in situ Apoptosis Detection Kit (Chemicon International, Inc, USA), as described below. For fixation of the sections, they were incubated in 4% paraformaldehyde for 15 min at room temperature, and in ethanol/acetic acid (2:1) solution for 5 min at −20 °C. DNA strand breaks were labeled with the digoxigenin-conjugated terminal deoxynucleotidyl transferase enzyme by incubation for 1 h at 37 °C. Then, the sections were incubated in anti-digoxigenin-fluorescein solution for 30 min at room temperature. Finally, the sections were mounted with Perma Fluor Aqueous Mounting Medium (Thermo Shandon, Pittsburgh, PA, USA) included in DAPI solution (1.0 μg/mL) and observed for fluorescence with a microscopic LSM system (Carl Zeiss, Co., Ltd., Germany).

2.10. Therapeutic experiment

PBS, AEPO (8 μg/kg), AEPO-liposomes (8 μg/kg as AEPO dose) or PEGylated liposomes were intravenously injected into t-MCAO rats immediately after the start of reperfusion. The volume of damaged area, the degree of brain swelling, and the functional outcome of rats were assessed at 24 h of reperfusion. For investigation of the functional outcome, the rats underwent a 21-point neurological score analysis prior to dissection of the brain, as described previously [27]. Then their brains were dissected, and the blood was collected to assess the hematopoietic effect of AEPO. The brains were sliced into 2-mm-thick coronal sections by using a rat brain slicer (Muromachi Kikai, Tokyo, Japan) and stained with 2, 3, 5-triphenyltetrazolium chloride (TTC, Wako Pure Chemical Ind. Ltd., Tokyo, Japan) for the measurement of brain cell death. The volume of damaged area was calculated by using an image-analysis system (NIH Image J). The damage regions were considered as completely white areas. Brain swelling was calculated as the ratio of volumes between right and left hemisphere sections.

2.11. 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 ± S.D.

3. Results

3.1. PEGylated liposomes accumulated in ischemic region after reperfusion

To determine therapeutic time window for PEGylated liposomal usage, we examined the time course of changes in the cerebral distribution of fluorescence-labeled PEGylated liposomes with DiIC$_{18}$ after reperfusion *ex vivo* (Fig. 1A–E). t-MCAO rats were injected with DiI-labeled PEGylated liposomes via a tail vein at 0, 1, 3, 6 or 24 h after the start of reperfusion; and then brain sections were prepared from them at 1 h after the injection. The fluorescence of the labeled liposomes in the brain sections was then observed with an *in vivo* imaging system. The accumulation of DiI-labeled PEGylated liposomes in the ischemic hemisphere was detected in and around the striatum immediately after the start of reperfusion. The most abundant localization of DiI-labeled PEGylated liposomes was observed when the liposomes were injected at 3 h after reperfusion (Fig. 1C). A small amount of liposomal fluorescence was observed when the liposomes were injected at 6 h of reperfusion (Fig. 1D), but hardly any was detectable when the injection was done at 24 h of reperfusion (Fig. 1E). Therefore, it appears that the region where the PEGylated liposomes accumulated in the ischemic hemisphere gradually spread as time passed until around 3 h after the start of reperfusion. Next, t-MCAO rats were injected with DiI-labeled PEGylated liposomes immediately after the start of reperfusion, and their brains were dissected at 24 h post injection to examine the cerebral distribution of the liposomes. The results revealed that the fluorescence of DiI-labeled PEGylated liposomes was detected in the ischemic hemisphere at 24 h (Fig. 1F). These data suggest that PEGylated liposomes were retained in the ischemic hemisphere for an extended period of time. Moreover, higher liposomal fluorescence intensity was observed in the brain sections taken at 24 h after injection than in those taken with at 1 h after injection (Fig. 1B, F), suggesting that the PEGylated liposomes gradually accumulated in the brain parenchyma due to the EPR effect. To quantify the accumulated amount of PEGylated liposomes that accumulated in the ischemic hemisphere, we labeled

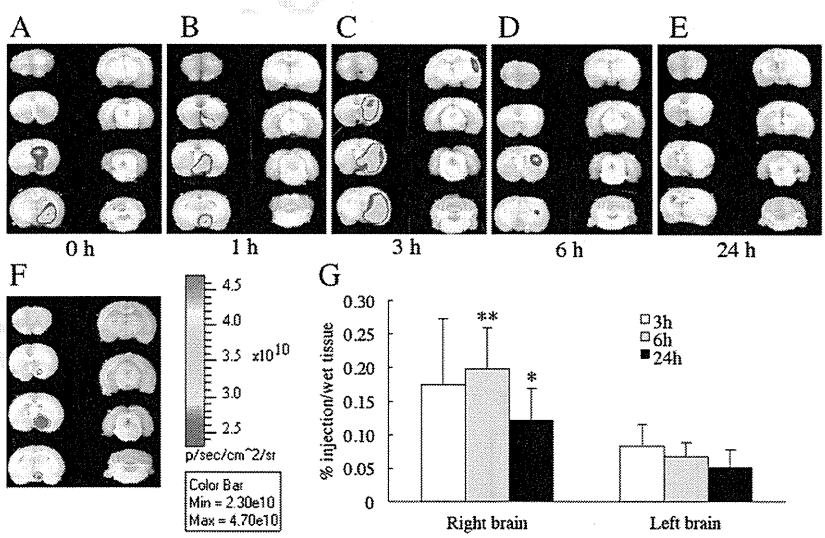

Fig. 1. Time course of PEGylated liposome localization in the brain of t-MCAO model rats. A–E) The t-MCAO rats were injected with DiI-labeled PEGylated liposomes (0.5 mL/rat *i.v.*) at 0, 1, 3, 6 or 24 h of reperfusion; and then the rats were sacrificed at 1 h after the injection. F) The t-MCAO rats were injected with DiI-labeled PEGylated liposomes (0.5 mL/rat *i.v.*) immediately after the start of reperfusion, and then their brains were dissected at 24 h after the injection. DiI-labeled PEGylated liposomes localized in the brain sections were observed with IVIS. The left hemispheres of the brain slices are the non-ischemic side; and the right hemispheres are the ischemic side. Bar shows the relative levels of fluorescence intensity, ranging from low (blue), to medium (green), to high (yellow, red). G) PEGylated liposomes were radiolabeled with [^{3}H]cholesteryl hexadecyl ether. t-MCAO rats were injected with [^{3}H]-labeled PEGylated liposomes immediately after the start of reperfusion. These rats were sacrificed at 3, 6 or 24 h of reperfusion, their brains were removed, and the radioactivity of the [^{3}H]-labeled PEGylated liposomes in the ischemic and non-ischemic hemispheres of the brain was determined. The columns indicate the mean ± SD, and the significant differences are as indicated: $^*p<0.05$, $^{**}p<0.01$ vs. corresponding value for non-ischemic hemisphere. Fluorescence data represent of 5 independent animal experiments, all of which demonstrated a similar profile of responses. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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PEGylated liposomes with a radioisotope and injected them into t-MCAO model rats immediately after the start of reperfusion, and then sacrificed the animals at 3, 6 or 24 h after the injection (Fig. 1G and Fig. S1). The results revealed that PEGylated liposomes had a significantly higher degree of accumulation in the ischemic hemisphere compared with their amount in the non-ischemic hemisphere at 6 ($p=0.007$) and 24 h ($p=0.02$), but not at 3 h ($p=0.08$), after the injection. Because cardiac perfusion was not performed in this experiment, both hemispheres included the radioactive PEGylated liposomes in the bloodstream. Thus, actual differences in the accumulation between ischemic and nonischemic hemispheres would be much larger. These data also indicate that PEGylated liposomes gradually accumulated in the ischemic region in a time-dependent manner. In addition, there was no significant difference between the hemispheres in the accumulation of PEGylated liposomes injected at 3 h after the start of reperfusion (Fig. S2). Taken together, these data suggest that the therapeutic time window for the use of PEGylated liposomes in the treatment of cerebral I/R injury is up to around 3 h after the start of reperfusion, with the most effective injection time point being regarded as immediately after the start of reperfusion.

3.2. AEPO-liposomes showed cytoprotective activity toward differentiated PC12 cells

After AEPO had been conjugated to DSPE-PEG-NHS, the conjugates were incubated with PEGylated liposomes; and then the AEPO-liposomes were purified by gel filtration (Fig. 2A). The optimal amount of AEPO to be used to modify the liposomes was determined by changing the molar ratio of AEPO to DSPE-PEG-NHS (Fig. S4A). Modification ratio of AEPO to PEGylated liposomes was approximately 35%, hence, 0.31 μg of AEPO was modified to 1 μmol of PEGylated liposomes. The average particle size and ζ-potential of AEPO-liposomes were 129 nm and 0.29 mV, respectively (Fig. S4B). The particle size of AEPO-liposomes showed little change in the presence of serum (Fig. S5). To examine the pharmacological activity of AEPO-liposomes, we evaluated the cytoprotective effects of them on PC12 cells by performing an MTT assay. PC12 cells are known to differentiate into nerve-like cells when treated with NGF; and the depletion of NGF induces programmed cell death (apoptosis) in the differentiated PC12 cells [28]. Depletion of NGF has also been observed in the striatum and cortex of the ischemic hemisphere of t-MCAO rats for 24 h of reperfusion [29,30]. In accordance with these findings, in the absence of NGF, the number of surviving differentiated PC12 cells was decreased to 32.1% of the number in the presence of the growth factor (Fig. 2B). Treatment of the cells with AEPO or AEPO-liposomes significantly suppressed the cell death observed in the absence of NGF (0.1 nmol/L AEPO vs. NGF (−) $p=2.5\times10^{-5}$; 0.01, 0.1 or 1.0 nmol/L AEPO-liposomes vs. NGF (−) $p=7.8\times10^{-5}$, 8.0×10^{-5}, 2.5×10^{-5}, respectively). The suppression of cell death brought about by the treatment with AEPO-liposomes occurred in a dose-dependent manner. The addition of PEG liposomes to PC12 cells in the absence of NGF did not significantly affect cell survival, indicating that the non-modified PEGylated liposomes were not neuroprotective.

3.3. Accumulation of AEPO in ischemic region was enhanced by liposomal DDS

AEPO was labeled with ^{125}I to trace the biodistribution of AEPO and AEPO-liposomes in the t-MCAO model rats. The rats were injected with ^{125}I-labeled AEPO or ^{125}I-labeled AEPO-liposomes via a tail vein immediately after the start of reperfusion, and then their distribution was evaluated at 3 and 24 h of reperfusion by measuring the accumulated radioactivity in each organ (Fig. 3A–D). The retention of AEPO in the bloodstream at 3 and 24 h after injection was significantly prolonged by liposomalization ($p=0.002$ and $p=0.04$, respectively; Fig. 3A–B). AEPO-liposomes showed higher accumulation in the liver than AEPO at both time points ($p=2.1\times10^{-5}$ and $p=0.008$ respectively). Fig. 3C and D show the amount of accumulation in the brain at 3 and 24 h after injection. The amount of AEPO-liposomes accumulated in the ischemic hemisphere was higher than that of AEPO at both times ($p=2.4\times10^{-5}$ and $p=0.004$, respectively). In addition, AEPO-liposomes accumulated significantly more in the ischemic hemisphere than in the non-ischemic hemisphere ($p=0.007$ and $p=0.04$, respectively); whereas AEPO showed no difference between the ischemic and non-ischemic hemispheres. Thus, liposomalization of AEPO prolonged the blood circulation time and increased the accumulation and retention of AEPO in the ischemic hemisphere at both 3 and 24 h of reperfusion.

3.4. AEPO-liposomes ameliorated neuronal apoptosis following cerebral I/R injury in rats subjected to t-MCAO

To evaluate the anti-apoptotic activity of AEPO-liposomes in the t-MCAO model rats, we observed TUNEL-positive cells in frozen brain

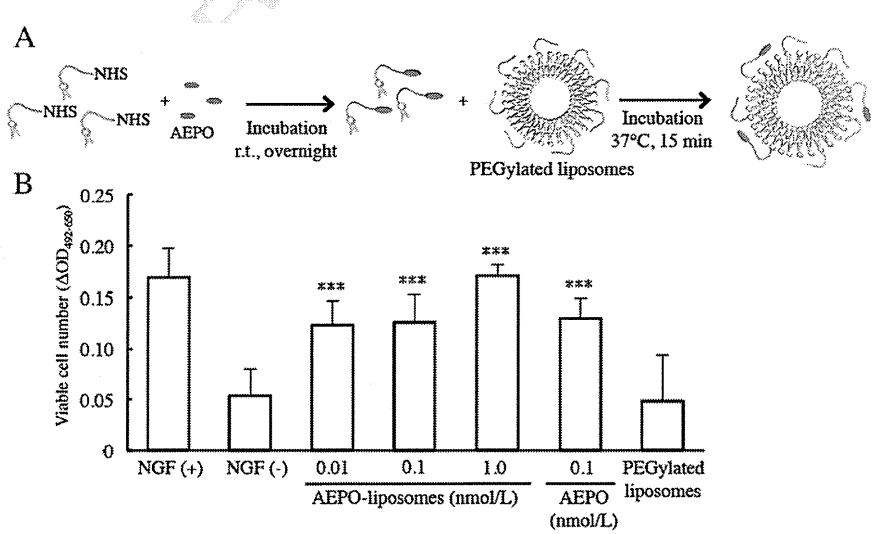

Fig. 2. Cytoprotective effect of AEPO-liposomes on PC12 cells. A) The strategy of modification of PEGylated liposomes with AEPO. B) PC12 cells were caused to differentiate by the addition of NGF at 100 ng/mL to culture medium supplemented with 0.5% HS. Following 5 days in culture for differentiation, the culture medium was changed to that without NGF, and each liposomal sample was added to the culture medium. After 5 additional days in culture, the number of viable cells was determined by performing the MTT assay. Data are presented as the mean ± S.D. ($n=6$). Statistical differences were calculated by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests (comparison with NGF-free group). (***$p<0.001$ vs. NGF [−]).

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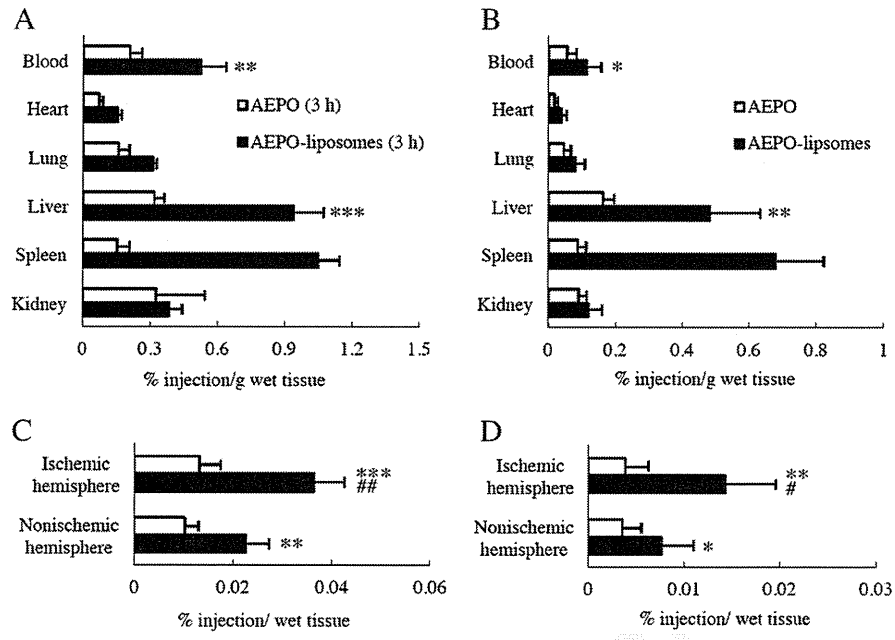

Fig. 3. Biodistribution of AEPO-liposomes in the t-MCAO model rats. AEPO was labeled with ^{125}I. The t-MCAO model rats were injected with ^{125}I-labeled AEPO or ^{125}I-labeled AEPO-liposomes via a tail vein. Biodistribution of each sample was determined by measuring the radioactivity in each organ at 3 (A and C) or 24 h (B and D) after the injection. Data are presented as the mean ± S.D. ($n=5$). Significant differences are indicated as follows: * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. AEPO value, # $p<0.05$, ## $p<0.01$ vs. nonischemic hemisphere.

sections of striatum and cerebral cortex by confocal microscopy (Fig. 5A–D). In the non-ischemic hemisphere, almost no TUNEL-positive cells were observed in either the striatum or the cerebral cortex. However, in the ischemic hemisphere of the control (PBS-treated) group, many cerebral cells in both the striatum and the cerebral cortex were TUNEL positive. In contrast, in the AEPO-liposome-treated group, the number of TUNEL-positive cells in the striatum of the ischemic hemisphere was significantly reduced compared that of the control group ($p=0.02$; Fig. 5A, C). On the other hand, no significant difference in TUNEL-positive cell number was found in the cerebral cortex ($p=0.16$; Fig. 5B, D).

The therapeutic effect of AEPO-liposomes on the cerebral I/R injury in the t-MCAO model rats was examined by evaluating the volume of damaged brain, degree of brain swelling, and motor activity of rats at 24 h after the start of reperfusion (Figs. 4E–G, 5). As judged by TTC staining, AEPO-liposomes greatly reduced cerebral cell death compared with the control (PBS) and AEPO ($p=2.3\times10^{-5}$ and $p=0.003$, respectively; Fig. 5E, F). In particular, TTC-defined cerebral lesion in the striatum was strongly suppressed by the treatment with AEPO-liposomes. The volume of damaged brain was not changed by the PEGylated liposomes, indicating that PEGylated these liposomes were neither neuroprotective nor augmented the cerebral I/R injury. Brain edema, a life-threatening complication caused by cerebral I/R, was determined based on the difference between the volume of the right cerebral hemisphere and that of the left one. One hour of ischemia and 24 h of reperfusion increased ischemic hemisphere volume compared with the volume for the sham group (Fig. 5G). However, the brain swelling was significantly suppressed by the treatment with AEPO-liposomes ($p=1.9\times10^{-5}$). Moreover, AEPO-liposomes clearly improved neurological function at 24 h of reperfusion ($p=0.03$; Fig. 6). These results taken together indicate that AEPO-liposomes have the potential to improve stroke outcome and a patient's prognosis. It is also significant that these liposomes did not increase the hematocrit value (an indicator of blood viscosity), as such an increase is suggestive of a poor cerebral stroke outcome (Table S1). This indicates that liposomalization of AEPO did not stimulate hematopoiesis, despite affording an increase in the AEPO level in the blood circulation.

4. Discussion

We previously reported that obvious damaged area appeared at 3 h of reperfusion in our t-MCAO model rats [31]. In the present study, using the same model rats, we observed that PEGylated liposomes injected after the start of reperfusion accumulated in the brain parenchyma quickly thereafter. These results suggest that liposomal drug delivery to an ischemic region after reperfusion is possible before the occurrence of obvious brain damage. A number of neuroprotective drugs have failed in clinical trials due to inadequate setting of the therapeutic time window [32]. We speculated that the therapeutic time window of nanoparticles would be up to about 3 h after the start of reperfusion, since the accumulation of PEGylated liposomes in the ischemic hemisphere was low in the case of the injection given at 6 h of reperfusion.

Our results indicated that there was little accumulation of PEGylated liposomes in the ischemic region when injected more than 6 h after reperfusion had begun. One possible reason for this poor accumulation is the interruption of blood flow. It has been reported that I/R impedes the microcirculation [33]. Two hours of ischemia and 6 h of reperfusion have been shown to induce the contraction of pericytes by causing oxidative-nitrative stress. This phenomenon may limit drug delivery to an ischemic region. This finding suggests that pericyte contraction may interrupt the circulation of nanoparticles in the ischemic hemisphere. This hypothesis would thus explain why PEGylated liposomes given at 24 h after the start of reperfusion did not accumulate in the ischemic region. Elimination of this oxidative-nitrative stress might possibly prolong the therapeutic time window of liposomal agents.

Several reports have described the usefulness of AEPO for the treatment of cerebral stroke in rodent models. Wang *et al.* showed that the intraperitoneal injection of AEPO (80 μg/kg) before focal ischemia reduces infarct volume in their hypoxic-ischemic model rats by suppressing ERK activation and up-regulating SNAP-25.[25] Another report showed that AEPO (44 μg/kg) administered intravenously at the restoration of cerebral bloodstream in focal ischemia model rats decreases infarct volume measured at 24 h after the injection [24].

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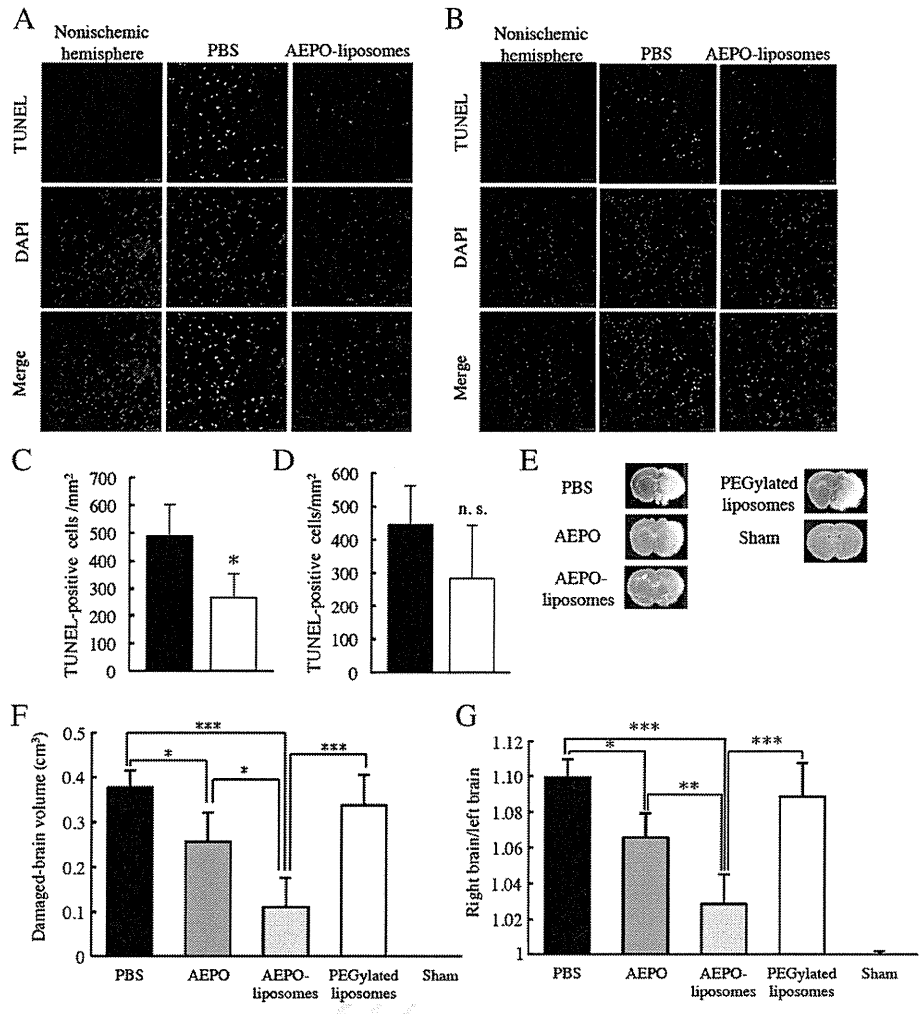

Fig. 4. Therapeutic effect of AEPO-liposomes on brain injury in the t-MCAO rats. The t-MCAO rats were injected via a tail vein with PBS or AEPO-liposomes immediately after the start of reperfusion. (as AEPO dosage of 8 μg/kg). Frozen sections of the brain in the t-MCAO model rats were prepared at 24 h after the injection of each sample, and then the sections were stained with TUNEL reagents and DAPI. The fluorescence images in the striatum (A) and the cortex (B) were observed by confocal laser scan microscopy. Quantitative data of apoptotic cerebral cells in the striatum (C) and the cortex (D) were obtained as the mean of 4 independent experiments. Solid columns indicate PBS control; and open columns, AEPO-liposomes. t-MCAO rats were injected via a tail vein with PBS, AEPO, AEPO-liposomes or PEGylated liposomes immediately after the start of reperfusion (as AEPO dosage of 8 μg/kg). E) The brains were dissected and stained with TTC at 24 h after the injection. Infarct volume (F) and the degree of brain swelling (G) were calculated by using Image J. Data are the mean ± S.D. (D, E; n = 4, G-H; n = 7). Significant differences are indicated as follows: *$p<0.05$, **$p<0.01$, ***$p<0.001$ as indicated by the brackets.

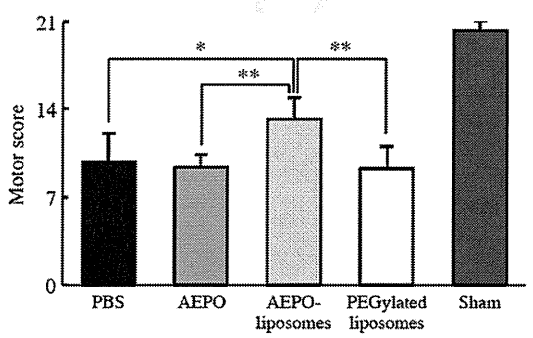

Fig. 5. Motor activity of t-MCAO model rats after treatment with AEPO-liposomes. t-MCAO model rats were injected via a tail vein with PBS, AEPO, AEPO-liposomes or PEGylated liposomes immediately after the start of reperfusion (as AEPO dosage of 8 μg/kg). At 24 h after the injection, the rats were assessed points in a 21-point neuropathological scoring system. Data are presented as the mean ± S.D. ($n = 7$). Significant differences are indicated as follows: * $p<0.05$, ** $p<0.01$, *** $p<0.001$ as indicated by the brackets.

We attempted to improve the outcome of cerebral stroke by enhancing the effect of low-dose (8 μg/kg) AEPO by using liposomal DDS technology. AEPO suppressed the infarct volume by approximately 30%, in contrast to the more than 70% achieved by the AEPO-liposomes. AEPO showed a cytoprotective effect on cerebral I/R injury via intravenous injection in spite of its short half-life in the bloodstream, probably because a brief exposure of neuronal cells to EPO is sufficient to cause a neuroprotective effect *in vitro* [19]. However, a long exposure to EPO is more effective than a short one for protecting neuronal cells [19]. AEPO-liposomes showed high accumulation and long retention in the ischemic hemisphere owing to a prolonged time in the blood circulation and the EPR effect. Therefore, we suggest that the significant neuroprotective effect of AEPO-liposomes should be attributed to the activation of many EPORs at the early stage after the start of reperfusion and to the long exposure of the cerebral cells to high concentration of AEPO.

Ischemic cerebral edema consists of cytotoxic edema and vasogenic edema resulting from dysfunction of the cellular osmotic pressure and disruption of the BBB, respectively. EPO has been shown to reduce astrocyte swelling by inhibiting the permeability of astrocyte aquaporin 4 after ischemia and also to protect neuronal cells possibly

through reducing cell swelling [34]. These findings suggest that AEPO might attenuate astrocyte swelling and neuronal cellular edema, resulting in suppression of neuronal cell death. In comparison with the other groups, the I/R rats treated with AEPO-liposomes significantly recovered neurological function (as assessed by motor score) at 24 h after an injection given immediately after the start of reperfusion. AEPO-liposomes mainly suppressed cerebral cell death in the striatum, which is the principal input nucleus of the basal ganglia receiving motor information from the cerebral motor cortex. Thus, the suppression of cell death in the striatum by the treatment with AEPO-liposomes resulted in the improvement of the motor abilities of the t-MCAO model rats. Nanoparticles appear to be suitable for delivering drugs in and around the striatum after cerebral ischemia, since the cerebral distribution of PEGylated liposomes corresponded to the region recovered by the treatment with AEPO-liposomes. This finding is of considerable interest as previous studies have found that some small molecular agents aid recovery mainly in the cerebral cortex [35,36]. Thus, a combination therapy using such agents together with liposomal drugs might be useful for the treatment of cerebral I/R injury. Our results indicate that AEPO-liposomes reduce progression of brain damage after recovery of blood flow from cerebral ischemia. AEPO-liposomes may be a useful adjunctive therapy after t-PA treatment in clinical practice.

5. Conclusions

The study has found that PEGylated liposomes injected immediately after reperfusion accumulated in the ischemic regions at an early stage after I/R and were retained there for at least 24 h after the start of reperfusion. Furthermore, AEPO-PEGylated liposomes significantly reduced cerebral I/R injury in t-MCAO model rats. Therefore, nanoparticles such as liposomes are potentially useful as a drug delivery carrier for the treatment of cerebral ischemia–reperfusion injury.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.jconrel.2012.02.004.

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A single injection of liposomal asialo-erythropoietin improves motor function deficit caused by cerebral ischemia/reperfusion

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ABSTRACT

Modification of the liposomal surface with a targeting molecule is a promising approach for the targeted delivery of therapeutics. Asialo-erythropoietin (AEPO) is a potent tool for targeting an ischemic region by binding to the EPO receptors on neuronal cells. Additionally, it shows a strong cytoprotective effect against programed cell death. Hence, AEPO-modified liposomes appear likely to have both a neuronal-targeting character and a neuroprotective effect on cerebral ischemic injury. In this study, we assessed the targeting ability of AEPO-modified PEGylated liposomes (AEPO-liposomes) to ischemic region and their improvement effect on neurological deficits induced by ischemia/reperfusion (I/R) in transient middle cerebral artery occlusion (t-MCAO) rats. Immunohistological analysis showed that the AEPO-liposomes given immediately after reperfusion extravasated into the ischemic region and attached strongly to neuronal cells. Also, neuronal nuclei (NeuN) staining was clearly visible only in the AEPO-liposome-treated group, suggesting that AEPO-liposomes protected neuronal cells from ischemia/reperfusion-induced damage. Moreover, a single administration of low-dose AEPO-liposomes significantly improved the neurological deficit compared to vehicle and free-AEPO treatment at 7 days after injection. In conclusion, AEPO-liposomes have clear potential as a neuroprotectant after stroke and as a DDS device targeting ischemic regions.

1. Introduction

Cerebral ischemia/reperfusion (I/R) injury is a secondary impairment occurring after recovery from cerebral stroke. It is a complex disorder caused by oxidative damage, inflammation, glutamate neurotoxicity, and cerebral edema (Gursoy-Ozdemir et al., 2004; Huang et al., 2006). Because the injury is closely related to stroke outcome, effective therapies for it are urgently needed, but have yet to be developed (Ginsberg, 2009; Ford, 2008). A considerable amount of research on the mechanism of the impairment has been reported (Eltzschig and Eckle, 2011; Amantea et al., 2009; Kiewert et al., 2008). Recently it has been revealed that the brain damage caused by cerebral I/R injury is affected not only by necrosis but also by apoptosis (Dirnagl et al., 1999; Xu et al., 2006). Thus, anti-apoptotic agents are anticipated to be useful for treating or preventing the injury.

Asialo-erythropietin (AEPO), a metabolite of erythropoietin (EPO), has been shown to have a cytoprotective effect against apoptotic cell death in neuronal cells by binding to the EPO receptor on the cellar surface and activating several signal pathways (Erbayraktar et al., 2003; Digicaylioglu and Lipton, 2001; Arcasoy, 2008; Brines and Cerami, 2005). However, EPO may also worsen cerebral stroke outcome, as it increases hematocrit and the production of hyperreactive platelets. In contrast, AEPO does not affect hematocrit (Savino et al., 2006), making it a promising agent for the treatment of cerebral I/R injury. However, the half-life of AEPO is extremely short, resulting in low accumulation in the ischemic region. Therefore, multiple doses or a continuous infusion of AEPO is needed to achieve an adequate long-term therapeutic effect.

We have recently developed AEPO-modified liposomes (AEPO-liposomes) to increase blood circulation and enhance the therapeutic effect of AEPO (Ishii et al., 2012). Indeed, the liposomalization of AEPO succeeded in prolonging blood circulation and increasing the amount of AEPO accumulation in the ischemic region. Therefore, AEPO-liposomes may be effective for the treatment of I/R injury at a lower dose, and with a lower number of doses. However, the influence of AEPO modification of the liposomal surface on cerebral distribution is poorly understood. In this study, we evaluated in detail the cerebral distribution of AEPO-liposomes and

Abbreviations: AEPO, Asialo-erythropoietin; AEPO-liposomes, AEPO-modified PEGylated liposomes; I/R, ischemia/reperfusion; NeuN, neuronal nuclei; t-MCAO, transient middle cerebral artery occlusion.

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their therapeutic effect on motor functional deficit when administered as a single low-dose intravenous injection immediately after reperfusion in transient middle cerebral artery occlusion (t-MCAO) model rats.

2. Materials and 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. t-MCAO model rats

t-MCAO model rats were prepared as described previously (Nagasawa and Kogure, 1989). In brief, anesthesia was induced with 3% isoflurane and maintained with 1.5% isoflurane during cerebral stroke surgery. 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 internal carotid artery (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 was closed and anesthesia was discontinued. MCAO was maintained for 1 h. Success of the surgery was judged by the appearance of hemiparesis and an increase in body temperature. Reperfusion was performed by withdrawing the filament about 10 mm at 1 h after the occlusion under isoflurane anesthesia.

2.3. Preparation of AEPO-liposomes

PEGylated liposomes composed of distearoylphosphatidylcholine (DSPC), cholesterol, and distearoylphosphatidylethanolamine (DSPE)-PEG (20/10/1 as molar ratio) and AEPO-liposomes were prepared as described previously (Ishii et al., 2012). In brief, distearoylphosphatidylethanolamine (DSPE)-PEG-*N*-hydroxysuccinimide (NHS) dissolved in borate buffer (pH 8.4) was mixed with AEPO solution, and the mixture was incubated for 1 day at room temperature to prepare DSPE-PEG-AEPO conjugates. A 20 mM solution of PEGylated liposomes was prepared, and then 1 mL of the liposomes was incubated with 0.5 mL of the DSPE-PEG-AEPO conjugates for 15 min at 65 °C. The AEPO-modified liposomes (AEPO-liposomes) were purified by gel filtration with Sepharose™ 4 Fast Flow (Amersham Biosciences, Sweden). The AEPO concentration of AEPO-liposomes was measured by HPLC. Based on liposomal size, and lipid and AEPO concentrations, the calculated number of AEPO molecules per one liposome was about 6 for AEPO-liposomes prepared under the present protocol. To observe cerebral distribution of these liposomes, DiI-C_{18} (Molecular Probes Inc., Eugene, OR, USA) was mixed with an initial lipid solution for fluorescence labeling.

2.4. Cerebral distribution of AEPO-liposomes

PEGylated liposomes and AEPO liposomes were fluorescently labeled with DiI-C_{18}. These liposomes were intravenously injected into the t-MCAO model rats immediately after reperfusion. Their brains were dissected at 3 or 24 h after the injection and sliced into 2 mm thick coronal sections with a rat brain slicer (Muromachi Kikai, Tokyo, Japan). All sections were put in glass slides, and the fluorescence of DiI was observed with an in vivo imaging system (IVIS, Xenogen Corp., Alameda, CA). Thereafter, these sections were put on 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 with cryostat (HM505E, Microm, Walldorf, Germany) for further staining.

2.5. Immunostaining for CD31

The sections were incubated in 1% bovine serum albumin-containing PBS for 10 min at room temperature for protein blocking, biotinylated anti-mouse CD31 rat monoclonal antibody (BD Pharmingen, Franklin Lakes, NJ, USA) for 18 h at 4 °C, and then 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 fluorescently observed with a microscopic LSM system (Carl Zeiss, Co., Ltd., Germany).

2.6. NeuN staining

The frozen sections made as described above were incubated with 1% bovine serum albumin-containing PBS for 10 min at room temperature, and then with anti-neuronal nuclei (NeuN) Alexa fluor 488-conjugated monoclonal antibody (Millipore, Billerica, MA, USA) for 3 h at room temperature. Finally, the sections were mounted with Perma Fluor Aqueous Mounting Medium (Thermo Shandon, Pittsburgh, PA, USA) in DAPI solution (1.0 μg/mL, Molecular Probes, Eugene, OR, USA) and the fluorescence was observed with a microscopic LSM system (Carl Zeiss, Co., Ltd., Germany).

2.7. Therapeutic experiment

PBS, AEPO (8 μg/kg) or AEPO-liposomes (8 μg/kg as AEPO dosage) were intravenously injected into t-MCAO model rats immediately after reperfusion. The motor function outcome of the rats was assessed at 1, 2, 3, 5 and 7 days after reperfusion. To investigate the motor function outcome, the rats underwent a 21-point neurological score analysis as described previously (Hunter et al., 2000). Maximum score is 21. Both normal rats and sham-operated rats showed 21.

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 ± S.D.

3. Results

3.1. Extravasation of AEPO-liposomes into the ischemic hemisphere

Cerebral distribution of AEPO-liposomes given immediately after reperfusion was observed at 3 and 24 h after reperfusion. The accumulation of DiI-labeled PEGylated liposomes and AEPO-liposomes in the ischemic hemisphere was confirmed with a fluorescence imaging system (Fig. 1A and B). In both t-MCAO and control groups, neither the intravenous injection of PEGylated liposomes nor of AEPO-liposomes resulted in detectable DiI fluorescence in the non-ischemic hemisphere (Fig. 2A and B). At 3 h after injection, PEGylated liposomes showed widespread diffusion in the ischemic region (Fig. 2A). Similarly, AEPO-liposomes had leaked into brain parenchyma from the cerebral vessels. Interestingly, AEPO-liposomes had also accumulated in the cerebral vessels in the ischemic hemisphere. At 24 h after injection, the fluorescence of both the DiI-labeled PEGylated liposomes and the

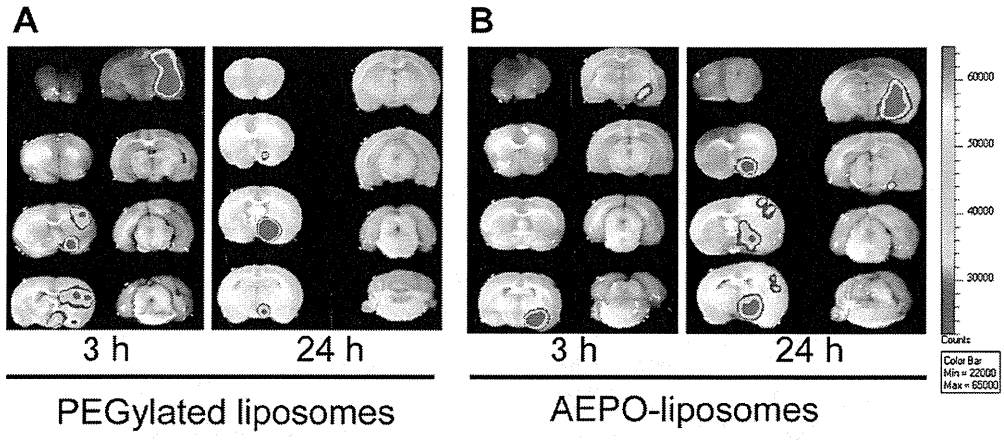

Fig. 1. Imaging of fluorescence-labeled PEGylated liposomes and AEPO-liposomes in t-MCAO brain slices. t-MCAO model rats were injected with DiI-labeled PEGylated liposomes and AEPO-liposomes (0.5 mL/rat *i.v.*) immediately after reperfusion; then the rats were sacrificed at 3 and 24 h after the injection. Each type of liposome localized in the brain sections was observed with IVIS. The left hemispheres of the brain slices are the non-ischemic side; the right hemispheres are the ischemic side. Bar shows the relative levels of fluorescence intensity, ranging from low (blue), to medium (green), to high (yellow, red). The localization of DiI-labeled PEGylated liposomes in the ischemic hemisphere is shown in A. Right and left panels indicate the fluorescence imaging in the brain section at 3 and 24 h after injection, respectively. (B) Localization of DiI-labeled AEPO-liposomes in the ischemic hemisphere is shown. Right and left panels indicate the fluorescence imaging in the brain section at 3 and 24 h after injection, respectively. Fluorescence data were obtained from 5 independent animal experiments, all of which demonstrated a similar profile of responses.

DiI-labeled AEPO-liposomes were observed in brain parenchyma (Fig. 2B). Previously, we have revealed that injected PEGylated liposomes showed almost no accumulation in the ischemic region when they were injected 6 h after reperfusion (Ishii et al., 2012). Taken together, these results indicate that AEPO-liposomes given immediately after reperfusion accumulate in the ischemic region at an early stage and are retained in brain parenchyma for at least 24 h after injection.

3.2. Accumulation of AEPO-liposomes in neuronal cells

To examine in more detail the cerebral distribution of AEPO-liposomes in the t-MCAO model rats, NeuN staining of neurons was performed on frozen sections (Fig. 3). Because PEGylated liposomes and AEPO-liposomes accumulated predominantly in the striatum containing the ischemic core region when the liposomes were injected immediately after reperfusion, their distribution in this region was observed. PEGylated liposomes were evenly distributed in the intercellular space in the ischemic hemisphere (Fig. 3A and C). On the other hand, AEPO-liposomes not only spread into the intercellular space, but also accumulated densely in the neuronal cells. At 24 h after reperfusion, the visibility of the NeuN staining had decreased in both the control and PEGylated liposome groups (Fig. 3C). However, the NeuN staining in the AEPO-liposome group was still clearly visible. In the cerebral cortex, which was far from the ischemic core, only a small amount of PEGylated liposomes was observed at 3 and 24 h after reperfusion (Fig. 3B and D). Also, localization of AEPO-liposomes in neuronal cells was barely detectable at both times.

3.3. Amelioration of motor functional deficit by treatment with AEPO-liposomes

Cerebral I/R injury is accompanied by a neuropathological disorder, resulting in motor function deficits. A single administration of AEPO alone resulted in no significant change in motor function in the t-MCAO model rats during 1 week, compared with the control group (Fig. 4). In contrast, AEPO-liposomes significantly decreased the motor function deficits. In particular, the paralysis of the right hind leg was greatly improved at 1 day after reperfusion with AEPO-liposome treatment. At 7 days after reperfusion, only the rats treated with AEPO-liposomes had recovered from paralysis of the right forepaw. These recoveries contributed to the high score in the rats treated with them. There were no significant differences in body weight change or hematocrit value in any of the groups during the 7 days.

4. Discussion

A nanocarrier modified with AEPO has the potential to be an active targeting device. Expression of EPO receptors in neuronal cells is upregulated via hypoxia-inducible factor 1α (HIF-1α) after cerebral ischemia (Rabie and Marti, 2008; Buemi et al., 2005; Morishita et al., 1997). Our results showed that AEPO-liposomes accumulated more densely in neuronal cells than PEGylated liposomes did. This indicates that the modification of a nano DDS carrier with AEPO is a useful method for delivering the drug to neuronal cells after an ischemic event. Additionally, it has been reported that EPO receptor expression in vascular endothelial cells increases after an ischemic event (Contaldo et al., 2007; Rabie and Marti, 2008). In the present study, despite PEGylated liposomes hardly accumulating in the cerebral vascular endothelial cells, AEPO-liposomes clearly accumulated in these cells in the ischemic hemisphere. On the other hand, a merged image of AEPO-liposomes and cerebral vessels was not observed in the non-ischemic hemisphere. This suggests that AEPO-modified nanocarriers possess specific targeting activity towards cerebral vessels damaged by ischemic or I/R events. Taken together, this evidence indicates that nanocarriers modified with AEPO may be useful for the treatment of ischemic disease.

In the cerebral cortex, the DiI fluorescence of these liposomes was barely detectable ex vivo at 3 and 24 h after injection (Fig. 1A and B). Additionally, AEPO-liposomes significantly reduced the number of TUNEL-positive cells in the striatum in the ischemic hemisphere (Ishii et al., 2012). On the other hand, no significant difference was found in the number of TUNEL-positive cells in the cerebral cortex. Therefore, drug therapy based on nanotechnology for the treatment of cerebral I/R injury may be suitable for targeting the ischemic core and the regions surrounding it.

AEPO has been studied as a therapeutic agent for cerebral I/R injury in animal stroke models (Wang et al., 2004; Mori et al., 2008). A previous report showed that continuous infusion of AEPO

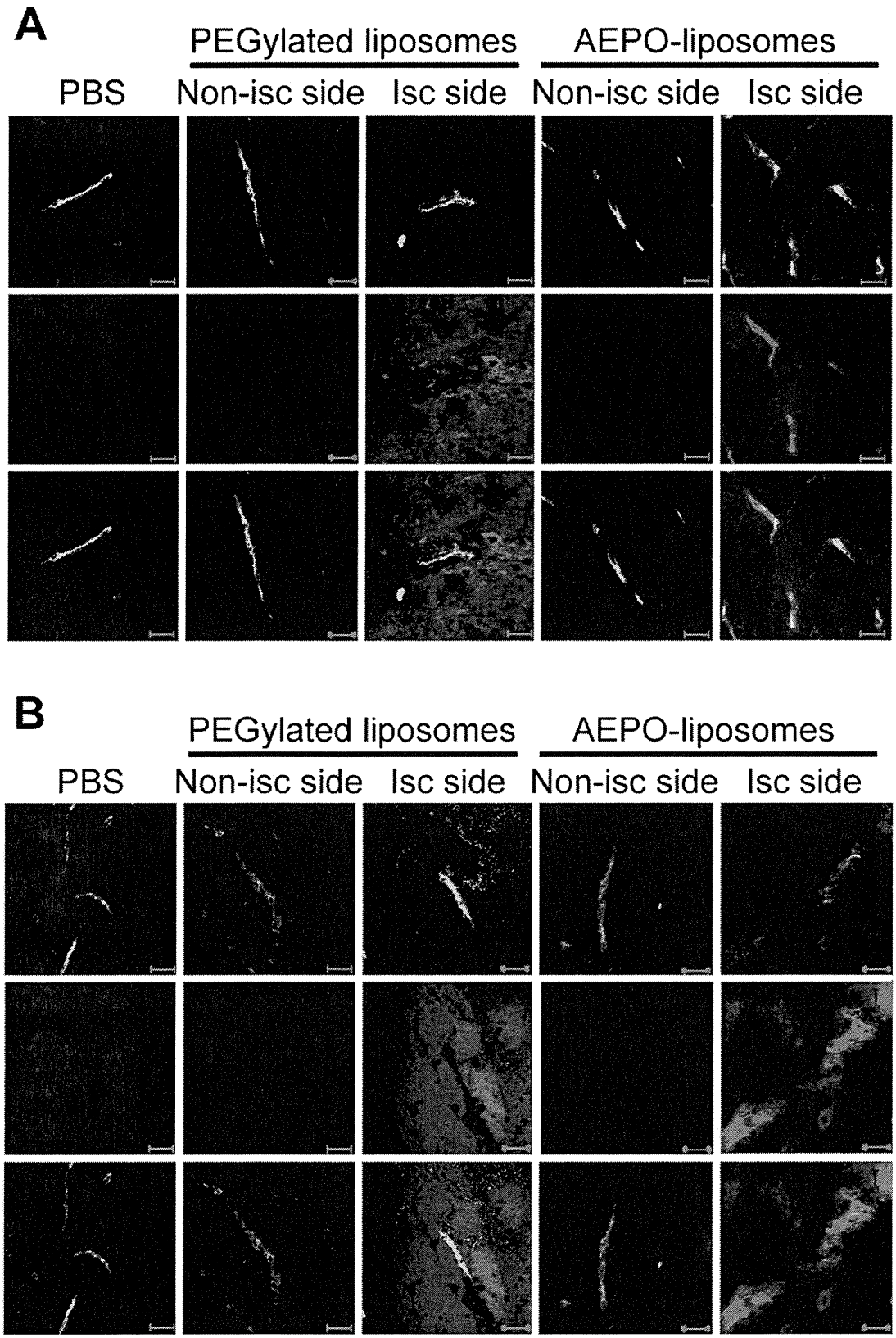

Fig. 2. Leakage of AEPO-liposomes into cerebral parenchyma in the t-MCAO model rats. Frozen sections of the brain in t-MCAO model rats were prepared from each brain slice that was observed using fluorescence by IVIS in Fig. 1. Immunostaining for CD31 was performed on the sections for histological analysis. The fluorescence images in each frozen section were observed by confocal laser scan microscopy. A and B indicate the cerebral distribution of each type of liposome in the frozen brain sections at 3 and 24 h after injection, respectively. Green shows cerebral vessels, red shows the fluorescence of DiI, and yellow shows colocalization of vessels and each liposomes. Isc side indicates ischemic hemisphere; Non-isc side indicates non-ischemic hemisphere. Scale bar, 20 μm.

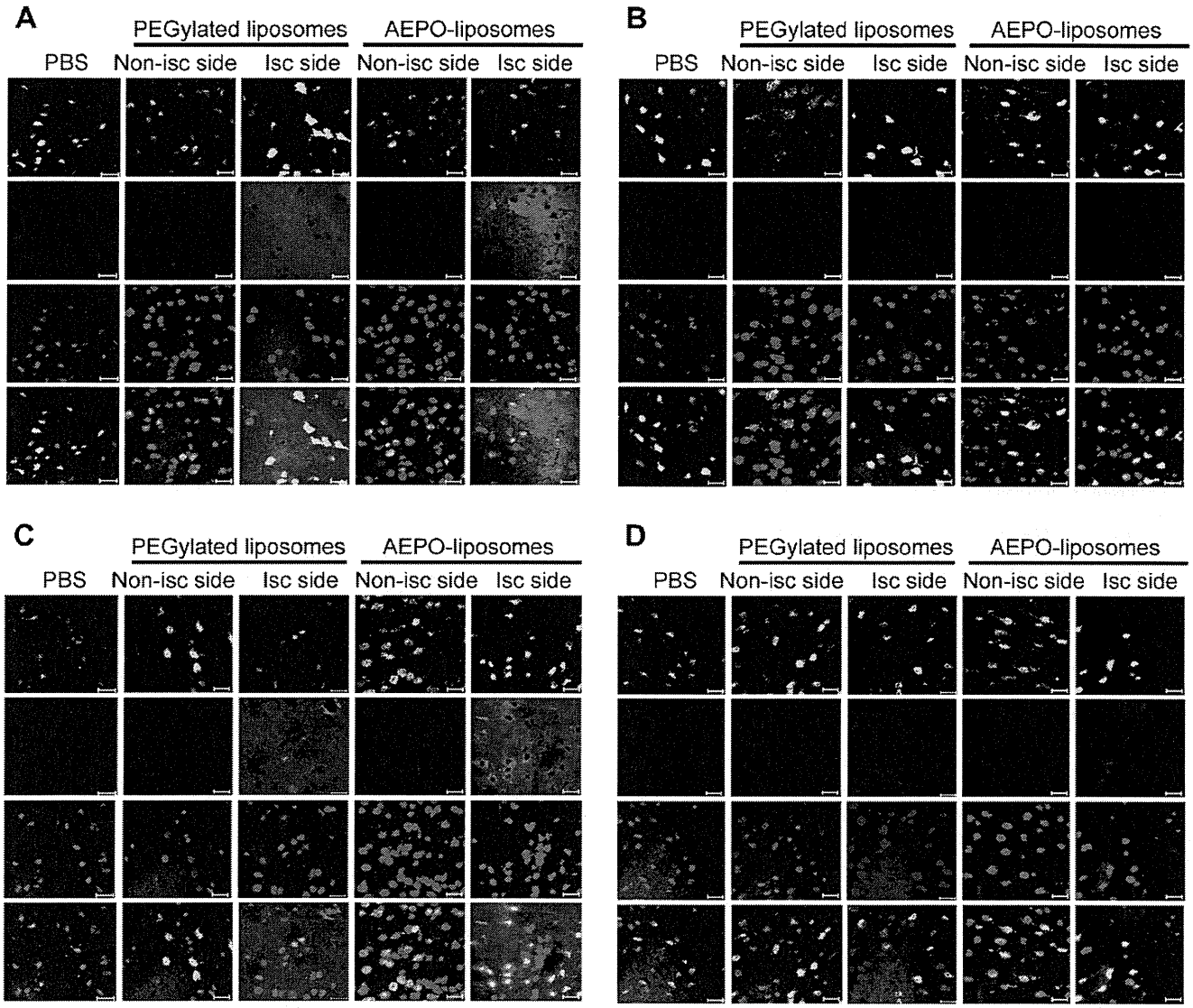

Fig. 3. Accumulation of AEPO-liposomes in neuronal cells. The frozen brain sections were stained with Alexa fluor 488-conjugated monoclonal antibody for NeuN. The fluorescence images in the striatum (A, C) and the cortex (B, D) were observed by confocal laser scan microscopy. A and B indicate the cerebral distribution of each type of liposome in the frozen brain sections 3 h after injection. C and D indicate that at 24 h after injection. Green shows neuronal cells, red shows the fluorescence of DiI, and blue shows nuclei stained with DAPI. Isc side indicates ischemic hemisphere; non-isc side indicates non-ischemic hemisphere. Scale bar, 20 μm.

(20 μg/kg per 24 h for 4 days) ameliorates this injury (Price et al., 2010). Another showed that AEPO (44 μg/kg) administered intravenously at the restoration of the cerebral bloodstream in focal ischemia model rats decreases infarct volume measured at 24 h after the injection (Erbayraktar et al., 2003). In the present study, we have demonstrated that AEPO-modified liposomes at a low dose (8 μg/kg) improved motor function of t-MCAO model rats when the liposomes were given in a single injection via tail vein immediately after reperfusion. This indicates that liposomalization of AEPO allows for a reduction in both the volume and the number of doses of AEPO required by enhancing the accumulation of the agent in the ischemic region.

NeuN immunoreactivity has been reported to decrease following cerebral ischemia (Unal-Cevik et al., 2004), and loss of this immunoreactivity is regarded as an indicator of neuronal injury (Liu et al., 2009), although NeuN immunoreactivity may not correlate with neuronal death. In our histological experiment, NeuN staining in the AEPO-liposome-treated group was more obvious than that in the control and PEGylated liposome-treated group. This suggests that AEPO-liposomes suppress the neuronal damage induced by ischemia and reperfusion due to the activation of the anti-apoptotic signal pathway and the proteins in neuronal cells. Aside from this effect, the vascular protective effects of EPO on ischemic or I/R injury have been shown in several reports (Chong et al., 2002; Santhanam and Katusic, 2006). Additionally, EPO reduces the capillary perfusion failure induced by an I/R event (Contaldo et al., 2007), Therefore, AEPO-liposomes may also protect endothelial cells from ischemia/reperfusion-induced damage and contribute to the suppression of cerebral perfusion deficit in I/R injury. These effects appear to have contributed to the protection of neuronal cells from ischemia/reperfusion-induced damage and to the amelioration of motor functional deficit seen in this study. Previously, we observed that AEPO-liposomes significantly decreased the number of TUNEL-positive cells and infarct volume in t-MCAO model rats, although these results did not conclusively demonstrate that AEPO-liposomes protected neuronal cells. In the present study, we

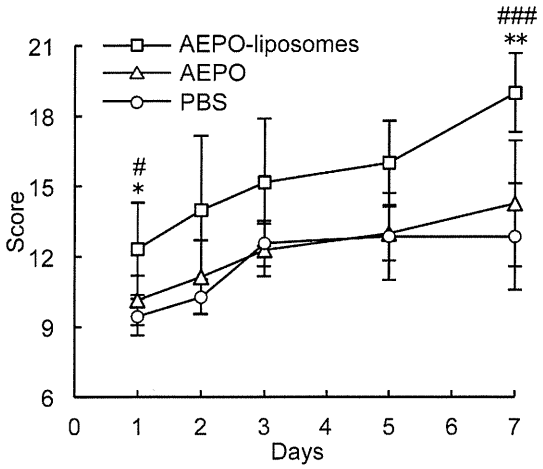

Fig. 4. Amelioration of functional deficit with a single administration of AEPO-liposomes. t-MCAO model rats were injected via a tail vein with PBS, AEPO, or AEPO-liposomes immediately after the start of reperfusion (AEPO dosage of 8 μg/kg). At 1, 2, 3, 5 and 7 days after the injection, the rats were assessed using a 21-point neuropathological scoring system. The details of this system are described in Section 2. Data are presented as the mean ± S.D. ($n = 7$). Significant differences are indicated as follows: # $p < 0.05$, ### $p < 0.001$ vs. control; * $p < 0.05$, ** $p < 0.01$, vs. AEPO.

have presented evidence that AEPO-liposomes do in fact diminish neuronal cell damage in t-MCAO model rats.

5. Conclusion

The present study has demonstrated that AEPO-liposomes administered intravenously immediately after reperfusion leaked into and accumulated in cerebral parenchyma at an early stage after injection, due to a disruption in the integrity of the blood–brain barrier early in the cerebral ischemic event. Consequently, liposomal AEPO protected neuronal cells from damage in the ischemic region. Moreover, a single administration of AEPO-liposomes induced substantial amelioration of motor function deficit in t-MCAO model rats 7 days after reperfusion, indicating that liposomalization is a potentially useful strategy for enhancing the cytoprotective effect of AEPO against cerebral I/R injury.

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REVIEW

Cardioprotection From Ischemia/Reperfusion Injury

– Basic and Translational Research –

Tetsuo Minamino, MD, PhD

Because ischemic heart diseases (IHDs) are a major cause of mortality and heart failure, novel therapeutic approaches are expected to improve the clinical outcomes of patients with IHDs such as acute myocardial infarction and ischemic heart failure. Brief episodes of nonlethal ischemia and reperfusion before sustained ischemia or at the onset of reperfusion can reduce ischemia-reperfusion injury. These ischemic conditioning phenomena are termed "ischemic preconditioning" and "ischemic postconditioning", respectively. Furthermore, brief episodes of nonlethal ischemia and reperfusion applied to the organ or tissue distal to the heart reduce myocardial infarct size, known as "remote ischemic conditioning". The cardioprotection afforded by these ischemic conditionings can be used to treat patients with acute myocardial infarction or cardiac operations. Extensive research has determined that autacoids (eg, adenosine, bradykinin opioid) and cytokines, their respective receptors, kinase signaling pathways and mitochondrial modulation are involved in ischemic conditioning. Modification of these factors by pharmacological agents mimics the cardioprotection by ischemic conditioning and provides a novel therapeutic intervention for IHDs. Here, the potential mechanisms of ischemic conditioning and its "proof-of-concept" translational studies are reviewed. In the near future, large, multicenter, randomized, placebo-controlled, clinical trials will be required to determine whether pharmacological and ischemic conditioning can improve the clinical outcomes of patients with IHDs. (*Circ J* 2012; **76:** 1074–1082)

Key Words: Pharmacological conditioning; Postconditioning; Preconditioning; Proof-of-concept clinical studies; Remote conditioning

Despite the recent advances in therapies, ischemic heart diseases (IHDs) are a major cause of mortality and heart failure in western countries and Japan.[1,2] Thus, developing novel drugs or interventions to improve the clinical outcomes of patients with IHDs is a world-wide unmet medical need. Because myocardial infarct size is recognized as a determinant of acute and long-term prognosis in patients with acute myocardial infarction (AMI),[3] reducing the size of the infarct is a therapeutic goal. Early reperfusion can prevent the myocardial damage due to ischemia and reduce infarct size.[4] This concept was quickly introduced for patients with AMI by the use of primary percutaneous coronary intervention (PCI) and thrombolytic therapy.[5] Although reperfusion can salvage myocardium after sustained ischemia, the reperfusion itself paradoxically induces myocardial injury named "reperfusion injury", which attenuates the benefits of myocardial reperfusion[6,7] (Figure 1).

Over 20 years ago, Murry et al first demonstrated that brief episodes of nonlethal ischemia and reperfusion before sustained ischemia reduce myocardial infarct size, and it was termed "ischemic preconditioning".[8] The infarct-size limiting effects of ischemic preconditioning have been consistently confirmed in many species and different models of ischemia-reperfusion (IR) injury. Brief episodes of nonlethal IR at the onset of reperfusion also reduce myocardial infarct size, known as "ischemic postconditioning.[9] The therapeutic goal of ischemic postconditioning is to attenuate "reperfusion injury" (Figure 1). After these landmark studies, extensive basic investigation has elucidated the underlying mechanisms of ischemic conditioning and led to their translation into the clinical setting by pharmacological agents.[10] Here, I will review the potential mechanisms of ischemic conditioning and the "proof-of-concept" translational studies.

Ischemic Preconditioning

Ischemic preconditioning confers different forms of cardioprotection and can reduce infarct size, lethal arrhythmia and contractile dysfunction.[11–13] Originally, Murry et al hypothesized that ATP preservation during ischemia is the major cardioprotective mechanism underlying ischemic preconditioning, but this hypothesis is not sufficient to explain its cardioprotection.[14] Currently, the major effects of ischemic preconditioning are assumed to prevent cell death due to reperfusion injury. Different factors such as autacoids (eg, adenosine, bradykinin, opioids), their respective receptors, kinase signaling

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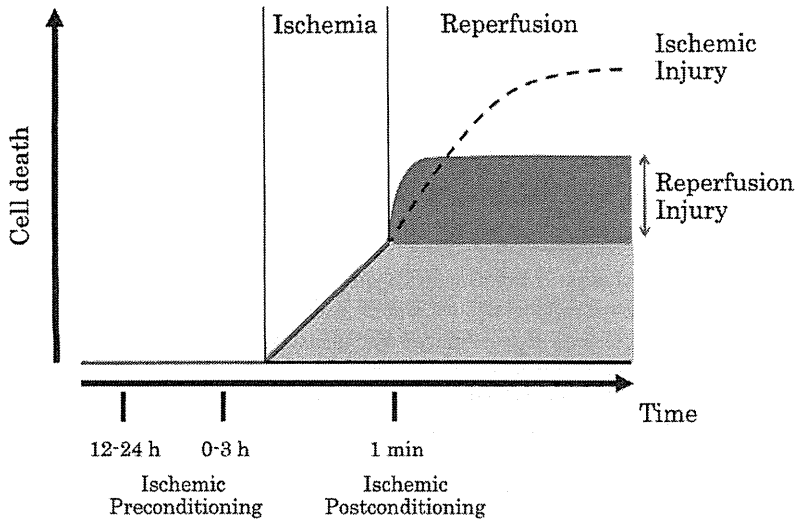

Figure 1. Myocardial ischemia/reperfusion (IR) injury and ischemic conditioning. Myocardial IR injury consists of 2 phases. During ischemia, cell death within the myocardial risk areas increases as the duration of ischemia extends if the ischemic myocardium is not reperfused (broken black curve). Although reperfusion can salvage myocardium after sustained ischemia, the reperfusion itself paradoxically induces myocardial injury during the early stage of reperfusion (red curve). "Early" and "delayed" ischemic preconditioning may reduce both myocardial ischemia and reperfusion injury. Ischemic postconditioning or pharmacological conditioning reduces the reperfusion injury (blue curve). (Adapted from Garcia-Dorado and Piper[7] with permission.)

pathways and mitochondria modulation are implicated in the cardioprotective effects of ischemic preconditioning (Figure 2). Nonlethal ischemia results in the production of endogenous autacoids such as adenosine, opioids, bradykinin. These autacoids initiate numerous signaling pathways that activate protein kinases through their respective receptors (Figure 2). These cardioprotective signaling pathways, including extracellular-regulated kinase (ERK)1/2, phosphatidylinositol 3 kinase (PI3K)/Akt, protein kinase C and protein kinase G, lead to the inactivation of mitochondrial glycogen synthase kinase-3β (GSK-3β). The inactivation of GSK-3β inhibits the opening of the mitochondrial permeability transition pore (mPTP), which plays a crucial role in myocardial necrosis.[15,16] Reactive oxygen species (ROS) production in mitochondria, where the mitochondrial ATP-dependent potassium channels play an essential role, is also involved in the cardioprotective mechanisms of ischemic preconditioning.[17] Although these findings are consistently observed in experimental models, applying ischemic preconditioning in the clinical setting is restricted to scheduled cardiac operation and elective PCI.[18] A meta-analysis showed that ischemic preconditioning may provide additional myocardial protection over cardioplegia alone.[19] However, cardiovascular surgeons do not like to repeatedly clamp and unclamp the aorta in patients with advanced atherosclerosis.

The cardioprotective effects of ischemic preconditioning disappear 2–3 h after the onset of the preconditioning insult, but reappear 24 h later. This phenomenon is recognized as "delayed" ischemic preconditioning.[20,21] A major difference in the cardioprotective mechanisms of early and delayed preconditioning is that early ischemic preconditioning results in the modification or turnover/translocation of existing molecules,[15,22] whereas delayed ischemic preconditioning is exerted by newly synthesized cardioprotective proteins. The triggers and mediators of early and delayed ischemic preconditioning are largely common and lead to the activation of transcriptional factors (Figure 2). They transcribe the de novo synthesized proteins involved in delayed ischemic preconditioning, including manganese superoxide dismutase, heat stress proteins and inducible nitric oxide synthase.[21] A potential clinical example of delayed ischemic preconditioning is "pre-infarct angina" by which patients who have suffered from repeated episodes of angina can preserve postischemic left ventricular function.[23] However, the clinical application of delayed ischemic preconditioning has not been fully investigated.

Ischemic Postconditioning

In 2003, Zhao et al demonstrated that brief episodes of coronary occlusion and reperfusion at the onset of reperfusion following 60 min of coronary occlusion reduced myocardial infarct size by 40% in canine hearts.[9] The protocols for ischemic postconditioning have been extensively investigated and the cardioprotective effects afforded by ischemic postconditioning have been confirmed in many species, including humans.[24,25] At the same time, the existence of reperfusion injury is strongly supported by the cardioprotection afforded by the intervention during reperfusion. One proposed mechanism through which ischemic postconditioning attenuates reperfusion injury is the prevention of rapid changes in intracellular pH and robust ROS generation. In the ischemic/reperfused myocardium, the ionic environment dramatically changes. Within a few minutes of myocardial ischemia, the interstitial and intracellular pH values rapidly decrease due to the accumulation of protons. Upon reperfusion, these interstitial protons are promptly washed out and intracellular low pH is corrected through the sarcolemmal Na^+/H^+ exchanger, which results in a massive Na^+ influx.[26] Intracellular Na^+ accumulation stimulates the passive, inverted action of the sarcolemmal Na^+/Ca^{2+} exchanger and in turn allows intracellular Ca^{2+} overload, which causes myocardial cell death or myocardial contractile dysfunction.[27,28] Therefore, the rapid normalization of intracellular pH enhances myocardial damage in the early stage of reperfusion and a gradual correction of low intracellular pH by acidic reperfusion would be cardioprotective through inhibition of the opening of mPTP,[29] preventing the activation of Ca^{2+}-dependent protease[30] and reducing the gap junction communication involved in spreading cell death.[31] The cardioprotective effects of ischemic postconditioning are associated with the maintenance of low intracellular pH during reperfusion and are comparable to the effects of acidic reperfusion.[32] Furthermore, during the early stage of reperfusion, there is robust ROS production in vascular endothelium, cardiomyocytes and mito-

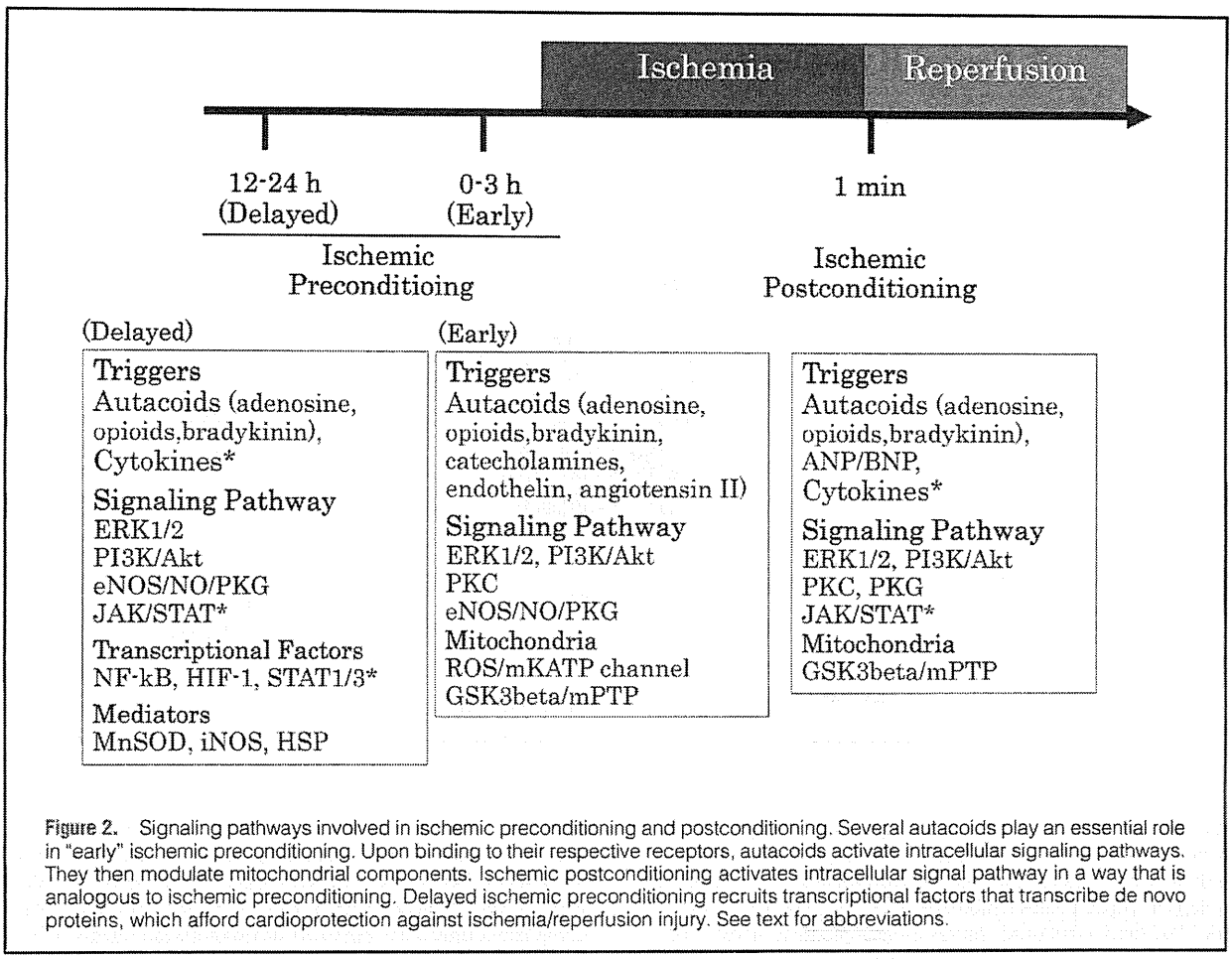

Figure 2. Signaling pathways involved in ischemic preconditioning and postconditioning. Several autacoids play an essential role in "early" ischemic preconditioning. Upon binding to their respective receptors, autacoids activate intracellular signaling pathways. They then modulate mitochondrial components. Ischemic postconditioning activates intracellular signal pathway in a way that is analogous to ischemic preconditioning. Delayed ischemic preconditioning recruits transcriptional factors that transcribe de novo proteins, which afford cardioprotection against ischemia/reperfusion injury. See text for abbreviations.

Table 1. Clinical Trials of Ischemic/Pharmacological Conditioning in Patients With STEMI

	Outcome	Reference
Conditioning		
Postconditioning	Decrease (IS), improved LVEF at 12 months	25, 39
Remote conditioning	Decrease (IS)	86
Pharmacological agents		
Adenosine	Decrease (IS)	44
Atrial natriuretic peptide	Decrease (IS), improved LVEF at 6–12 months	49
Cyclosporine A	Decrease (IS), improved LVEF at 6 months	62
Erythropoietin		
High dose	No change (IS, LVEF)	52, 53, 53
Low dose	Improved LVEF at 6 months	55, 56
Nicorandil	No change (IS)	49
Statin	No change (IS)	67
Protein kinase C inhibitor	No change (IS, LVEF)	68

STEMI, ST-elevation myocardial infarction; IS, infarct size; LVEF, left ventricular ejection fraction.

chondria. ROS generation is suppressed in the postconditioned heart.[33,34]

In addition to the effects of ischemic postconditioning on ionic changes and ROS production, ischemic postconditioning activates intracellular signal transduction in a way that is analogous to ischemic preconditioning. Autacoids (eg, adenosine, bradykinin and opioids), natriuretic peptides (atrial and brain natriuretic peptides) and cytokines play a crucial role in postconditioning[35] (Figure 2). These autacoids activate a kinase signaling pathway known as the reperfusion injury risk kinases (RISK) pathway, which consists of the PI3K/Akt and ERK1/2 pathways.[36] The activation of RISK pathway inactivates GSK3β, which inhibits mPTP opening at reperfusion. The inhibition of mPTP opening is the final common target

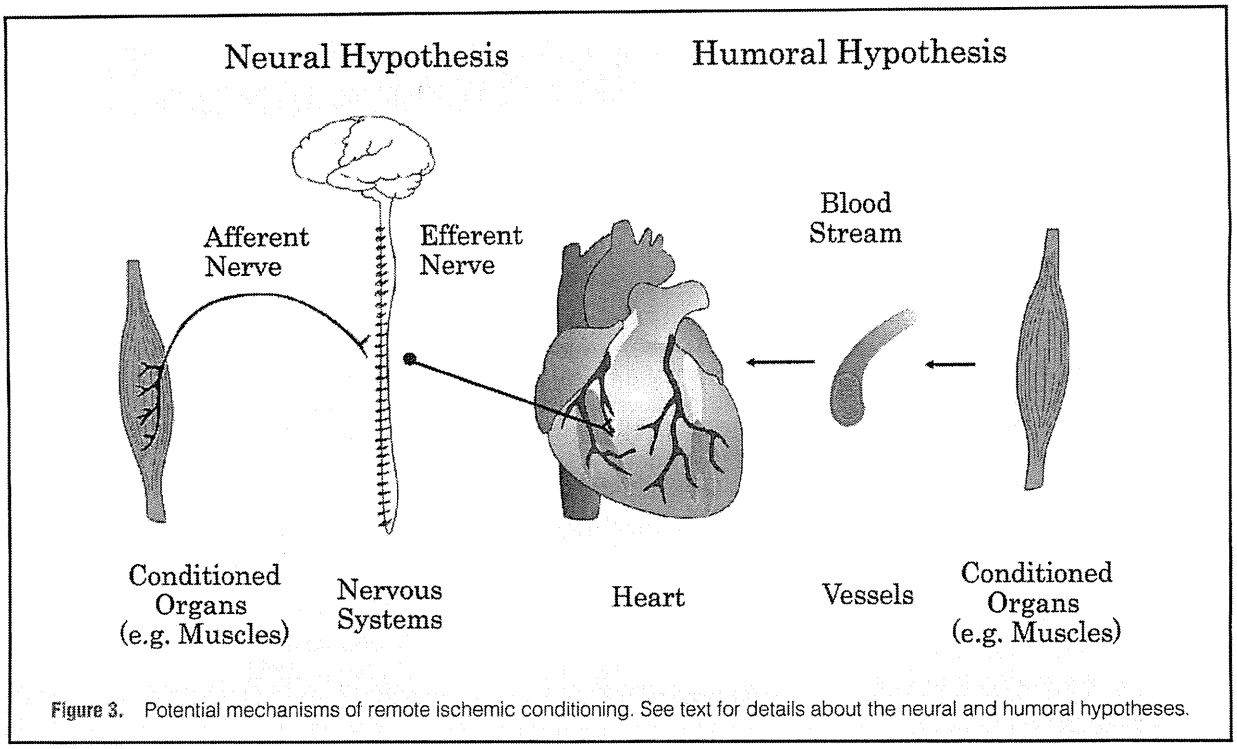

Figure 3. Potential mechanisms of remote ischemic conditioning. See text for details about the neural and humoral hypotheses.

through which the signaling pathways can protect against necrosis.[15] Activation of the JAK-STAT pathway by cytokines has also been implicated in the cardioprotective effects induced by ischemic postconditioning.[37,38] This pathway is named the "survivor activating factor enhancement (SAFE)" pathway; however, it is not fully understood how SAFE pathway is involved in the cardioprotection afforded by ischemic postconditioning. In contrast to ischemic preconditioning, ischemic postconditioning can be easily applied in patients with AMI undergoing primary PCI. A small number of "proof-of-concept" studies have showed that a postconditioning procedure reduced myocardial infarct size[25] and improved left ventricular ejection fraction (LVEF) at 1 year.[39] Prospective and randomized studies are now ongoing to evaluate the infarct-size-limiting effects of ischemic postconditioning in patients with ST-segment elevation myocardial infarction (STEMI) who are undergoing primary PCI.[40,41]

Coronary blood flow must be interrupted in order to apply ischemic postconditioning, which increases the time required for the procedure and could potentially cause atherosclerotic emboli. Pharmacological manipulation of autacoids, their receptors, kinase signaling pathways and modulation of the mPTP opening, all of which are involved in ischemic postconditioning, could be easily utilized to treat patients with AMI undergoing primary PCI (Table 1). Adenosine is a representative autacoid that is involved in both ischemic preconditioning and postconditioning, and its administration at the onset of reperfusion provides myocardial protection from IR injury in animal models.[42] The results of a randomized, double-blinded, placebo-controlled multicenter trial of a 3-h adenosine infusion as an adjunct to thrombolytic reperfusion in the treatment of anterior wall STEMI (AMISTAD-II) have been reported.[43,44] Clinical outcomes, including new congestive heart failure, first re-hospitalization for chronic heart failure and death, were not significantly improved with adenosine administration, although infarct size was reduced in response to a high-dose infusion.[44] Post-hoc analysis revealed that adenosine infusion within the first 3.17 h after the onset of anterior wall STEMI enhanced early and late survival, and reduced the composite clinical endpoints of death or chronic heart failure at 6 months.[45] In the J-WIND study, a multicenter, randomized clinical trial was conducted to test the acute effect of either the sarcolemmal KATP channel opener, nicorandil, or the recombinant human atrial natriuretic peptide (ANP), carperitide, as an adjunct to successful PCI.[46,47] The administration of carperitide, but not nicorandil, produced a small but significant 15% reduction in myocardial infarct size and an improvement in LVEF.[48] Experimental studies showed that erythropoietin, a hematopoietic cytokine, reduces myocardial infarct size and prevents cardiac remodeling in the chronic stage.[49,50] In patients with STEMI, the administration of a high dose of erythropoietin did not improve LVEF or reduce infarct size;[51–53] however, the use of erythropoietin was related to fewer major adverse cardiovascular events in 1 study.[52] In contrast, a low dose of erythropoietin appears to be cardioprotective.[54,55] Platelet activation by a high-dose of erythropoietin and the existence of an optimal dose for limiting infarct size will explain the dose-dependent discrepancy of erythropoietin-induced cardioprotection.[56–58] Therefore, a large-scale, double-blinded, placebo-controlled study is being conducted to clarify the effects of a low dose of erythropoietin on cardiac function after 6 months in patients with AMI who received successful PCI in Japan (UMIN000005721). Pharmacological inhibitors of mPTP reduce myocardial infarct size in experimental models.[59,60] Recently, Piot et al demonstrated that the mPTP inhibitor, cyclosporine A, administered as an intravenous bolus immediately before coronary artery reperfusion by PCI, resulted in a 40% reduction in enzyme release and prevented cardiac remodeling.[61,62] The data are promising and large, multicenter, randomized, placebo-controlled, clinical trials are

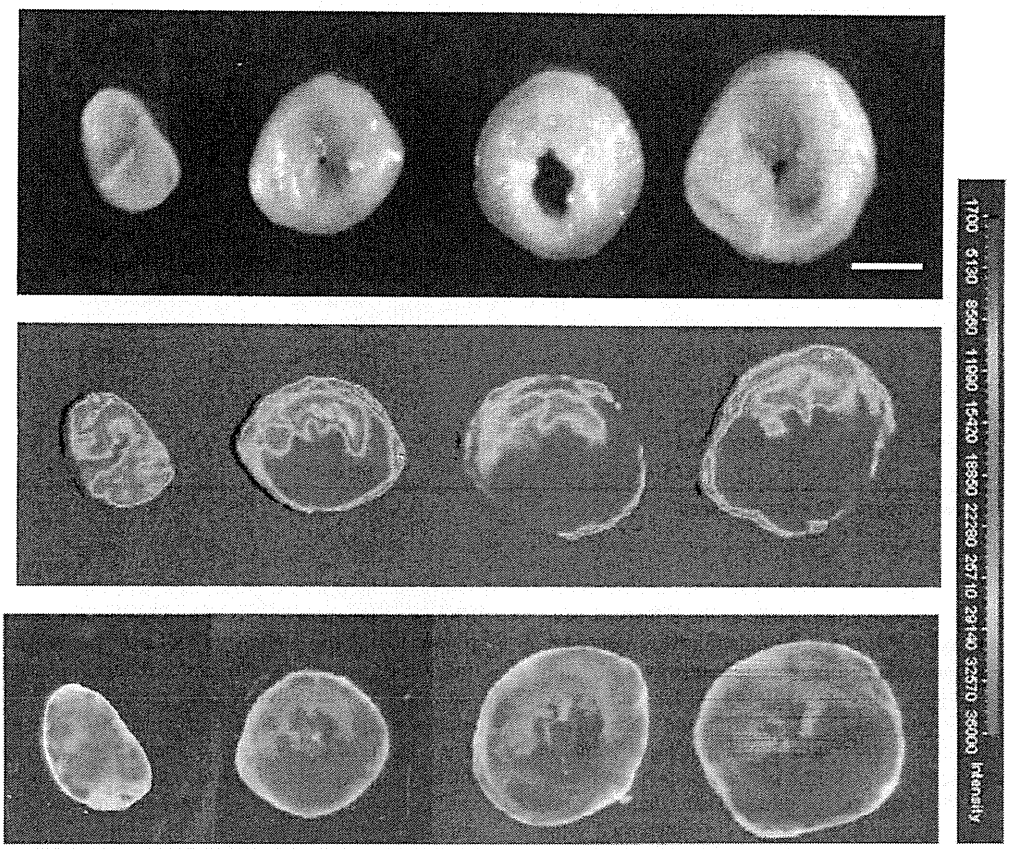

Figure 4. Detection of fluorescent-labeled nano-sized particles in ischemic/reperfused myocardium. Representative photographs at 3 h after myocardial infarction of hearts from rats that received fluorescent-labeled nano-sized liposomes. (Upper panels) Short axis cross-section of each heart (bright field). (Middle panels) Same sections analyzed under a fluorescence microscope. (Lower panels) Same sections stained with triphenyltetrazolium chloride. Note that high fluorescent areas correspond to the infarcted and border areas. Scale bar=5 mm. (Adapted from Takahama and Minamino[60] with permission.)

required to elucidate the improvement in clinical outcomes.

To date, most clinically tested agents that induce cardioprotection, except adenosine and cyclosporine A, have failed to reduce infarct size in the clinical setting[63–65] (Table 1). These negative results of "proof-of-concept" studies can be attributed to multiple factors.[66–68] Pharmacological intervention as an adjunct to primary PCI is estimated to be effective for only 25% of AMI patients with an infarct size larger than 20% of the left ventricle and who have adverse symptoms.[68,69] Proper patient selection is required to evaluate the benefit of pharmacological conditioning. In addition to the ischemic risk zone, infarct size is also determined by the duration of ischemia. If the duration of ischemia extends beyond 60 min, the infarct-size limiting effects of ischemic postconditioning are largely attenuated in experimental models.[24] Thus, some proportion of patients in the study may have already been beyond the appropriate time-window within which myocardial salvage can be achieved. Another important point is the timing of drug administration. Reperfusion injuries such as robust ROS production, Ca^{2+} overload and mPTP opening occur within the first few minutes of myocardial reperfusion.[70] In the cyclosporine A study, this compound was administered just before coronary artery reperfusion by PCI, whereas most drugs were administered after successful reperfusion therapy. Finally, we need to consider confounders such as sex and age and comorbidities such as hypercholesterolemia, diabetes and hypertension, which are not present in animal studies as compared with clinical reality.[71] For example, pharmacological postconditioning with cyclosporine A failed to provide cardioprotection in the prediabetic but normoglycemic heart of Zucker obese rats.[72] Erythropoietin fails to exert infarct-size limiting effects in hypertensive hypertrophied hearts.[73] Thus, both appropriate study design and execution are required to translate future novel cardioprotecive agents into the clinical setting.[66,67]

Remote Ischemic Conditioning

Brief episodes of nonlethal ischemia and reperfusion applied to the organ or tissue distal to the heart reduce myocardial infarct size, which is known as "remote ischemic conditioning".[74,75] Transient upper or lower limb ischemia is a simple noninvasive stimulus with important potential clinical applications and high-cost performance. Furthermore, the remote ischemic conditioning procedure can be applied before and during sustained ischemia[76] and at the onset of reperfusion.[77] An experimental study showed that the infarct-size-limiting effects

Table 2. MicroRNAs Involved in Hypertrophy/Failure and Ischemia/Reperfusion

MicroRNA	Hypertrophy/ failure[94]	Ischemia[95]	Ischemic preconditioning[95]	Ischemic postconditioning[97]
miR-1	↓	↓	↑	↑
miR-9	↓	ND or NC	ND or NC	ND or NC
miR-17	ND or NC	↓	ND or NC	ND or NC
miR-21	ND or NC	↓	↑	ND or NC
miR-23	↑	ND or NC	ND or NC	ND or NC
miR-24	ND or NC	↓	↑	ND or NC
miR-26	↓	ND or NC	ND or NC	ND or NC
miR-30	↓	ND or NC	ND or NC	ND or NC
miR-92a	ND or NC	↑	ND or NC	ND or NC
miR-126	ND or NC	↓	ND or NC	ND or NC
miR-133	↓	↓	ND or NC	↑
miR-138	ND or NC	↓	ND or NC	ND or NC
miR-155	ND or NC	↓	ND or NC	ND or NC
miR-199a	↑	↑	ND or NC	ND or NC

NC, not changed; ND, not determined.

of remote conditioning are comparable to the effects of ischemic postconditioning.[78] It remains unclear how remote ischemic conditioning exerts cardioprotection; however, 2 major hypotheses have been proposed (Figure 3). The neural hypothesis states that autacoids released from the ischemic remote organ affect the local afferent neural pathway, which in turn, activates the efferent neural pathways to trigger end-organ protection. The humoral hypothesis states that autacoids released from the ischemic remote organ are transported to the end organ, resulting in the activation of kinase signaling pathways in the end organ. Remote ischemic preconditioning is associated with the activation of PI3K/Akt[79] or STAT5[80] in the heart. The clinical application of remote ischemic conditioning was tested in patients undergoing CABG, but the results were inconsistent.[81,82] Multicenter randomized double-blinded controlled clinical trials to clarify the effects of remote conditioning on clinical outcomes and the incidence of atrial fibrillation in patients with CABG are now ongoing.[83,84] Recently, remote ischemic conditioning before hospital admission was shown to increase myocardial salvage measured by myocardial perfusion imaging and have a favorable safety profile in patients with AMI.[85]

Future Directions

Recent advances in nanotechnology open up new possibilities in the development of drug delivery systems (DDS) for the treatment of patients with IHDs. DDS enhance the therapeutic concentrations of the drugs in diseased tissues and reduce the side effects.[86] Nano-sized particles can passively accumulate in tissues where vascular permeability is enhanced.[87] This concept is particularly applicable for developing anti-cancer and anti-inflammatory drugs, because vascular permeability is enhanced in tumors and inflamed tissues.[88,89] In the rat IR model, after the intravenous administration of fluorescence-labeled nano-sized particles, high fluorescent areas corresponded to infarcted and border, but not non-ischemic areas in the rat heart, suggesting that the nano-sized particles specifically accumulated in the myocardial infarct and border, but not in non-ischemic tissue (Figure 4).[90] These findings suggest that ischemic/reperfused myocardium has enhanced permeability and that nano-sized liposomes can accumulate there. In a rat IR model, the intravenous administration of nano-sized liposomes containing adenosine, but not free adenosine, at the onset of reperfusion significantly reduced myocardial infarct size and lethal arrhythmia during repefusion.[91] Encapsulated adenosine in nano-sized liposomes enhances the cardioprotective effects of adenosine and attenuates the hypotension induced by the systemic administration of adenosine. Targeting cardioprotective agents to ischemic/reperfused tissues using nano-sized liposomes may maximize the effect of the drug and minimize its side effects.[92,93] Liposomes are a promising DDS for developing new treatments for patients with AMI who have undergone successful PCI.[92]

MicroRNAs have emerged as important regulators of gene expression that affects cardiovascular function.[94] MicroRNAs regulate gene expression through the degradation and translational inhibition of target messenger RNAs. IR stimuli alter the expression of microRNAs.[94] Recent studies revealed that microRNAs are implicated in cardiac pathology including hypertrophy and failure[95] and IR injury[96] (Table 2). Therefore, microRNAs are novel promising therapeutic targets for IHDs. Cheng et al demonstrated that ischemic preconditioning up-regulates microRNA 21, which protects the heart against IR injury.[97] Yin et al showed that an injection of microRNAs induced by ischemic preconditioning in the heart exerted cardioprotective effects against IR injury, which is comparable to that induced by the late phase of ischemic preconditioning.[98] With advances in nanotechnology, microRNAs are potentially good candidates for targeting ischemic/reperfused myocardium with nano-sized liposomes.[99]

Perspectives

Basic and translational research examining the therapeutic potential of ischemic conditioning are now actively ongoing. We need to continue to investigate the molecular mechanisms of ischemic conditioning, improve DDS, design study protocols to consider the timing and dose of drug administration and select patients who can benefit from pharmacological intervention. These efforts will lead to solving the unmet medical need for therapeutic drugs and interventions that improve the clinical outcomes of patients with IHD.

Acknowledgments

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