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Messenger RNA-based therapeutics for brain diseases: An animal study for augmenting clearance of beta-amyloid by intracerebral administration of neprilysin mRNA loaded in polyplex nanomicelles

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ABSTRACT

Alzheimer's disease (AD) pathogenesis is considered to be the metabolic imbalance between anabolism and clearance of amyloid-beta (Aβ), and the strategy of breaking the equilibrium between soluble and insoluble forms of Aβ is likely to help prevent the progression of AD. Neprilysin (NEP) plays a major role in the clearance of Aβ in the brain, and its supplementation using viral vectors has shown to decrease Aβ deposition and prevent pathogenic changes in the brain. In this study, we developed a new therapeutic strategy by mRNA-based gene introduction. mRNA has the advantages of negligible risk of random integration into genome and not needing to be transcribed precludes the need for nuclear entry. This allows efficient protein expression in slowly-dividing or non-dividing cells, such as neural cells. We constructed mRNA encoding the mouse NEP protein and evaluated its ability degrade Aβ. *In vitro* transfection of NEP mRNA to primary neurons exhibited Amyloid Precursor Protein (APP) degradation activity superior to that of NEP encoding plasmid DNA. We then evaluated the *in vivo* activity of NEP mRNA by intracerebroventricular (i.c.v.) infusion using a cationic polymer-based PEGylated nanocarrier to form polyplex nanomicelles, which have been shown to have a high potential to deliver mRNA to various target tissues and organs. Nanomicelles carrying a GFP-NEP fusion mRNA produced efficient protein expression in a diffuse manner surrounding the ventricular space. An ELISA evaluation revealed that the mRNA infusion significantly augmented NEP level and effectively reduced the concentration of Aβ that had been supplemented in the mouse brain. To the best of our knowledge, this is the first study to demonstrate the therapeutic potential of introducing exogenous mRNA for the treatment of brain diseases, opening the new era of mRNA-based therapeutics.

1. Introduction

Alzheimer's Disease (AD) manifests as progressive dementia, affecting millions of people and causing tremendous economic and social burdens. However, the complete pathogenic mechanisms of AD are still unclear, and no fundamentally effective therapy is available for clinical AD patients. The most accepted model of AD pathogenesis is the metabolic imbalance between anabolism and clearance of amyloid-beta (Aβ). Although Aβ may have some beneficial physiological roles [1], much evidence demonstrates that the neuronal death observed in AD patients correlates with the over-production of Aβ, leading to the deposition of Aβ in the forms of extracellular senile plaques and intracellular neurofibrillary tangles [2,3]. Moreover, many studies indicate that the loss of memory function is correlated with the soluble form of Aβ monomer or oligomer, which exists in equilibrium with the fibril form of insoluble Aβ [4,5]. Indeed, in experimental animal models, intracerebroventricular (i.c.v.) infusion of soluble Aβ gave rise to the neuronal damage in the hippocampus, causing impairment of spatial learning and memory functions in a dose-dependent manner [6,7].

In this context, the strategy of breaking the equilibrium between the monomer or oligomer form of soluble Aβ and the fibril form of insoluble Aβ is likely to help alleviate or prevent the pathogenic progression of AD. Neprilysin (NEP), a protease that degrades Aβ, is a good candidate for this purpose. NEP is a membrane protein composed of ~750 residues (~110 kDa), capable of degrading Aβ monomers and oligomers [8]. Theoretically, the degradation of Aβ by NEP would be effective for regulating the initiation and progression of the early stage of AD, resulting in reduced Aβ deposition and preventing the pathogenic changes in the brain [9,10]. A key problem is that NEP needs to be administered by

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gene introduction because NEP is not a secreted protein. Animal studies are generally performed using NEP-expressing viral vectors such as lentivirus or adeno-associated virus [9,10]. However, considering the slowly progression of AD pathology, frequent dosing of NEP could be necessary, and the safety of the gene delivery method becomes more important.

In this regard, messenger RNA (mRNA) administration is a promising alternative for introducing NEP. Compared to conventional methods of gene delivery using DNA, mRNA-based gene delivery has several advantages. First, mRNA will not be integrated into the host cell genome, which avoids the possibility of insertional mutagenesis. Second, mRNA does not need to enter the nucleus, making it more effective in very slowly-dividing or non-dividing cells, such as neural cells [11,12]. In addition, mRNA may allow for greater control over the level of protein expression, as it does not include promoter sequences or require transcription. Finally, mRNA does not contain additional sequences such as plasmid backbone or promoters originally derived from naturally occurring viruses. These points highlight the safety and effectiveness of mRNA for gene introduction into neural cells. Nevertheless, the use of mRNA still has some issues to be addressed, such as instability under physiological conditions and the strong immunogenicity through recognition by Toll-like receptors. Therefore, a drug delivery system (DDS) capable of protecting the mRNA against nucleases and preventing recognition by TLRs is mandatory for achieving therapeutic outcomes.

In this study, we performed animal experiments to establish the feasibility and efficacy of mRNA for the treatment of AD. NEP-expressing mRNA was used for treating the pathological conditions of Aβ accumulation in the brain. The mRNA was administered using our carrier that had been proven to be applicable for *in vivo* mRNA delivery based on the self-assembly of polyethylene glycol (PEG)-polyamino acid block catiomer with DNA or RNA to form polyplex nanomicelles. The mRNA is incorporated into the core of the nanomicelles by association with a functionalized polyamino acid, poly[N′-[*N*-(2-aminoethyl)-2-aminoehtyl] aspartamide] (PAsp(DET)), and surrounded by a PEG outer layer. PAsp(DET) has a unique two-step protonation behavior due to the DET side chains, in which DET shows a distinctive *gauche to anti* conformational transition from pH changes from 7.4 to 5.0, resulting in pH-dependent destabilization of the cell membrane and endosomal escape [13,14]. In addition, PAsp(DET) degrades to non-toxic metabolites after releasing mRNA in the cells, effectively preventing cumulative toxicity after mRNA introduction [15]. This nanomicelle system has successfully introduced mRNA into the target tissues, including neural tissues [16,17], by preventing nuclease digestion and recognition of the mRNA by the innate immune system. As will be shown, the mRNA effectively provided NEP for the brain, resulting in the significant decrease of Aβ concentration in the brain, and shows the therapeutic potential of mRNA-based gene therapy for treating brain diseases.

2. Materials and methods

2.1. Construction of vector for in vitro transcription (IVT) and preparation of IVT mRNA

A DNA plasmid carrying the mouse NEP cDNA sequence (pNEP) (catalog number: MC206432, Origene Technologies, Inc.) was used for sub-cloning the NEP gene into pAcGFP1 vector (Clontech Laboratories, Inc.) to create pGFP-NEP fusion protein vector (Fig. S1A). For the production of template DNA from pSP73 vector (Promega Corporation), chemically synthesized poly(d(A/T) fragments containing 120 bps were cloned downstream of the cDNA region [18] and the NEP or GFP-NEP genes were further sub-cloned into this vector, which were named pSP73-NEP and pSP73-GNEP, respectively (Fig. S1B). Then, the vectors were linearized with SpeI, blunted with *E.coli* DNA polymerase I, purified with gel electrophoresis, and served as templates for *In Vitro* Transcription (IVT) using the mMESSAGE mMACHINE T7 Ultra Kit (Ambion, Invitrogen, Carlsbad, CA) to generate mRNA encoding NEP (mNEP) or GFP-NEP fusion protein (mGNEP). The mRNAs encoding Luc2 or GLuc were similarly constructed from the vectors encoding *Photinus pyralis* luciferase (pGL4; Promega Corporation) or Gaussia luciferase (pCMV-GLuc; New England Biolabs). Prior to the experiments, all transcribed mRNA were purified by QIAquick PCR purification kit (Qiagen) and analyzed for size and purity with the Agilent RNA 6000 Nano Assay on a BioAnalyzer 2100 (Agilent Technologies).

2.2. Cell culture, primary neuron isolation, in vitro transfection and NEP activity assay

HuH-7, N2A/PS/APP cells, 293/PS/APP cells, and mouse primary neurons were cultured and isolated as described in the Supplementary methods. The subsequent *in vitro* transfection and NEP activity assay were carried out as described in the Supplementary methods. All animal experiments were approved by the University of Tokyo, Committee for the Use and Care of Animals.

2.3. ELISA measurements of human amyloid precursor protein (APP), NEP, Aβ-40, Aβ-42, green florescent protein (GFP) and preparation of Aβ-40, Aβ-42 supplement

All ELISA kits were purchased commercially and assays were conducted in compliance with the manufacturer's instructions as detailed described in the Supplementary methods.

2.4. Immunocytochemical (ICC) staining

N2A cells and primary neurons were transfected with mGNEP, followed by ICC staining at 24 h post-transfection according to the standard protocol. Briefly, cells were cultured, transfected, and then serially fixed, permeabilized, and blocked in 8-well chamber slides. Cells were stained with rabbit anti GFP primary antibody (Invitrogen), followed by Alexa 488-conjugated goat anti rabbit secondary antibody (Jackson ImmunoResearch Inc.), then counter stained with DAPI (VECTASHIELD®, VECTOR Laboratories, Inc.) and observed by two photon microscope (LSM 510, Carl Zeiss).

2.5. Preparation of polyplex nanomicelles

A PEGylated block catiomer was previously developed [16] through a simple and affordable synthetic procedure based on an aminolysis and amine reactions to generate N-substituted polyaspartamides bearing 2 repeating units of aminoethylene and referred to as PEG (M.W. = 12,000)-poly{*N*-[*N*-(2-aminoethyl)-2-aminoethyl]aspartamide}, (PEG-PAsp(DET)) (Fig. S2A). The degree of polymerization of the PAsp(DET) portion was determined to be 72 by ^{1}H NMR analysis. To prepare the polyplex nanomicelles, PEG-PAsp(DET) and mRNA (or pDNA) were separately dissolved in HEPES buffer, and mixed at a volume ratio of 1:2, subsequently measured with dynamic light scattering (DLS) (Malvern) and transmission electron microscope (TEM) (Hitachi) for the evaluation of physicochemical properties, such as particle size, polydispersity index (PDI) and zeta-potential (Fig. S2B). Data showed nanomicelles containing Luc2 plasmid DNA (pDNA) or mRNA with particle size = 86.85 nm and 99.12 nm, PDI = 0.127 and 0.197, zeta-potential = 3.85 ± 0.22 mV and 17.3 ± 1.08 mV, respectively. The 2 repeating units of aminoethylene cationic groups confer block copolymer the electrostatic capability to interact with mRNA and form tiny nanomicelles, which showed uniform particle size and distribution (Fig. S2). The concentration of mRNA was set to 300 μg/ml and that of PEG-PAsp(DET) was adjusted to obtain a residual molar ratio of amino groups in polymers to phosphate in mRNA (N/P ratio) of 3.