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Horizontal gene transfer from macrophages to ischemic muscles upon delivery of naked DNA with Pluronic block copolymers

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ABSTRACT

Intramuscular administration of plasmid DNA (pDNA) with non-ionic Pluronic block copolymers increases gene expression in injected muscles and lymphoid organs. We studied the role of immune cells in muscle transfection upon inflammation. Local inflammation in murine hind limb ischemia model (MHLIM) drastically increased DNA, RNA and expressed protein levels in ischemic muscles injected with pDNA/Pluronic. The systemic inflammation (MHLIM or peritonitis) also increased expression of pDNA/Pluronic in the muscles. When pDNA/Pluronic was injected in ischemic muscles the reporter gene, Green Fluorescent Protein (GFP) co-localized with desmin^{+} muscle fibers and CD11b^{+} macrophages (MØs), suggesting transfection of MØs along with the muscle cells. P85 enhanced (~4 orders) transfection of MØs with pDNA *in vitro*. Moreover, adoptively transferred MØs were shown to pass the transgene to inflamed muscle cells in MHLIM. Using a co-culture of myotubes (MTs) and transfected MØs expressing a reporter gene under constitutive (cmv-luciferase) or muscle specific (desmin-luciferase) promoter we demonstrated that P85 enhances horizontal gene transfer from MØ to MTs. Therefore, MØs can play an important role in muscle transfection with pDNA/Pluronic during inflammation, with both inflammation and Pluronic contributing to the increased gene expression. pDNA/Pluronic has potential for therapeutic gene delivery in muscle pathologies that involve inflammation.

1. Introduction

Direct intramuscular (*i.m.*) injection of naked plasmid DNA (pDNA) for skeletal muscle transfection was pioneered by J.A. Wolff in 1990 [1]. As of today *i.m.* injection of pDNA makes up around 18% of worldwide gene therapy human clinical trials [2]. These trials are related to DNA vaccines, Duchene muscular dystrophy, hind limb ischemia and cardiac ischemia [3–5]. The *i.m.* injection of pDNA has shown excellent safety profiles, but low gene expression levels, which limits its use in various therapeutic applications. To overcome this limitation, we and others have proposed a very simple and inexpensive approach of co-administering naked pDNA with non-ionic Pluronic block copolymers ("Pluronics" or "poloxamers") that were shown to drastically increase the levels and duration of muscle gene expression [6,7]. Pluronics consist of non-ionic ethylene oxide (EO) and propylene oxide (PO) blocks arranged in a basic triblock A-B-A structure: EO_x-PO_y-EO_x. They do not form complexes with the pDNA [8]. So far the mechanisms by which Pluronic increases the transfection with naked pDNA in skeletal muscles remained not well understood.

It has been known that skeletal muscles have a remarkable ability to regenerate after tissue injury, which coincides with the inflammatory events and presence of immune cells, in particular, macrophages (MØs). These cells play a key role in the process of skeletal muscle regeneration [9–13]. Due to constant persistence throughout the inflammatory response, MØs help muscle

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membrane repair in a cell-to-cell contact dependent manner [14]. Similarly, we know that direct *i.m.* injection of pDNA also triggers an inflammation response due to first, the physical injury caused by the needle, and second, the exposure to unmethylated CpG islands in bacterially derived pDNA. A previous study reported an increase in muscle transfection of pDNA during hind limb ischemia [15]. It was already noted before that Pluronic effects pDNA expression in the muscle were lacking in immune-deficient athymic mice, which implied the role of immune cells in muscle transfection [8]. Moreover, co-administration of Pluronic with pDNA in the muscle greatly increased expression of the transgene along with the cDNA and mRNA levels in distal lymphoid organs, such as draining lymph nodes and spleen, with the transgene being co-localized there with antigen presenting cells (APCs) [16].

Most disease pathologies involve recruitment of inflammatory cells to the tissues and secretion of pro-inflammatory factors either locally or systemically. Since the therapeutic genes in the pDNA constructs are driven by constitutive cytomegalovirus (cmv) promoter with multiple NF-kb binding sites [17], inflammation may influence the gene expression. However, many studies involving *i.m.* injections of pDNA have been conducted in healthy muscles even when the ultimate goal was to use this method for therapeutic protein expression in disease-affected tissues. Therefore, studies of the gene expression using relatively new platforms such as Pluronic block copolymers in various disease models will further help understanding the limits and opportunities for application of these platforms in gene therapy.

The aim of this study was to evaluate the effect of local and systemic inflammation on muscle gene expression after *i.m.* delivery of naked pDNA with or without Pluronics. To induce local inflammation, we used a murine hind limb ischemia model (MHLIM) and for systemic inflammation both MHLIM and peritonitis. In previous work, we ranked the potency of various Pluronics on the expression of a plasmid containing a luciferase reporter gene under the control of cmv promoter, and found that Pluronic P85 (P85) and SP1017 were the most effective and safe [16]. Here we examine the effects of these copolymers on the MØ ability to take up and express pDNA and horizontally transfer the transgene to the muscle cells both during *in vitro* coculture and adoptive transfer to healthy and disease affected animals.

2. Materials and methods

2.1. Plasmids

The gWIZ™ high expression vectors encoding the reporter genes, luciferase (gWIZ™ Luc) and green fluorescent protein (GFP; gWIZ™ GFP), both under control of an optimized human cmv promoter followed by intron A from the cmv immediate-early gene were used throughout the study (Gene Therapy Systems, San Diego, CA). The pDRIVE5Lucia-mDesmin and pDRIVE5GFP-mDesmin (InvivoGen, San Diego, CA) encodes luciferase and GFP reporter proteins respectively, transcribed under the control of murine desmin promoter for muscle-specific expression. All plasmids were expanded in E. coli DH5α and isolated using Qiagen's EndoFree Plasmid Giga Kit (Qiagen, Valencia, CA) according to the supplier's protocol, reconstituted in phosphate buffered saline (PBS) and stored at −20 °C until use.

2.2. Block copolymers

Pluronic L61 (batch # WPNT-511B), P85 (batch # WPNT-511B), F127 (batch # WPNT-511B), were a gift of the BASF Co. (Mount Olive, NJ). A mixed composition of L61 (0.25% w/v) and F127 (2% w/v), SP1017 (2.25% w/v) was obtained from Supratek Pharma Inc. (Montreal, Canada) or prepared using the corresponding copolymers.

2.3. pDNA/Pluronic formulations

The pDNA formulations were prepared as described [16] and used immediately for *i.m.* injections.

2.4. Cells

RAW264.7 immortalized mouse MØs cell line and C2C12 immortalized myoblasts (MBs) cell line were purchased from ATCC and cultured at 37 °C and 5% CO_2. To obtain terminally differentiated skeletal myotubes (MTs), C2C12 MBs were plated in 96-well plates (50,000 cells/well) in complete media (CM) containing Dulbecco's minimal essential media (DMEM) and 10% fetal bovine serum (FBS). After 24 h or ~100% confluence, CM was replaced with 200 μl/well differentiation media (DM) containing DMEM and 2% horse serum (HS) to facilitate fusion of MBs [18]. DM was changed every 12 h thereafter until day 5–10 when healthy long differentiated MTs were formed.

2.5. Animals

All animal experiments were carried out with approval of the University of Nebraska Medical Center (UNMC) and University of North Carolina at Chapel Hill (UNC–CH) Institutional Animal Care and Use Committee and in accordance with the NIH Guide for Laboratory Animal Use. Female Balb/c mice (6- to 8-weeks-old, Charles River Laboratories, Wilmington, MA) were used throughout this study. The animals were kept in groups of five and fed *ad libitum*.

2.6. Inflammation models and scheme of experiments

MHLIM was generated by surgical procedure as described [19,20]. Briefly, under general anesthesia, the femoral artery (FA) of right hind limb was completely excised after ligation at its proximal origin as a branch of the external iliac artery and before the point distally where it bifurcates into the saphenous and popliteal arteries. Saphenous artery and saphenous vein were also excised. An interval of 10 days was allowed for postoperative recovery before DNA administration in ischemic *tibialis anterior* (*TA*) muscle by direct *i.m.* injections. In some groups MHLIM surgery was performed on ipsilateral hind limb and test articles were simultaneously injected in contralateral *TA* muscles of the same mouse. Peritonitis was induced by intraperitoneal (*i.p.*) injections of filter-sterilized λ-carageenean (CGN; 1 mg/200 μl PBS) on the 1st and 2nd day and followed by test articles injections in *TA* muscles on the 3rd day. In some groups pDNA and Pluronic were administered in separate legs in both healthy and peritonitis model.

2.7. pDNA injections

Animals were anesthetized by *i.p.* injection of mixed solution of ketamine (100 mg/kg) and xylazine (25 mg/kg) (Sigma, St. Louis, MO). Single injection of pDNA in 50 μl of Hank's balanced salt solution (HBSS) alone or 50 μl of the block copolymer solution in HBSS was administered directly into right *TA* muscle of the mice using 28G 1 cc sterile syringe (BD Bioscience, Franklin Lakes, NJ).

2.8. Luciferase activity in vivo

Unless indicated otherwise mice were euthanized at the time points indicated in the figure legends and tissues were processed.