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Multifunctional envelope-type nano device for controlled intracellular trafficking and selective targeting *in vivo*

Kazuaki Kajimoto, Yusuke Sato, Takashi Nakamura, Yuma Yamada, Hideyoshi Harashima *

Faculty of Pharmaceutical Sciences, Hokkaido University, Japan

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ABSTRACT

Nanomedicine is expected to be a basic technology for using nucleic acids as a drug, in which treating the cause of diseases represent the ultimate therapy. However, a sophisticated delivery system is required for efficient delivery of RNA/DNA, since these compounds need precise control of intracellular trafficking as well as biodistribution. Here we report on the use of a multifunctional envelope-type nano device (MEND) which is capable of intracellular trafficking such as endosomal escape, delivery to mitochondria, as well as active targeting to selective tissues/cells *in vivo*. In this review, we focused on the controlled intracellular trafficking of antigens for advanced immunotherapy, and then introduced a mitochondrial delivery system as an organelle targeting system for unmet medical needs. We also provide a successful *in vivo* delivery of siRNA to the liver based on a newly designed pH-responsive cationic lipid. Finally we will discuss an important role of an active targeting system using a peptide ligand to adipose vasculature. These progresses in drug delivery system will break through the barriers exist in our body, tissues and cells and open a window for future Nanomedicine.

Contents

1. Introduction

In the last decade of the 20th century, long circulation liposomes encapsulating Doxorubicin appeared in clinics as an innovative drug

* Corresponding author at: Laboratory for Molecular Design of Pharmaceutics, Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-12, Nishi-6, Kita-ku, Sapporo, Hokkaido 060–0812, Japan. Tel.: +81 11 706 3919; fax: +81 11 706 4879.
E-mail address: harasima@pharm.hokudai.ac.jp (H. Harashima).

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delivery system (DDS). This DDS relied on the enhanced permeability and retention (EPR) effect [1] and this strategy is classified as passive targeting [2]. While this represented a breakthrough in history of DDS, a paradigm-shift occurred in the field of drug discovery and development in the 21st century, where antibody-drugs emerged as blockbusters and occupied top marketing drugs. Therefore, it is recognized that a new strategy is required to produce new drugs which address unmet medical needs such as cancer, central nervous disorders, immunodeficiencies, etc., and Nanomedicine is expected to be a breakthrough technology in the development of new medicines, since the functional compounds should be delivered to the site of action via an intelligent delivery system which can control intracellular trafficking as well as biodistribution using molecular mechanisms identified so far [3].

We have been in the process of developing a multifunctional envelope-type nano device (MEND) which has been inspired by viral vectors which evolved in nature. MEND is designed so as to control intracellular trafficking of nano carriers as well as to control tissue distribution [4]. Octaarginine (R8) was introduced into MEND to enhance cellular uptake, since R8 was found as a cell penetrating peptide [5]. Surface modification of MEND with R8 in the form of stearyl-R8 not only enhanced cellular uptake but also endosomal escape after taken up via macropinocytosis, when modified with high density R8 [6]. In case of low density R8 modification, R8-MEND was assigned to degradation pathway after taken up via clathrin-mediated endocytosis [6]. As a result, high density R8-MEND could induce as high transfection activities as adenovirus in dividing cell lines [7]. In parallel, we have challenged to overcome PEG-dilemma when we deliver nano particles to tumor tissues based on EPR-effect [8]. PEGylation is so effective for nano particles in long blood circulation, however, it remarkably inhibit the escape from endosomes in tumor cells. We proposed a few strategies to overcome this dilemma by introducing cleavable PEG into PEG-lipid. Cleavable PEG successfully degraded in tumor tissue and PEG free MEND could induce transfection activities or silencing effect in tumor cells [9], however, the efficacy was not enough to move into clinics.

In this review, we provide a summary of our recent progress controlling the intracellular trafficking of peptide antigens as well as lipid antigen in immune cells for efficient vaccination by focusing on endosomal escape. MITO-Porter was introduced as an organelle targeting system, which is an envelope-type fusion-based mitochondrial delivery system, intended to deliver not only small molecular compounds but also proteins/nucleic acids for functional regulation of mitochondria [10]. It is also important to control *in vivo* biodistribution for a view point of efficacy as well as toxicity. *In vivo* siRNA delivery would be one of the most expected technologies for successful application of nucleic acids into clinics. Recently, we succeeded in designing a pH-responsive cationic lipid for siRNA delivery to the liver and optimized the system to induce efficient silencing in liver. Finally, we will focus on a powerful active targeting system to the vasculature in adipose tissue where, we thought, the passive targeting strategy does not work [11]. Tissue selective targeting systems promise to expand a new field of therapy via Nanomedicine.

2. Application for cancer immunotherapy

2.1. Delivery of a nucleic acid adjuvant

Cellular immunity is indispensable for the efficient elimination of tumors. Cytotoxic T lymphocytes (CTL) mainly function as terminators against tumor cells in cellular immunity. CTL is activated by cytokines and antigen presentation via the major histocompatibility complex I (MHC-I) on antigen presenting cells (APCs). APCs degrade cytosolic antigens via proteasomes and present antigen derived peptides on MHC-I molecules. It is necessary to deliver tumor associated antigens to the cytosol in APCs, because the tumor associated antigens used in cancer immunotherapy are basically exogenous antigens. We previously reported that liposomes (R8-Lip) modified with octaarginine (R8) efficiently delivered antigens to the cytosol of APCs and induced a high level of MHC-I antigen presentation [12]. In this report, subcutaneous immunization of R8-Lip encapsulating ovalbumin (OVA), a model antigen, showed significant antitumor effects in E.G7-OVA lymphoma-bearing mice. However, the efficient cytosolic delivery of an antigen alone is not sufficient to induce a promising cellular immunity, because no significant difference in antitumor effects was observed between R8-Lip encapsulating OVA and the R8/OVA complex. The R8/OVA complex is a mixture of R8 and OVA, and was disadvantageous in terms of intracellular trafficking [13,14]. It is likely that the R8/OVA complex does not deliver substantial levels of antigen to the cytosol.

There is another factor associated with the induction of efficient cellular immunity in addition to the cytosolic delivery of antigen. Activation of APCs, namely maturation of APCs, is also required. Therefore, we incorporated an adjuvant into R8-Lip. Polyinosine-polycytidylic acid (polyI:C) is an adjuvant and activates APCs via recognition by the toll-like receptor (TLR) 3 and the melanoma differentiation-associated gene 5 (Mda5) [15]. The resulting R8-Lip incorporating polyI:C and OVA (R8-Lip/PIC/OVA) drastically enhanced CTL activity compared to the R8/OVA complex incorporating polyI:C (R8/PIC/OVA-Com). In addition, the CTL activity caused by R8-Lip/PIC/OVA was significantly higher than that for complete Freund's adjuvant (CFA) with OVA. CFA is one of the strongest adjuvants available for research. We further examined the preventative and therapeutic antitumor effects of the preparation in E.G7-OVA lymphoma-bearing mice. Mice immunized with R8-Lip/PIC/OVA showed significant preventative antitumor effects compared to that of R8/PIC/OVA-Com. In the experiment dealing with the therapeutic effect, only R8-Lip/PIC/OVA immunization induced a significant therapeutic antitumor effect, although R8/PIC/OVA-Com immunization showed no therapeutic effect compared to the control group. These results indicate that the topological control of polyI:C has a great influence on the induction of cellular immunity *in vivo*. The encapsulation of polyI:C into the inner phase of R8-Lip may protect polyI:C from degradation after its administration compared with R8/PIC/OVA-Com. It is also likely that the enhancement of cytosolic delivery of polyI:C by R8-Lip promoted the recognition via Mda 5 in the cytosol in addition to TLR3 in endosome, resulting in the difference of induction of cellular immunity. The combination of antigen and adjuvant is indispensable for efficient induction of cellular immunity. In the future, the topological control of adjuvant in a delivery system was found to also be important, when other useful adjuvants were used.

2.2. Lipid antigen mediated immune responses

As discussed above, the MHC molecule binds the antigen protein-derived peptide and presents it to T cells. This has been a central dogma in modern immunology. However, we now know that a new paradigm exists for antigen presentation, which is the recognition of non-peptide antigens present on CD1 molecules by T cells [16]. The human CD1 family is composed of five members, namely, CD1: CD1a, CD1b, CD1c, CD1d and CD1e. CD1 molecules are prominently expressed in APCs and bind lipids such as fatty acids, glycolipids and lipopeptide antigens of foreign or self origin [17]. These lipid antigens are mainly found in bacteria, particularly mycobacterium. Thus, an immune system Q3 via lipid antigen presentation by CD1 molecules would be expected to function as a vaccine against bacterial infections. However, it has been difficult to develop and utilize lipid antigens as a vaccine, because lipid antigens are poorly soluble in water. Thus, we incorporated lipid antigens into R8-Lip for delivering them to APCs *in vitro* and *in vivo* [18–21]. Glucose monomycolate (GMM), a highly hydrophobic glycolipid of the cell wall of mycobacteria, is a well defined lipid antigen that is present on human CD1b molecules [22]. The R8-Lip incorporating GMM elicited a delayed-type hypersensitivity (DTH) in guinea pigs and the DTH response was skewed to Th1 [18]. The R8-Lip incorporating GMM also induced GMM-specific T cell responses and Th1-skewed tissue responses in the mycobacteria-infected rhesus macaques [20,21].

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