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Localization of exogenous DNA to mitochondria in skeletal muscle following hydrodynamic limb vein injection

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ABSTRACT

Mitochondrial genetic disorders are a major cause of mitochondrial diseases. It is therefore likely that mitochondrial gene therapy will be useful for the treatment of such diseases. Here, we report on the possibility of mitochondrial gene delivery in skeletal muscle using hydrodynamic limb vein (HLV) injection. The HLV injection procedure, a useful method for transgene expression in skeletal muscle, involves the rapid injection of a large volume of naked plasmid DNA (pDNA) into the distal vein of a limb. We hypothesized that the technique could be used to deliver pDNA not only to nuclei but also to mitochondria, since cytosolic pDNA that is internalized by the method may be able to overcome mitochondrial membrane. We determined if pDNA could be delivered to myofibrillar mitochondria by HLV injection by PCR analysis. Mitochondrial toxicity assays showed that the HLV injection had no influence on mitochondrial function. These findings indicate that HLV injection promises to be a useful technique for *in vivo* mitochondrial gene delivery.

1. Introduction

Mitochondrial dysfunction has been implicated in a variety of human diseases [1–3]. It is noteworthy that such disorders are mainly associated with tissues that have high energy requirements, such as the brain, heart, muscle and liver. It is now well accepted that mutations and defects in the mitochondrial genome form the basis of these diseases [4–7]. Therefore, mitochondrial gene therapy and diagnosis would be expected to have substantial medical benefits. However, their utility has not yet been realized because mitochondrial gene delivery technology is the bottleneck. Even through *in vitro* experiments have been reported, actual studies of mitochondrial gene delivery are very few [8–11]. The use of a variety of applications for nuclear gene delivery *in vitro*/*in vivo* have been reported [11–16], in attempts to accelerate progress in the field of nuclear gene therapy. Thus, successful mitochondrial gene transfer would largely contribute to mitochondrial gene therapy.

Skeletal muscle represents an attractive target tissue for mitochondrial gene therapy as well as nuclear gene therapy, because mitochondrial diseases, including mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) [5] and myoclonic epilepsy and ragged-red fiber disease (MERRF) [7] are largely associated with mitochondrial genomic dysfunction in skeletal muscle. Vascular delivery procedures have recently been used to deliver plasmid DNA (pDNA) to the skeletal muscle of rodents and nonhuman primates by hydrodynamic limb vein (HLV) injection [17–20]. The hydrodynamic injection method originally was reported by Liu et al. [21] and Zhang et al. [22], and was used to achieve effective nuclear transgene expression in hepatocytes in mice by the rapid injection of large volumes of naked pDNA into the tail vein. Recently, Yan et al. reported on a facile hydrodynamic injection method via the retro-orbital sinus [23].

In the HLV injection procedure, a tourniquet is used to limit the delivery area to one limb per injection and naked pDNA is rapidly injected into the vein in the anterograde direction [20]. A sufficient volume of saline is used to facilitate extravasation of the pDNA from the vasculature and into the muscle tissue through multiple physical barriers. It has been suggested that hydrodynamic force could induce the transient opening of cellular membrane to permit pDNA to be internalized into cells, the subsequent localization of pDNA into the nucleus may be achieved via some mechanisms such as active nuclear import, cell division or transient opening of nuclear membrane. More recently, it has been reported that the HLV injection of condensed pDNA was even more effective than naked pDNA in achieving nuclear transgene expression in skeletal muscle [24–26]. Based on these reports,

Abbreviations: Ch, channel; CLSM, confocal laser scanning microscopy; COX, cytochrome c oxidase; DAPI, 4′, 6-Diamidino-2-phenylindole; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; FISH, fluorescent *in situ* hybridization; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; HLV, hydrodynamic limb vein; MELAS, Mitochondrial myopathy encephalopathy, lactic acidosis and stroke-like episodes; MERRF, Myoclonic epilepsy and ragged-red fiber disease; MIB, mitochondrial isolation buffer; MTDR, MitoTracker Deep Red 633; PBS (-), phosphate-buffered saline; pDNA, plasmid DNA; q-PCR, quantitative real-time PCR; RLU, relative light units; TMRM, Tetramethylrhodamine.

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we hypothesized that the HLV injection technique might be useful for delivering pDNA into, not only the nucleus, but also the mitochondria.

The purpose of this study was to validate the possibility of mitochondrial DNA delivery in skeletal muscle of rats by HLV injection (Fig. 1a). We first verified, using PCR analysis, that the HLV injection technique could be used to deliver naked pDNA into myofibrillar mitochondria. We also investigated the effects of injection volume and dose of pDNA on mitochondrial delivery by HLV injection. A combination of immunostaining for mitochondrial proteins and fluorescent *in situ* hybridization to detect pDNA (Immuno-FISH) permitted us to visualize the pDNA that was delivered to skeletal muscle tissue. Finally, we assessed the mitochondrial toxicity in skeletal muscle following HLV injection in terms of cytochrome c oxidase (COX) activity and mitochondrial membrane potentials.

2. Materials and methods

2.1. Materials

The pcDNA3.1 (+)-luc plasmid was constructed by inserting the firefly luciferase gene (*Hind* III-*Xba* I fragment) of the pGL3-Control plasmid (Promega, Madison, WI, USA) into the pcDNA3.1 (+) plasmid (Invitrogen, Carlsbad, CA, USA) pretreated with the same restriction enzymes. The luciferase gene in the pDNA is expressed under the control of the cytomegalovirus promoter. The pDNA was purified using an Endfree Plasmid Giga Kit (Qiagen GmbH, Hilden, Germany). Oligonucleotides were purchased from Sigma Genosys Japan (Ishikari, Japan) in purified form. Primary antibodies against COX IV, Lamin A + C or Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Abcam (Cambridge, UK). 4′, 6-Diamidino-2-phenylindole (DAPI) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals used were commercially available reagent-grade products.

2.2. Experimental animals

Female Wistar Hannover rats (7–9 weeks old) were purchased from Sankyo Labo Service (Sapporo, Japan). Rats with body weight in the range 150–180 g were used in all experiments. All animal protocols were approved by the institutional animal care and research advisory committee at the Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan.

2.3. Hydrodynamic injection into the limb vein of rats

Rats were anesthetized with a pentobarbital (37.5 mg/kg) solution via an intraperitoneal injection. Prior to each pDNA injection, a tourniquet was placed on the upper hind limb to restrict blood flow into and out of the hind limb. Basically, a suspension of naked pDNA (3 mL) containing 184 μg of pDNA was injected in 20 s from a distal site of the dorsalis pedis vein. At 2 min after the injection, the tourniquet was released.

2.4. Luciferase assay of muscle tissue after injection

At 24 h postinjection, rats were sacrificed and the limb muscles were harvested and separated into three groups (quadriceps, hamstring, crural muscles). Each of the muscle tissues were minced with scissors and frozen in liquid nitrogen. Approximately 250 mg samples of frozen muscle tissue were then homogenized using a PreCellys (bertin technologies, Montigny-le-Bretonneux, France) in 1 mL of lysis buffer (100 mM Tris–HCl, 2 mM EDTA, 0.1% Triton X-100, pH 7.8). After centrifugation at 16,630 *g* for 10 min at 4 °C, a 20 μL aliquot of the supernatant was used for a luciferase assay using the Luciferase Assay System (Promega, Madison, WI, USA). Protein concentrations were determined with a BCA protein assay kit (Pierce, Rockford, IL). Luciferase activities are expressed as relative light units (RLU) per mg of protein.

2.5. Isolation of mitochondria from rat skeletal muscle

At 24 h postinjection, the rats were sacrificed and the crural muscles were harvested and minced with scissors, and 1 mL of ice-cold mitochondrial isolation buffer [MIB: 250 mM sucrose, 2 mM Tris–HCl, 1 mM EDTA, pH 7.4] was then added. The suspension was homogenized using a PreCellys, and centrifuged at 800 *g* for 5 min at 4 °C. A 500 μL of supernatant was transferred into ice-cold tubes containing 500 μL of

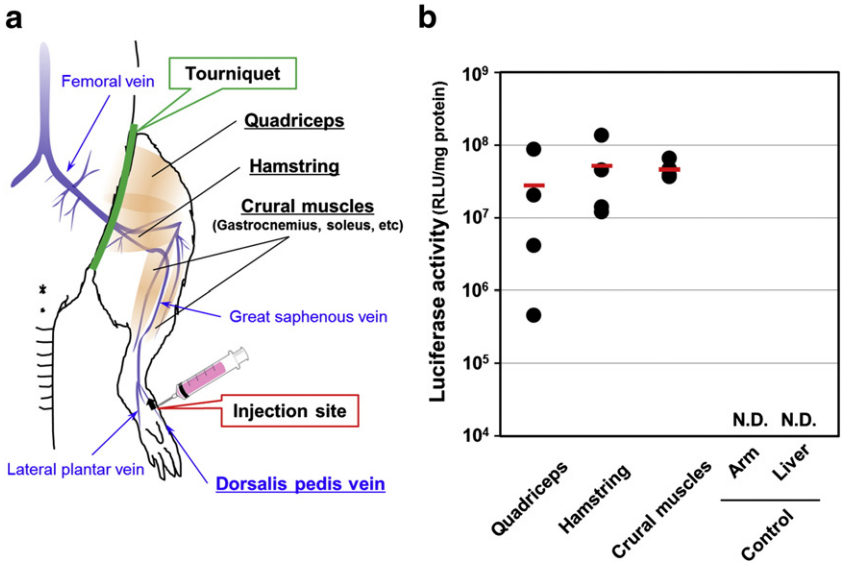

Fig. 1. Schematic images of gene delivery to skeletal muscle by HLV injection and evaluation of nuclear transgene expression in part of skeletal muscles. (a) Schematic diagram of HLV injection of pDNA into the distal hind limb of rats. pDNA was intravenously injected into the dorsalis pedis vein under the conditions that restrict blood flow with a tourniquet. At 24 h postinjection, rats were sacrificed, the limb muscles were harvested and separated into three groups (quadriceps, hamstring, crural muscles). (b) Gene expression in part of skeletal muscles. Luciferase activities were measured at 24 h after HLV injection of pDNA coding luciferase. Bars represent the mean values (n = 4). Statistical analysis among the quadriceps, hamstring and crural muscles was performed by a one-way ANOVA ($p = 0.69$). We also measured luciferase activities in the arm and liver as controls. N.D., not determined.