Regular Article

Weak Electric Current Treatment to Artificially Enhance Vascular Permeability in Embryonated Chicken Eggs

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Technologies that overcome the barrier presented by vascular endothelial cells are needed to facilitate targeted delivery of drugs into tissue parenchyma by intravenous administration. We previously reported that weak electric current treatment (ET: 0.3–0.5 mA/cm^2) applied onto skin tissue in a transdermal drug delivery technique termed iontophoresis induces cleavage of intercellular junctions that results in permeation of macromolecules such as small interfering RNA and cytosine-phosphate-guanine (CpG) oligonucleotide through the intercellular space. Based on these findings, we hypothesized that application of ET to blood vessels could promote cleavage of intercellular junctions that artificially induces increase in vascular permeability to enhance extravasation of drugs from the vessels into target tissue parenchyma. Here we investigated the effect of ET (0.34 mA/cm^2) on vascular permeability using embryonated chicken eggs, which have blood vessels in the chorioallantoic membrane (CAM), as an animal model. ET onto the CAM of the eggs significantly increased extravasation of intravenously injected calcein (M.W. 622.6), a low molecular weight compound model, and the macromolecule fluorescein isothiocyanate (FITC)-dextran (M.W. 10000). ET-mediated promotion of penetration of FITC-dextran through vascular endothelial cells was also observed in transwell permeability assay using monolayer of human umbilical vein endothelial cells without induction of obvious cellular damage. Confocal microscopy detected remarkable fluorescence derived from injected FITC-dextran in blood vessel walls. These results in embryonated chicken eggs suggest that ET onto blood vessels could artificially enhance vascular permeability to facilitate extravasation of macromolecules from blood vessels.

Key words embryonated chicken egg; weak electric current treatment; macromolecule; blood vessel; vascular permeability; drug delivery

INTRODUCTION

Targeted delivery of drugs such as macromolecules and nanoparticles into parenchyma of normal tissue following intravenous injection is a significant challenge in drug delivery since vascular-endothelial barriers such as the blood–brain barrier (BBB) strictly limit the penetration of substances through paracellular routes.[1)] Under certain disease conditions (*e.g.*, cancer, ischemic stroke), increased permeability of disintegrated blood vessels allows leakage of macromolecules and nanoparticles into tissue parenchyma.[2–4)] In particular, the enhanced permeability and retention (EPR) effect is known to be a useful phenomenon for passive targeting of macromolecules and nanoparticles into tumor tissues.[5)] The EPR effect is based on the presence of highly permeable angiogenic vessels and immature lymphatic systems in tumors. However, it was previously reported that the magnitude of the EPR effect can vary across some species and cancer types.[6,7)] Multiple approaches to enhance the EPR effect have been attempted such as use of nitric oxide to augment vascular permeability *via* vasodilation, as well as a combination of bubble liposomes and ultrasound.[8,9)] Techniques to artificially enhance vascular permeability of normal blood vessels and angiogenic vessels in tumors could be valuable to facilitate delivery of drugs to the parenchyma.

Focusing on a physical technique to apply weak electric current, termed iontophoresis (IP), we previously reported that non-invasive and efficient transdermal delivery of macromolecules and nanoparticles could be achieved *via* IP using weak electric current treatment (ET; 0.3–0.5 mA/cm^2).[10)] We successfully performed intradermal delivery of small interfering RNA (siRNA), cytosine-phosphate-guanine (CpG) oligonucleotides, and nano-sized liposomes using IP, resulting in exertion of their functionalities *in vivo*.[11–13)] In analyses of the underlying mechanism of IP-mediated permeation of macromolecules and nanoparticles into skin tissues, we revealed that Ca^{2+}-mediated activation of intracellular signaling is elicited in skin cells by ET.[14)] Moreover, we showed that expression of the gap-junction protein connexin 43 (Cx43) is decreased and that depolymerization of filamentous actin, which is associated with tight junction, is induced. Together these events contribute to intercellular junction cleavage. However, since the influence of ET on cleavage of intercellular junction was evaluated in normal human epidermal keratinocyte (NHEK) and human Caucasian colon adenocarcinoma Caco-2 cells,[14)] the effect of ET on permeability of blood vessels is unclear. Based on our previous findings, we hypothesized that the effect of ET-mediated intercellular junction cleavage might also be artificially induced onto vascular endothelial cells. Then, it is expected that vascular permeability would transiently be increased by ET, which allows for leakage of therapeutic macromolecules through blood vessels to target tissue parenchyma.

Investigation of how ET can affect vascular permeability *in vivo* using rodent models such as mice and rats is challenging because few blood vessels are present on the surface of the

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body. Here we used embryonated chicken eggs as an alternate *in vivo* animal model, as these eggs have accessible vessels in the chorioallantoic membrane (CAM).[15] Agents can be administered intravenously to vessels in CAM of embryonated chicken eggs similarly to injection into rodents. Notably, previous studies reported that the activity and pharmacokinetics of several candidate therapeutic compounds were similar between eggs and animal studies.[16] Indeed, using embryonated chicken eggs as an alternate model, it was reported that the *in vivo* activities of antiangiogenic and radiosensitizing agents could successfully be evaluated in eggs inoculated with tumor cells.[17–19] The *in vivo* antioxidative activity of redox nanoparticles was also previously assessed.[20] Based on these findings, we considered that the influence of ET on vascular permeability and leakage of intravenously injected agents from blood vessels could be evaluated using the embryonated chicken eggs having the vascular structure in the CAM.

In the present study, to evaluate vascular permeability and the effect of ET in embryonated chicken eggs, we used calcein (M.W. 622.6) and fluorescein isothiocyanate (FITC)-dextran (M.W. 10000) to represent movement of low molecular weight compounds and macromolecules, respectively. Through these examinations, we investigated the possibility for application of ET to deliver therapeutic molecules to target tissue parenchyma *via* enhancement of vascular permeability.

MATERIALS AND METHODS

Ethical Issues All experiments using embryonated chicken eggs were evaluated and approved by the Animal and Ethics Review Committee of Tokushima University (No. T29-94).

Preparation of Embryonated Chicken Eggs Embryonated chicken eggs (Goto Hatchery, Inc., Gifu, Japan) were placed on an egg inverter (Shikoku Riken, Tokushima, Japan) and incubated in a humidified incubator at 37.6 °C for 15 d. On day 16, the eggs were candled with a halogen light to mark blood vessels on the CAM. Rectangular windows (one 0.5×2 cm and two 2×2 cm) were marked around the blood vessels and the eggshell was cut using a grinder and then removed with tweezers. The smaller window was used for intravenous administration of calcein (Dojindo, Kumamoto, Japan) or FITC-dextran (M.W. 10000; Tokyo Chemical Industry, Tokyo, Japan), whereas the two larger windows served as attachment points for Ag-AgCl electrodes (3M Health Care, Minneapolis, MN, U.S.A.) to perform ET to the CAM. The prepared eggs were used for the experiments described below.

Weak ET Nonwoven fabric ($0.5\,cm^2$) moistened with phosphate buffered saline (PBS) (−) was attached to Ag-AgCl electrodes having a surface area of $0.5\,cm^2$. The Ag-AgCl electrodes fitted with the nonwoven fabric containing PBS (−) were connected to the cathode and anode of a power supply (TTI ellebeau, Inc., model TCCR-3005, Tokyo, Japan). After attaching the electrodes onto the exposed CAM, ET was performed for 1 h at a constant current of $0.34\,mA/cm^2$ (0.17 mA).

Evaluation of Extravasation of Fluorescence-Labeled Tracers One hour after 1 h ET, liquid paraffin was dropped onto the eggshell membrane to allow visualization of the blood vessels under the membrane. Then, 100 μL of calcein (336.2 μg/mL in PBS) or 100 μL of FITC-dextran (M.W. 10000; 10 mg/mL in PBS) was intravenously administered to the eggs with a 30 G needle (Misawa Medical Industry, Ibaraki, Japan). At 1, 3, 6, and 24 h after sample injection, 100 μL of chorioallantoic fluid was collected with a syringe fitted with a 30G needle and fluorescence of the collected chorioallantoic fluid was measured using a Tecan Infinite M200 microplate reader (Salzburg, Austria).

Cell Culture The human umbilical vein endothelial cells (HUVECs) were purchased from PromoCell GmbH (Heidelberg, Germany). HUVECs were cultured in endothelial growth medium (EGM-2; PromoCell GmbH) composed of endothelial basal medium (EBM-2; PromoCell GmbH), 100 U/mL penicillin-100 μg/mL streptomycin (Gibco, MA, U.S.A.), and endothelial cell growth medium-2 supplement pack (PromoCell GmbH). The cells were cultivated at 37 °C in a 5% CO_2 incubator. The passage of HUVECs used in experiments was between 3 and 5.

Transwell Permeability Assay HUVECs were seeded onto a 6.5-mm Transwell plate with 3-μm pore polycarbonate membrane inserts (Corning, Kennebunk, ME, U.S.A.) at a density of 3×10^5 cells/insert. The inserts were pre-incubated with 0.1% gelatin in PBS for 1 h at 37 °C, and with EGM-2 overnight before cell seeding. The media in both upper and bottom compartments were replaced with fresh medium 48 h after seeding. At 72 h after seeding, the media in both compartments were removed, and EBM-2 containing 5 μM FITC-dextran (M.W. 10000) and fresh EBM-2 were added into the upper and lower compartment, respectively. Then, Ag-AgCl electrodes with $0.5\,cm^2$ surface areas were placed in upper compartments of the inserts, and the cells were exposed to ET with a constant current of $0.34\,mA/cm^2$ (0.17 mA) for 15 min. The experimental condition of ET in this study was the optimized condition for *in vitro* ET employed in our previous reports.[14,21,22] At 1, 3, and 6 h after ET, the media in the bottom compartment were collected, and FITC fluorescence was measured with a Tecan Infinite M200 microplate reader. After collection of the media 6 h after ET, the cells were trypsinized and the number of cells was counted after staining with trypan blue.

Hematoxylin–Eosin (H&E) Staining For histological analysis by H&E staining, the CAM and blood vessels of embryonated eggs were dissected 6 h after injection of FITC-dextran (M.W. 10000) that was followed by 1 h ET. The dissected samples were washed in PBS before embedding in optimal cutting temperature (OCT) compound (Sakura Finetek, Tokyo, Japan) and freezing in a dry ice/ethanol bath. The frozen sections were cut into 10 μm-thick sections with a cryostat (CM3050S; Leica Biosystems, Tokyo, Japan). The sections were fixed with 4% paraformaldehyde (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) for 10 min, washed with PBS and stained with Mayer's hematoxylin solution (FUJIFILM Wako Pure Chemical Corporation) for 10 min at room temperature, followed by staining with 1% eosin (FUJIFILM Wako Pure Chemical Corporation) for 1 min. The samples were then dehydrated with 80–100% ethanol, cleared with xylene, and mounted with hydrophobic mounting medium (Entellan New, Merck Millipore, Burlington, MA, U.S.A.) for observation with a phase contrast microscope (BZ-9000, Keyence, Osaka, Japan).

Confocal Laser Scanning Microscopy To observe fluorescence of calcein and FITC-dextran, 10 μm-frozen sections of the CAM and blood vessels prepared as described above were mounted with Perma Fluor Aqueous Mounting Medium

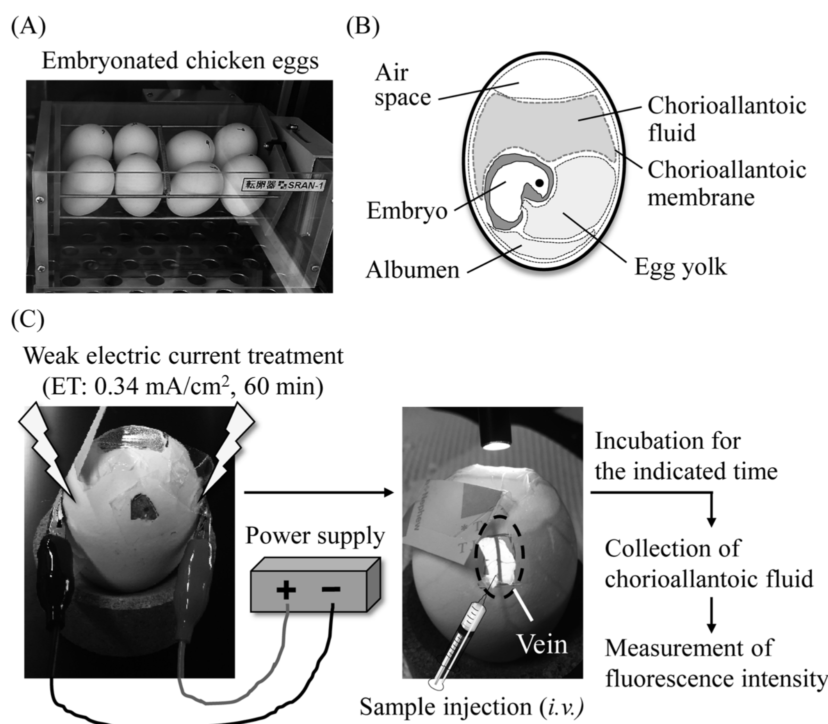

Fig. 1. Scheme of Weak Electric Current Treatment (ET) onto Embryonated Chicken Eggs

(A) The images of embryonated chicken eggs placed on an egg inverter in a humidified incubator. (B) The internal structure of the embryonated chicken eggs. (C) Graphic display of ET (0.34 mA/cm^2 for 1 h) onto embryonated chicken eggs with a power supply connected to Ag-AgCl electrodes. After 1 h of ET, embryonated chicken eggs were intravenously injected with calcein (M.W. 622.6) or FITC-dextran (M.W. 10000), and further incubated in the humidified incubator. After incubation for the indicated time, chorioallantoic fluid was collected from the eggs, and the fluorescence intensity (Excitation: 488 nm, Emission: 535 nm) was measured to evaluate extravasation of the injected samples as an indicator of vascular permeability.

(Thermo Fisher Scientific, Waltham, MA, U.S.A.). The fluorescence in the sections was observed by confocal laser scanning microscopy (LSM700, Carl Zeiss, Jena, Germany).

Statistical Analysis Statistical differences were determined using Student's *t*-test. Data are presented as the mean ± standard deviation (S.D.).

RESULTS AND DISCUSSION

Here we investigated how ET influences vascular permeability using the blood vessels in the CAM of embryonated chicken eggs. The embryonated chicken eggs were placed on an egg inverter and incubated prior to use in experiments (Fig. 1A). The internal structure of eggs is shown in Fig. 1B. ET was performed onto the eggs by attaching cathodal and anodal electrodes onto the CAM, as shown in Fig. 1C. To evaluate extravasation of fluorescence tracers from blood vessels after the intravenous injections, the chorioallantoic fluid, which is surrounded by the CAM, was collected after the sample injection, followed by measurement of fluorescence intensity of the chorioallantoic fluid. Application of CAM for evaluating extravasation of fluorescence-labeled agents from blood vessels was previously reported, in which report ultrasound was employed as a physical stimulation.[23)]

At first, the effect of ET on extravasation of calcein (M.W. 622.6), a low molecular weight compound model, from blood vessels was investigated. One hour after the intravenous (i.v.) injection of calcein into eggs exposed to ET, the fluorescence intensity of the chorioallantoic fluid collected from eggs exposed to ET (ET (+)) was nearly similar to that for eggs that were not exposed to ET (ET (−)) (Fig. 2). As the time after

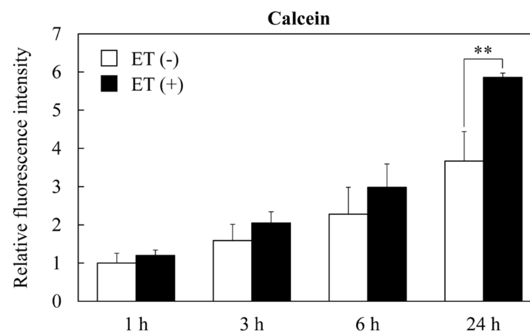

Fig. 2. Increase in Calcein Extravasation into Chorioallantoic Fluid by ET

At 1, 3, 6, and 24 h after intravenous injection of calcein (M.W. 622.6; 336.2 µg/mL in PBS), chorioallantoic fluid was collected and the fluorescence intensity was measured. Relative fluorescence intensity of chorioallantoic fluid collected from embryonated eggs at each time point to that collected 1 h after sample injection from eggs not exposed to ET (ET (−)) is shown. ET (+) indicates the group of embryonated eggs exposed to ET. Data are the mean ± S.D. ($n = 4$–5). Significant difference: ** $p < 0.01$.

injection increased, the fluorescence intensity of the chorioallantoic fluid increased for both the ET-treated and untreated eggs, although the fluorescence intensity tended to be higher for the ET (+) compared to ET (−) group 3 and 6 h after the sample injection. At 24 h after injection, the fluorescence intensity was significantly higher (1.6-fold) for the ET (+) group compared to the ET (−) group. In terms of cellular damage produced by ET, we previously confirmed that cell viability did not decrease either immediately or 24 h after ET of murine melanoma and fibroblast cells.[21,24)] These results suggested that leakage of low molecular compounds from blood vessels could be enhanced by ET of vessels in the CAM and that

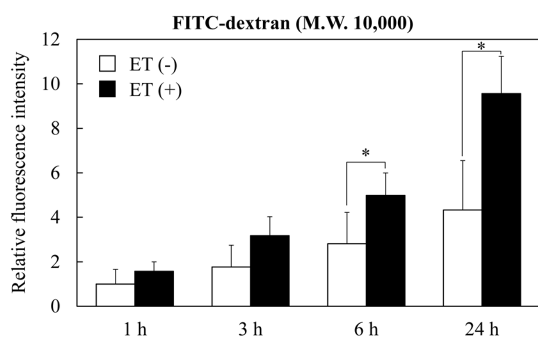

Fig. 3. ET Increased Extravasation of Macromolecules from Blood Vessels of Embryonated Chicken Eggs

At 1, 3, 6, and 24 h after i.v. injection of FITC dextran (M.W. 10000; 10 mg/mL in PBS), chorioallantoic fluid was collected and the fluorescence intensity of the fluid was measured. Relative fluorescence intensity of chorioallantoic fluid collected from embryonated eggs at each time point to that collected 1 h after sample injection from embryonated chicken eggs not exposed to ET (ET (−)) is shown. Data are the mean ± S.D. ($n = 5$). Significant difference: $*p < 0.05$.

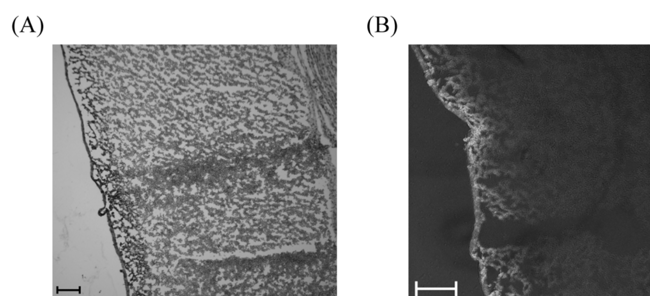

Fig. 4. Histological Analysis of Distribution of FITC-Dextran (M.W. 10000) Injected into Embryonated Chicken Eggs Exposed to ET

(A) HE staining and (B) confocal images of 10 μm-frozen sections of blood vessels dissected from embryonated eggs injected with FITC-dextran (M.W. 10000) following ET. For confocal images, merged images of FITC fluorescence and bright field are represented. Scale bars = 100 μm.

Table 1. Influence of ET on Permeability in HUVEC Monolayer

Time after ET	Relative fluorescence intensity		Significant difference
	ET (−)	ET (+)	
1 h	1.0 ± 0.26	1.19 ± 0.43	—
3 h	1.0 ± 0.31	1.41 ± 0.47	$p < 0.05$
6 h	1.0 ± 0.32	1.43 ± 0.47	—
Cell viability (%)	100 ± 35.1	98 ± 12.0	—

ET (0.17 mA, 15 min) was performed onto HUVEC monolayer formed on transwell inserts 30 min after the addition of EBM-2 containing 5 μM FITC-dextran (M.W. 10000). At 1, 3, and 6 h after ET, the fluorescence intensity of FITC in the media of the bottom compartment was measured. The relative fluorescence intensity of ET (+) to that of ET (−) in each time point are shown. The data are mean ± S.D. ($n = 9$–10). Cell viability was determined 6 h after ET by trypan blue staining.

increases in vascular permeability could be induced by ET of embryonated chicken eggs.

We next evaluated the influence of ET on vascular permeability by investigating extravasation of FITC-dextran (M.W. 10000), a representative macromolecule. Similar to the results for calcein, FITC fluorescence in the chorioallantoic fluid increased with increasing time after injection (Fig. 3). However, higher fluorescence intensity could be observed beginning at 1 h and also 3 h after sample injection in eggs exposed to ET (ET (+)) compared to those that were not exposed to ET (ET (−)). Notably, significantly higher fluorescence intensity was observed in ET-treated eggs (ET (+)) at 6 (1.8-fold) and 24 h (2.2-fold) after sample injection compared with untreated eggs (ET (−)), indicating that, like calcein, extravasation of FITC-dextran (M.W. 10000) from blood vessels was promoted by ET onto CAM.

We also investigated the effect of ET on penetration of FITC-dextran (M.W. 10000) in transwell permeability assay using HUVEC as a vascular endothelial cell. ET was performed onto the upper compartment of HUVEC monolayer formed on transwell inserts, and FITC fluorescence of the media in the bottom compartments was measured 1, 3, and 6 h after ET. The results showed that the relative fluorescence intensity was higher in the ET (+) group at each time point compared with ET (−) group, and the difference between ET (−) and ET (+) became larger from 1 through 6 h (Table 1). On the other hand, no obvious cellular damage was observed 6 h after ET. Similar results were obtained in our previous study, in which ET could promote penetration of Lucifer yellow (low molecular weight compound) and liposomes through Caco-2 cell monolayer.[14] The present results suggest that ET could promote macromolecule penetration through vascular endothelial cells without induction of obvious cellular damage. Although the effect of ET on vascular permeability was smaller than that seen in embryonated chicken eggs, this is considered due to the difference in the circumstances between *in vitro* and *in vivo*. We could observe only two-dimensional permeation of macromolecule *in vitro*, whereas three-dimensional permeation could occur *in vivo*. The differences in these circumstances could affect the results of macromolecule penetration.

For histological evaluation of the extravasation of fluorescent macromolecules from blood vessels, frozen sections of the CAM and vessels from embryonated chicken eggs were prepared 6 h after injection of FITC-dextran (M.W. 10000) following 1 h of ET. HE staining and confocal microscopic imaging showed that remarkable fluorescence derived from intravenously injected FITC-dextran could be observed in the blood vessel walls (Fig. 4). Since the CAM and blood vessels were washed with PBS prior to preparing the frozen sections, residual FITC-dextran in the blood vessels was likely to be completely removed. To further confirm ET-mediated leakage of FITC-dextran, the both ends of selected blood vessels were ligated prior to dissection and were collected 6 h after injection of the tracer molecule. The dissected vessels were then incubated in cell culture medium for 3 h and the FITC fluorescence in the supernatant was measured. Similar to the results of Fig. 2, the fluorescence intensity of the chorioallantoic fluid from the ET (+) group was higher than that for ET (−) group (data not shown). These results suggest that ET could

artificially induce an increase in vascular permeability to promote extravasation of macromolecules from blood vessels. Our previous study demonstrated that ET onto the skin could induce Ca^{2+}-mediated activation of intracellular signaling, and subsequent decrease in Cx43 expression and depolymerization of filamentous actin that resulted in intercellular junction cleavage.[14] Based on this finding, it is suggested that intercellular gaps between endothelial cells could be induced by ET onto the blood vessels in the CAM.

ET-mediated enhancement of macromolecule extravasation was time-dependently induced, and significant increase in fluorescence intensity of chorioallantoic fluid was observed 6 and 24 h after tracer injection (Fig. 3). Mechanistic studies in our previous report using *in vivo* rat skin showed that ET-mediated phosphorylation of Cx43, which leads to decrease in Cx43 protein expression, was significantly induced 3 and 6 h after ET onto skin.[14] At 6 h after ET, actin depolymerization was also remarkably elicited, and these events together contributed to nanoparticle permeation through skin tissue. Considering these results, it is speculated that ET-induced decrease in Cx43 expression and actin depolymerization in vascular endothelial cells of embryonated chicken eggs might occur in a time-dependent manner, similar to those observed in skin tissue. Accordingly, permeability increase in blood vessels was suggested to be induced by ET. Although specific mechanism of ET-mediated enhancement of vascular permeability is unclear, ET could also promote penetration of FITC-dextran (M.W. 10000) in transwell permeability assay using HUVEC monolayer (Table 1), similar to the assay using Caco-2 cell monolayer as reported previously.[14] We also previously demonstrated that, ET-mediated intercellular junction cleavage is triggered by Ca^{2+} influx into the cells, followed by activation of PKC. Subsequently, Cx43 phosphorylation and actin depolymerization are induced, by which permeability increase in cultured cell monolayer is caused. This series of cellular events is considered to be involved in ET-mediated increase in vascular permeability, although detailed analyses are needed in future.

In this study, we performed ET onto normal vessels of embryonated chicken eggs. However, it is expected that ET-mediated increase in vascular permeability can likely be applied for blood vessels under pathological conditions. For example, for cancer therapy using macromolecules or nanoparticles, species- or cancer-specific differences can influence the magnitude of the EPR effect.[6,7] Moreover, the presence of abundant stromal tissues could limit the accumulation of these drugs in tumor tissue in certain cancers.[25] To address these problems, regulation of vascular permeability and intercellular junction cleavage using ET could promote the accumulation of macromolecular and nano-particulate drugs through enhancement of the EPR effect. Additionally, we previously reported that ET can deliver exogenous macromolecules, such as siRNA and antibodies, into the cytoplasm *via* induction of endocytosis with unique characteristics.[21,26] We also revealed that ET-induced Rho GTPase activation *via* heat shock protein 90 and protein kinase C is involved in cellular uptake of siRNA.[22] Considering these previous findings and the results of this study, there is a possibility that ET might promote not only accumulation of macromolecular and nano-particulate drugs into tumor tissue *via* induction of intercellular junction cleavage, but also increase cellular uptake of those drugs by cancer cells. As another example, it was previously reported that treatment of the brain through the skull by weak electric current, which is termed transcranial direct current stimulation (tDCS), is effective in alleviating neurologically and neuropsychiatrically abnormal conditions such as ischemic stroke and major depression in humans.[27] Although the detailed mechanisms that produce these positive, ET-mediated effects in the brain are unclear, if ET can regulate the permeability of the BBB, tDCS could be a useful technique for drug delivery to the brain parenchyma. As such, application of ET as a drug delivery technology for cancer or diseases involving the brain should be interesting for future studies.

In conclusion, the present study demonstrated that ET onto the CAM of embryonated chicken eggs significantly increased extravasation of intravenously injected calcein (M.W. 622.6), a model of low molecular compound. Also, leakage of a macromolecule FITC-dextran (M.W. 10000) was also significantly enhanced by ET, and bright fluorescence derived from injected FITC-dextran was detected in the blood vessel walls in the confocal microscopic images. These results suggest that ET using weak electric current could artificially induce enhancement of vascular permeability to promote extravasation of macromolecules from the blood vessels. Also, these findings suggest that ET could have applications in drug delivery technologies that enable extravasation of drugs into target tissue parenchyma to enhance drug efficacy.

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Conflict of Interest The authors declare no conflict of interest.

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