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Research Paper

Treatment of stroke with liposomal neuroprotective agents under cerebral ischemia conditions

Tatsuya Fukuta [a,b], Takayuki Ishii [a], Tomohiro Asai [a], Akihiko Sato [a], Takashi Kikuchi [a], Kosuke Shimizu [a], Tetsuo Minamino [c], Naoto Oku [a,*]

[a] Department of Medical Biochemistry, University of Shizuoka, School of Pharmaceutical Sciences, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan
[b] Japan Society for the Promotion of Science (JSPS), 8 Ichiban-cho, Chiyoda-ku, Tokyo 102-8472, Japan
[c] Department of Cardiovascular Medicine, Osaka University, Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan

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ABSTRACT

Since the proportion of patients given thrombolytic therapy with tissue plasminogen activator (t-PA) is very limited because of the narrow therapeutic window, the development of new therapies for ischemic stroke has been desired. We previously reported that liposomes injected intravenously accumulate in the ischemic region of the brain via disruption of the blood–brain barrier that occurs under cerebral ischemia. In the present study, we investigated the efficacy of a liposomal neuroprotective agent in middle cerebral artery occlusion (MCAO) rats to develop ischemic stroke therapy prior to the recovery of cerebral blood flow. For this purpose, PEGylated liposomes encapsulating FK506 (FK506-liposomes) were prepared and injected intravenously into MCAO rats after a 1-h occlusion. This treatment significantly suppressed the expansion of oxidative stress and brain cell damage. In addition, administration of FK506-liposomes before reperfusion significantly ameliorated motor function deficits of the rats caused by ischemia/reperfusion injury. These findings suggest that FK506-liposomes effectively exerted a neuroprotective effect during ischemic conditions, and that combination therapy with a liposomal neuroprotectant plus t-PA could be a promising therapeutic strategy for ischemic stroke.

1. Introduction

Ischemic stroke is one of the high-mortality diseases and the leading cause of serious, long-term disability worldwide [1,2]. This event occurs when cerebral blood flow is markedly reduced by a thrombus. This loss of blood supply induces cerebral cell death in the ischemic area. Brain cells in the ischemic penumbra surrounding the ischemic core region are known to escape from cell death when the cerebral blood flow is rapidly improved [3]. In clinical settings, thrombolytic therapy such as tissue-type plasminogen activator (t-PA) is approved for the treatment of acute ischemic stroke [4,5]. However, due to the narrow therapeutic time window (<4.5 h) and safety concerns such as the risk of cerebral hemorrhage stemming from t-PA treatment, the number of applicable patients is very limited [6]. In addition, even when the cerebral blood flow is restored by thrombolysis, secondary cerebral damage known as ischemia/reperfusion (I/R) injury often occurs because of the production of deleterious substances such as reactive oxygen species (ROS) and inflammatory cytokines around the lesion area, resulting in poor prognosis [7,8]. Thus, drug candidates for neuroprotection against I/R injury have been studied in clinical trials to improve therapeutic outcomes. However, none of them have been approved worldwide due to their insufficient efficacy or to undesirable side effects [9,10].

The blood–brain barrier (BBB) is functionally constituted to maintain brain homeostasis by restricting the transport of molecules into the brain. However, it has been reported that the integrity of the BBB is compromised in the acute phase after cerebral ischemia [11–13]. By focusing on this phenomenon, we previously found that intravenous administration of a liposomal neuroprotectant immediately after reperfusion is effective for the treatment of cerebral I/R injury in transient middle cerebral artery occlusion (t-MCAO) rats [14,15]. In addition, we also demonstrated that, under the cerebral ischemic state, 100-nm liposomes injected intravenously into MCAO rats can accumulate in the ischemic core and penumbra region by passing through the disrupted BBB [16]. Imaging data on [^{18}F]-labeled PEGylated liposomes (PEG-liposomes) imaged by positron-emission tomography (PET) show that such liposomes gradually accumulate in the ischemic region despite a reduced blood flow [17]. From these findings,

* Corresponding author. Tel.: +81 54 264 5701; fax: +81 54 264 5705.
E-mail address: oku@u-shizuoka-ken.ac.jp (N. Oku).

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we hypothesized that liposomal delivery of a neuroprotective agent to the ischemic region might be effective for stroke treatment even under the ischemic condition. However, the effectiveness of liposomal neuroprotectants for I/R injury, which are administered during cerebral ischemia has not been investigated.

Therefore, in the present study, we investigated the effect of a liposome-delivered neuroprotective agent, FK506, injected before the restoration of blood flow on I/R injury in t-MCAO rats. For this purpose, we prepared PEGylated liposomes encapsulating FK506 (tacrolimus, FK506-liposomes) as a neuroprotectant [18]. FK506 has been reported to exert a neuroprotective effect in animal models of ischemic stroke by inhibiting the activation of calcineurin following excessive influx of Ca^{2+} into cells [19–21]. Our previous study demonstrated that liposomalization of FK506 enables a reduction in the dosage of FK506 and that FK506-liposomes injected immediately after reperfusion might be useful for the treatment of cerebral I/R injury [18]. In the present study, to examine the usefulness of administering a liposomal neuroprotectant prior to reperfusion therapy, FK506-liposomes were injected into t-MCAO rats before reperfusion.

2. Materials and methods

2.1. Materials

Distearoylphosphatidylcholine (DSPC), dipalmitoylphosphatidylcholine (DPPC), cholesterol, and distearoylphosphatidylethanolamine (DSPE)-PEG2000 were a gift from Nippon Fine Chemical (Hyogo, Japan). [^{3}H] Cholesteryl hexadecyl ether was purchased from Perkin Elmer (Boston, MA); and 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (DiI-C_{18}), from Molecular Probes (Eugene, OR, USA). Dihydroethidium (DHE) and 2,3,5-triphenyltetrazolium chloride (TTC) were obtained from Wako Pure Chemical (Tokyo, Japan). Other reagents were of analytical grade.

2.2. Animals

Male Wistar rats (8 weeks old, 180–220 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.

2.3. Middle cerebral artery occlusion rats

Middle cerebral artery occlusion (MCAO) rats were prepared as described previously [22]. In brief, anesthesia was induced with 3% isoflurane (Escain®, Pfizer, NY, USA) and maintained with 1.5% isoflurane during the MCAO surgery, with the anesthesia being performed with a small-animal anesthesia apparatus (Model TK-4, Bio Machinery, Chiba, Japan). Rectal temperature of rats was maintained at 37 °C with a heating pad (Unique Medical Co., Ltd., Tokyo, Japan). After a midline incision of the neck skin had been made, the right common carotid artery, external carotid artery, and internal carotid artery (ICA) were isolated. Then, the MCA was occluded by inserting a silicon-coated 4-0 nylon filament (Keisei Medical Industrial Co., Ltd., Niigata, Japan) into the right internal carotid artery and advancing it to the origin of the MCA. Silk thread was used for ligation to keep the filament at the inserted position in the MCA. After the operation, the neck skin was closed; and the anesthesia was then discontinued. In the case of preparing t-MCAO rats, cerebral blood flow was restored by withdrawing the filament about 10 mm under isoflurane anesthesia. Success of the MCAO surgery was judged by the appearance of both hemiparesis and an increase in body temperature to over 38 °C after 1 h of occlusion. A sham operation was performed by following the same experimental procedure without inserting the filament.

2.4. Preparation of liposomes

Plain liposomes composed of DSPC/cholesterol (2/1 molar ratio) and PEG-liposomes composed of DSPC/cholesterol/DSPE-PEG2000 (20/10/1 molar ratio) were prepared as follows: Lipids dissolved in chloroform were evaporated to form a thin lipid film by using a rotary evaporator. The lipid film was desiccated for at least 1 h under reduced pressure. The dried lipid film was then hydrated with phosphate-buffered saline (PBS, pH 7.4). The liposomal solution was freeze-thawed for 3 cycles with liquid nitrogen and then sonicated for 10 min at 65 °C with a bath-type sonicator (Yamato Scientific Co., Ltd., Tokyo, Japan). Finally, the size of the liposomes was adjusted by extrusion of the particles through polycarbonate membrane filters having 100-nm pores (Nuclepore, Cambridge, MA, USA). Then, the particle size and ζ-potential of liposomes were measured with a Zetasizer Nano ZS (MALVERN, Worcestershire UK, USA). For preparing [^{3}H]-labeled liposomes or fluorescence-labeled ones, [^{3}H]cholesteryl hexadecyl ether or DiI-C_{18}, respectively, was added to the initial lipid solution.

FK506-liposomes composed of DPPC and DSPE-PEG2000 (20/1 molar ratio) were prepared as described previously [18]. In brief, FK506 dissolved in methanol was added to a recovery flask with the above lipids dissolved in *tert*-butyl alcohol for freeze-drying. After lyophilization of the solution, the lyophilizate was hydrated with PBS at 50 °C. The liposomal solution was freeze-thawed for 3 cycles with liquid nitrogen. Liposomes were sized by extrusion through 100 nm-pore membrane filters, and then the particle size and ζ-potential of FK506-liposomes were determined with a Zetasizer Nano ZS. For removing the unencapsulated FK506, the liposomal solution was washed by centrifugation at 453,000g for 15 min (Hitachi, Tokyo, Japan). The FK506 concentration in the liposomal solution was determined by performing HPLC (Hitachi). FK506-liposomes were dissolved in tetrahydrofuran, and 20 μL of the solution was injected into an octadecylsilane (ODS) column (TSK gel ODS-80TM, 4.6 × 150 mm, Tosoh, Tokyo, Japan). The mobile phase was composed of acetonitrile and water (3:2 v/v). The HPLC conditions were as follows: Column oven, 60 °C; flow rate, 1 mL/min; and UV detection, 214 nm.

2.5. Biodistribution of liposomes in MCAO rats

MCAO rats were intravenously injected with [^{3}H]-labeled PEG-liposomes (74 kBq/rat; 5 μmol phospholipid/rat) after 1 h of occlusion. At 1 or 3 h after injection, the rats were sacrificed; and the plasma and various organs (heart, lung, liver, spleen, kidney, and brain) were collected. The radioactivity in them was measured with a liquid scintillation counter (LSC-7400, Hitachi Aloka Medical, Tokyo, Japan). The brain was separated into ischemic and non-ischemic (normal) sides.

2.6. Intracerebral distribution of liposomes

Plain liposomes were labeled with fluorescent dye DiI-C_{18}. These DiI-labeled plain liposomes (5 μmol phospholipid/rat) were intravenously injected into MCAO rats 1 h after occlusion. At 30 min after the injection of the liposomes, the rats were intravenously injected with 0.1 mL fluorescein isothiocyanate (FITC)-conjugated tomato lectin (*Lycopersicon esculentum*; 1 mg/mL; Vector Laboratories, Burlingame, CA) to label the perfused blood vessels. After 30 min, the rats were transcardially perfused with PBS, and then the brains were dissected and sliced into 2-mm thick coronal sections with a rat brain slicer (Muromachi Kikai, Tokyo,

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