapeutic time window is narrow, and the number of patients that this therapy is appropriate for is very small [2,3]. Hence, the development of a new and more widely applicable therapeutic strategy is required for the improvement of ischemic stroke outcome. One strategy with considerable potential is the use of a drug delivery system (DDS) to reduce ischemic damage by site-selected delivery of the drug.

Several reports have shown that the blood–brain barrier (BBB) is disrupted in the acute phase after an ischemic event [4–6]. Because this disruption causes an increase in vascular permeability, substances with higher molecular weight might be able to enter the brain parenchyma during this phase. In a previous report, we showed that FITC-dextran (150 kDa) quickly accumulated in the ischemic hemisphere of transient middle cerebral artery occlusion (t-MCAO) rats when FITC-dextran was intravenously injected immediately after reperfusion [7]. These findings suggest that BBB disruption occurs at an early stage after cerebral occlusion, and that macromolecules pass through the spaces between endothelial cells in the acute phase of cerebral ischemia. Correspondingly, nanoparticles might also be able to leak into brain parenchyma during the acute phase of cerebral ischemia. If this hypothesis is correct, nanoparticles such as liposomes are promising drug delivery carriers for neuroprotection against and diagnosis of cerebral ischemic disease.

In this study, we observed the cerebral distribution of nanoparticles in permanent MCAO (p-MCAO) rats to determine whether nanoparticles are applicable to the treatment and diagnosis of ischemic stroke.

Abbreviations: I/R, ischemia/reperfusion; p-MCAO, permanent middle cerebral artery occlusion; t-MCAO, transient middle cerebral artery occlusion.

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2. Methods

2.1. Animals

Male Wistar rats (170–210 g) were purchased from Japan SLC, Inc. (Shizuoka, Japan). The animals were cared for according to the Animal Facility Guidelines of the University of Shizuoka. All animal procedures were approved by the Animal and Ethics Review Committee of the University of Shizuoka.

2.2. MCAO rats

MCAO was induced experimentally in rats as described previously [8]. In brief, anesthesia was induced with 3% isoflurane and maintained with 1.5% isoflurane during cerebral stroke surgery by small animal anesthetizer (Model TK-4, Bio Machinery, Chiba, Japan). Rectal temperature was maintained at 37 °C with a heating pad. After a median incision of the neck skin, the right carotid artery, external carotid artery, and ICA were isolated with careful conservation of the vagal nerve. A 4–0 monofilament nylon filament coated with silicon was introduced into the right ICA and advanced to the origin of the MCA to occlude it. Silk thread was used for ligation to keep the filament at the site of insertion into the MCA. After the operation, the neck skin was closed and anesthesia was discontinued. p-MCAO rats were examined without withdrawing of the filament, while, in the case of t-MCAO, cerebral blood flow was restored by withdrawing the filament about 10 mm under isoflurane anesthesia after 2 h of ischemia. Success of the surgery was judged by the appearance of hemiparesis and an increase in body temperature at 1 h after occlusion. Sham-operated rats received the same experimental surgery without suture insertion. This surgery never induced the cerebral cell death and neurological deficit [9]. Non-operated rat did not receive any surgery.

2.3. Preparation of liposomes

Liposomes composed of distearoylphosphatidylcholine (DSPC) and cholesterol (2/1 M ratio) were prepared as follows. Lipids dissolved in chloroform were rotary-evaporated to form a thin lipid film. The film was then dried for over 1 h under reduced pressure. Next, the dried film was hydrated with 0.3 M sucrose buffer (pH 7.4). The liposome solution was freeze–thawed for 3 cycles with liquid nitrogen and then sonicated for 15 min at 65 °C. Finally, the particle size of the liposomes was adjusted by extrusion through 100 nm-pore size polycarbonate filters (Nuclepore, Cambridge, MA, USA). Liposome size was measured with ZETASIZER (Malvern Instruments, Worcs, UK). To monitor the cerebral distribution of liposomes, DiI-C_{18} (Molecular Probes Inc., Eugene, OR, USA) was mixed with the initial lipid solution for fluorescence labeling of the liposomes.

2.4. Laser Doppler perfusion imaging of cerebral blood flow

MCAO rats were anesthetized as described above. A skin incision was made on the head to expose the whole skull. A whole brain scan was performed using PeriScan PIM-III (Perimed AB, Stockholm, Sweden). The laser beam was set to lambda, and the scan range was set as 2.5 × 2.5. Laser Doppler perfusion imaging was performed at 30 min after occlusion. The ratio of the total blood perfusion in the ipsilateral hemisphere to that in the contralateral hemisphere was calculated.

2.5. Cerebral distribution of liposomes

DiI-labeled liposomes (10 mM as DSPC, 0.5 mL) were intravenously injected into MCAO rats at 1 or 2 h after the start of ischemia. The rats were sacrificed at 1 h after the injection, and the brain was sliced into 2-mm thick coronal sections with a rat brain slicer (Muromachi Kikai, Tokyo, Japan). All sections were put on glass slides, and DiI fluorescence was measured with an *in vivo* imaging system (IVIS, Xenogen Corp., Alameda, CA).

2.6. Histological analysis

DiI-labeled liposomes were intravenously injected into MCAO rats at 1 h after the induction of ischemia. At 1 h after injection, brain slices were placed on an optical cutting temperature compound (Sakura, Finetech., Co. Ltd., Tokyo, Japan), and then frozen in a dry ice-ethanol bath. These frozen sections were cut into 10 μm sections with a cryostat (HM505E, Microm, Walldorf, Germany). The sections were incubated in 1% bovine serum albumin-containing PBS for 10 min at room temperature for protein blocking, in biotinylated anti-mouse CD31 rat monoclonal antibody (BD Pharmingen, Franklin Lakes, NJ, USA) for 18 h at 4 °C, and then in streptavidin-Alexa fluor 488 conjugates (Molecular Probes Inc.) for 30 min at room temperature. Finally, the sections were mounted with Perma Fluor Aqueous Mounting Medium (Thermo Shandon, Pittsburgh, PA, USA), and their fluorescence was observed with a microscopic LSM system (Carl Zeiss, Co., Ltd., Germany).

2.7. Brain damage assessment in MCAO rats

The ischemic or ischemia/reperfusion damage in p-MCAO or t-MCAO rats, respectively, was assessed by morphometric analysis of the brain sections stained with TTC (Wako Pure Chemical Ind. Ltd., Tokyo, Japan). To assess cerebral cell death, p-MCAO rats were sacrificed 2 or 3 h after ischemia, and t-MCAO rats were sacrificed after 1.5 or 3 h reperfusion pre-treated with 2 h ischemia. The brains were sliced into 2 mm thick coronal sections using a rat brain slicer. The brain sections of p-MCAO and t-MCAO rats were stained with 2% TTC in PBS for 30 min at 37 °C, then fixed in 10% formalin neutral buffer solution. All sections were put on glass slides and photographed with a digital camera (OLYMPUS E-300). Damaged regions were defined as areas that were completely white.

2.8. Statistical analysis

Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests. Data are presented as mean ± SD.

3. Results

3.1. Imaging of cerebral blood flow in MCAO rats

Cerebral blood flow was observed in non-operated, sham-operated, and p-MCAO rats. There were no significant differences in blood perfusion between the ipsilateral hemisphere and contralateral hemisphere in non-operated rats (Fig. 1A and D). Compared with the contralateral hemisphere, sham surgery induced approximately 20% loss of ipsilateral cerebral perfusion due to internal carotid artery (ICA) ligation (Fig. 1B and D). In contrast, MCAO reduced ipsilateral cerebral perfusion by 60% at 30 min after the onset of ischemia (Fig 1C and D). Additionally, hemiparesis and increased body temperature were observed in the MCAO-operated rats. Based on these findings, it appears that the MCAO operation induced acute ischemia in the ipsilateral hemisphere.