Combination Therapy with Liposomal Neuroprotective Agents Plus Tissue Plasminogen Activator for the Treatment of Ischemic Stroke

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Purpose
Ischemic stroke is a high-mortality disease and a leading cause of serious disability. Tissue plasminogen activator (t-PA) is the only therapeutic agent for recovery from acute ischemic stroke worldwide. However, the number of patients given thrombolytic therapy with t-PA is very limited due to the narrow therapeutic time window (TTW; <4.5 h) and safety concerns such as a risk of cerebral hemorrhage derived from t-PA. Also, secondary cerebral ischemia/reperfusion (I/R) injury often occurs despite the restoration of cerebral blood flow by thrombolysis, resulting in the expansion of brain damage and poor prognosis for the patients. Therefore, development of more widely applicable and effective therapies that have a capability to extend the TTW of t-PA and suppress I/R injury has been desired. Our previous study demonstrated that delivery of neuroprotectants to ischemic region with liposomal drug delivery system (DDS) is possible via the disintegrated blood-brain barrier (BBB) after ischemic stroke, and that intravenous delivery of liposomal fasudil (Fasudil-Lip), a Rho-kinase inhibitor, is effective for the treatment of cerebral I/R injury. In the present study, we investigated the usefulness of a combination therapy with Fasudil-Lip plus t-PA for ischemic stroke.

Methods
As an ischemic stroke model, middle cerebral artery occlusion (MCAO) rats by photochemically induced thrombosis (PIT) were employed. Cerebral distribution of intravenously injected fluorescence-labeled PEGylated liposomes at various time after occlusion was examined with in vivo imaging system. The particle size of PEGylated liposomes was adjusted to around 100 nm in diameter by extrusion. Then, the TTW of t-PA treatment in MCAO rats by PIT was evaluated based on the cerebroprotective effect of it. MCAO rats were intravenously injected with t-PA at 0, 1, 2, or 3 h after occlusion. At 24 h after occlusion, brains of the rats were dissected and the efficacy of t-PA was assessed by 2, 3, 5-triphenyltetrazolium chloride (TTC) staining. Next, the effect of combined treatment with Fasudil-Lip and t-PA was evaluated. The time points of Fasudil-Lip and t-PA administration were set at 1 and 3 h after occlusion, respectively. The effect of t-PA and combination treatment on the BBB permeability was examined by quantifying amounts of extravasated Evans blue (EB) into the brain tissue 6 and 24 h after occlusion. EB was intravenously injected into the rats 1 h before dissecting them. Additionally, influence of t-PA and the combination treatment on activities of matrix metalloproteinase (MMP)-2, 9 was assessed by in situ zymography. The frozen brain sections of MCAO rats were prepared 6 and 24 h after occlusion, and then incubated with FITC-labeled dye-quenched gelatin. The fluorescence in the sections was then observed by confocal laser scanning microscopy. Finally, the therapeutic effect of t-PA/Fasudil-Lip treatment was evaluated 24 h after occlusion by TTC staining.

Results
Although the brain damage judged by TTC staining progressed with time after the onset of thrombotic occlusion, accumulation of the PEGylated liposomes was observed prior to an appearance of obvious damage. The treatment with t-PA by 2 h after occlusion showed significant cerebroprotective effect after 24-h occlusion. However, the damage was widely observed when t-PA was administered after 3-h occlusion, suggesting that the TTW of t-PA is almost 2 h after occlusion in the rat model used in this study. The t-PA administration after 3-h occlusion markedly increased in the amounts of EB leakage into the brain parenchyma, whereas Fasudil-Lip treatment prior to t-PA significantly suppressed t-PA-induced permeability increase in the BBB after 24-h occlusion. In addition, the confocal images showed that the fluorescence signals derived from MMP-2, 9 activation were remarkably increased by t-PA administration. Importantly, the combination treatment with Fasudil-Lip and t-PA suppressed the MMP-2, 9 activation, especially in the peri-infarct region. Finally, the therapeutic effect of t-PA/Fasudil-Lip combined treatment was investigated. The results showed that Fasudil-Lip treatment alone showed dose-dependent therapeutic response. Notably, the combination treatment exerted significant neuroprotective effect compared with each treatment alone.

Conclusion
The present study showed that intravenously injected PEGylated liposomes accumulated in the ischemic region before the progression of brain damage in MCAO rats by PIT. In addition, the treatment with Fasudil-Lip prior to t-PA administration remarkably suppressed the increase in the BBB permeability stemming from t-PA, and also inhibited MMP-2, 9 activation. Moreover, the t-PA/Fasudil-Lip combined treatment showed significantly higher neuroprotective effect compared with each treatment alone. These results suggest that combination treatment with Fasudil-Lip plus t-PA has a potential to decrease in the risk of t-PA-induced cerebral hemorrhage and to extend the TTW of thrombolytic therapy with t-PA. Taken together, the present study suggests that combination therapy with liposomal DDS plus t-PA could be a useful therapeutic option for ischemic stroke.