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Lipid-mediated delivery of peptide nucleic acids to pulmonary endothelium

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Abstract

Peptide nucleic acid (PNA) is a DNA/RNA mimic in which the phosphodiester (PO) linkage is replaced with a peptide bond. It has a number of unique properties compared to currently used oligonucleotides including higher affinity towards RNA or DNA target, resistance to nucleases or proteases, and minimal non-specific interactions with proteins. Clinical applications of PNA, however, are limited by its inefficient intracellular delivery. In this study, we have shown that delivery of PNA to pulmonary endothelium in intact mice can be greatly improved via hybridization with a short PO oligonucleotide that serves as a carrier to form complexes with cationic liposomes. We have also shown for the first time that unlike a CpG DNA oligo that is highly proinflammatory, a CG-containing PNA is inert in triggering TNF-α response in cultured macrophages and in mice. Thus delivery of PNA to pulmonary endothelium may prove to be a therapeutically useful for the treatment of pulmonary vascular diseases.

Keywords: Peptide nucleic acid; Delivery; Cationic liposomes; Lung; Endothelium; Cytokine response

Delivery of antisense oligodoexynucleotides (ODN) to pulmonary endothelium may hold promise as a new therapy for the treatment of a number of pulmonary diseases including acute respiratory distress syndrome and pulmonary hypertension [1,2]. It may also provide a useful research tool in studying the function of novel genes in pulmonary endothelium [3]. Currently, the ODN that are most frequently used in antisense studies have a phosphorothioate (PS) linkage. Despite the demonstrated success of using PS ODN in down-regulating a variety of genes in vitro and in vivo, systemic administration of PS ODN is sometimes associated with a number of undesired side-effects [4]. These include non-specific binding to proteins, prolongation of partial thromboplastin time, and complement activation [4]. Recently several new types of chemistries have been developed to modify the ODN aimed at decreasing the PS backbone-associated toxicities [5–7]. One of the new oligomers that have been developed is PNA [6,7].

PNA is a DNA/RNA mimic in which the PO linkage is replaced with a peptide bond [6,7]. There are a number of potential advantages of using PNA for gene silencing compared to PS ODN: (1) PNA hybridizes more efficiently than DNA (or RNA) to the complementary sequence (RNA or DNA) [7], (2) PNA can also exert anti-gene activities by binding to a specific sequence in double stranded DNA and forming extremely stable helix invasion complexes [7], (3) PNAs are stable in serum as well as in various cell extracts and towards proteases and peptidases [7], and (4) PNA may be associated with significantly reduced toxicity due to its neutral backbone and thus minimal non-specific interactions with proteins [7,8]. A major limitation for PNA application is its poor cellular uptake. A number of methods have been proposed to improve its intracellular delivery including: (a) conjugation with a targeting ligand (Zhou and Li, unpublished data), (b) conjugation

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with a penetration peptide [9,10], and (c) derivatization with a lipid and incorporation into liposomes [11]. Recently, Corey's group has developed a method that is highly efficient in intracellular deliver of PNA in vitro using cationic liposomes [12]. This involves annealing of PNA with a short, biologically inert PO ODN that subsequently serves as a carrier to form complexes with cationic liposomes [12]. The carrier ODN are dissociated from PNA and degraded following intracellular delivery while the PNA exerts its sequence-specific antisense activity. In this study we examine whether a similar strategy can be utilized to deliver PNA to lung endothelial cells in intact mice via systemic route. Its immunotoxicity was also compared to PS ODN delivery.

Materials and methods

Chemicals. 1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP) was purchased from Avanti Lipids (Alabaster, AL). PNA monomers, Fmoc-T-OH, Fmoc-C(Bhoc)-OH, Fmoc-G(Bhoc)-OH, and Fmoc-A(Bhoc)-OH were obtained from Applied Biosystems (Foster City, CA). Fmoc-XAL-PEG-PS resin was also from Applied Biosystems. All other chemicals were of reagent grade.

ODN. ODN of PS linkage was purchased from Midland Certified Reagents Company (Midland, TX) and its sequence is 5′-TCCATG ACGTTCCTGATGCT (ODN 1668) [13]. The two inert carrier ODN have a PO linkage and their sequences are 5′-AGCATCAGG-3′ and 5′GGAGCATGA-3′, respectively.

Synthesis of PNA. Two PNAs (5′-TCCATGACGTTCCTGAT GCT-3′ and 5′-CCGTGGTCATGCTCC-3′) were synthesized and used for TNF-α response and distribution study, respectively. For the latter study, PNA was labeled with rhodamine (Rho). PNAs were synthesized using an Applied Biosystems Expedite 8909 Synthesizer using Fmoc protected monomer reagents and XAL-PEG-PS resin from Applied Biosystems. Rho-labeled PNA was synthesized as the following. Briefly, Fmoc-XAL-PEG-PS resin was deprotected by 2 ml of 20% piperidine, followed by extensive washing. Coupling of Lys (Rho) was achieved via reaction with 5 molar equivalent Fmoc-Lys(Rho)-OH in the presence of HBTU, HOBt, and DIPEA. The resin was extensively washed and loaded onto Expedite 8909 synthesizer. PNA was then synthesized as described above. The crude Rho-PNA was obtained after cleavage by TFA with 5% m-cremsol scavenger. It was purified by reversed-phase HPLC and kept at −20 °C until use.

Annealing of PNA with PO carrier ODN. Hybridization of PNA with ODN was performed according to the method described by Doyle et al. [14]. Briefly, equal moles of PNA and ODN were mixed in PBS and annealing of PNA and ODN was performed in a thermal cycler according to the following temperature profile. Reductions in temperatures occurred in 1 min with hold times indicated (°C, min): 95, 5; 85, 1; 75, 1; 65, 5; 55, 1; 45, 1; 35, 5; 25, 1; and 15 °C, 1 min, hold at 15 °C. The annealed PNA:ODN duplexes were directly used without further purification.

Preparation of liposomes. DOTAP liposomes were prepared as follows. The lipid in chloroform was dried as a thin layer in a 100-ml round-bottomed flask which was further dried under vacuum for 2 h. The lipid film was hydrated in 5% dextrose in water to give a final concentration of 10 mg DOTAP/ml. Preparation of small unilamellar vesicles by extrusion was performed as follows. The lipid solution was briefly sonicated, followed by incubation at 50 °C for 10 min and then sequentially extruded through polycarbonate membranes with the following pore sizes: 1.0, 0.6, and 0.1 μm. The size of liposomes was measured by dynamic laser scattering using a Coulter N4SD particle sizer (Hialeah, FL).

Lung uptake of PNA following i.v. injection of DOTAP/PDN:ODN complexes. PNA:ODN duplexes were mixed with cationic liposomes at a charge ratio of 4/1 (+/−) and the mixture was incubated at RT for 20 min, prior to injection at a dose of 25 μg Rho-labeled PNA per mouse. At 30 min after injection, mice were killed and lungs were perfused intravascularly with PBS followed by 2% paraformaldehyde in PBS and inflated with this fixative to near total lung capacity. The lungs were rinsed with cold PBS and immersed in 30% sucrose in PBS at 4 °C overnight. The lungs were then quickly frozen in OCT with dry ice. Five-micrometer lung cryo-sections were then cut. Following three washes in PBS containing 0.5% bovine serum albumin and 0.15% glycine (PBG buffer) sections were incubated in a 1:100 dilution of rat anti-mouse PECAM (PharMingen, San Diego) for 1 h at RT, washed with PBG three times, and labeled with Alexa 488-labeled goat anti-rat IgG for 1 h at RT. Following 3 further washes with PBG the sections were stained with Hoescht dye 33258 (Sigma) for 30 s and mounted in Gelvatol (Monsanto, St. Louis). Cells were visualized using an Olympus Provis microscope (Olympus, Tokyo, Japan) using a triple pass (blue/green/red) cube, which allows excitation at 384 nm and collection at 540 nm. Images were collected using an Optronics Magnifier Camera (Santa Barbara, CA) or with a Leica TCS NT confocal microscope with a 60× oil immersion objective at 1024 × 1024 pixel resolution.

In vivo toxicity assays. Groups of six mice received tail vein injection of dextrose, DOTAP liposome, or liposomes complexed with PNA:ODN, PO carrier ODN, or PS ODN 1668. The doses for DOTAP, PNA, and ODN were 212, 25, and 25 μg per mouse, respectively. Twenty-four hours after injection, mice were anesthetized and blood was collected by retroorbital bleeding. The serum levels of transaminases (alanine aminotransferase [ALT] and aspartate aminotransferase [AST]) were then examined. Serum samples were analyzed by the Antech Diagnostics (Farmingdale, NY).

In a separate experiment, groups of six mice received tail vein injection of liposomes complexed with PNA:ODN or ODN 1668 as described above. Mice were bled from retroorbital sinuses under anesthesia 2 h following the injection. Serum levels of mouse tumor necrosis factor (TNF)-α were determined with a specific immunoassay kit for mouse TNF-α (R&D, Minneapolis, MN).

TNF-α induction in cultured macrophages exposed to PNA, free or complexed with DOTAP liposomes. RAW cells were plated in a 24-well plate at a cell density of $1–2 \times 10^5$ cells/well and then allowed to culture overnight. Cells were washed three times with serum-free medium and PNA (1 or 2 μg/well) or PNA:ODN (2 μg PNA/well) complexed with DOTAP liposomes were added to cells. Other controls include 1668 ODN (1 μg/well) and medium. Four hours later, the transfection medium was replaced with complete medium and cells were cultured for another 12 h. TNF-α levels in the supernatants of treated cells were determined with a specific immunoassay kit for mouse TNF-α (R&D, Minneapolis, MN).

Statistical analysis. Data are expressed as means ± standard deviation and analyzed by the two-tailed unpaired Student's *t* test using the PRISM software program (GraphPad Software, San Diego, CA). Data were considered significant if $P < 0.05$(*) and very significant if $P < 0.01$(**).

Results

Cationic liposomes mediated efficient delivery of PNA: ODN to pulmonary endothelium in intact mice

Rho-labeled PNA (5′-CCGTGGTCATGCTCC-3′) was annealed with a carrier ODN (5′GGAGCATGA-3′) and the resulting duplexes were complexed with DOTAP liposomes. Successful hybridization of PNA with

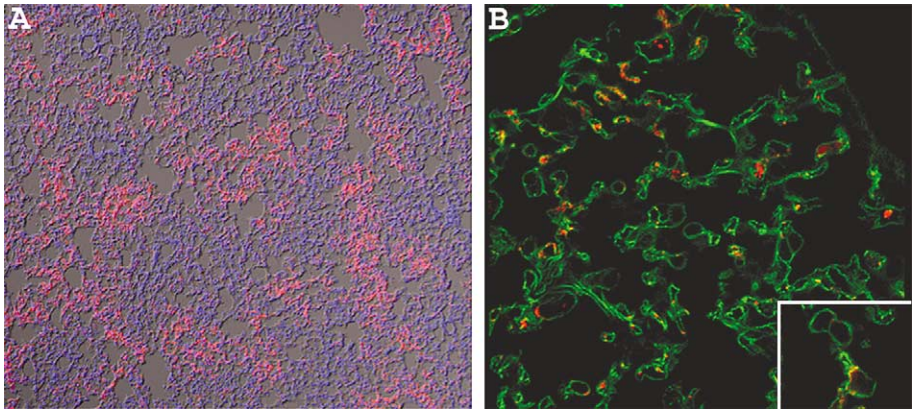

Fig. 1. Distribution of PNA in mouse lungs 30 min following i.v. administration of Rho-PNA:ODN complexed with DOTAP lipoosmes. (A) A representative image taken with a fluorescence microscope. In a separate experiment, sections were incubated in a 1:100 dilution of rat anti-mouse PECAM and then labeled with Alexa 488-labeled goat anti-rat IgG for 1 h at RT. (B) A representative image taken with a confocal fluorescence microscope.

carrier ODN was confirmed by PAGE analysis (data not shown). DOTAP liposomes were used since a previous study showed that this formulation was highly efficient in delivering ODN to lung endothelial cells in intact mice [15]. Fig. 1A shows the distribution of Rho-labeled PNA 30 min following i.v. injection. PNA was efficiently accumulated in the alveolar capillary region. The type of cells that took up PNA was further analyzed by anti-platelet endothelial cell adhesion molecule-1 (PECAM-1) immunofluorescence staining of lung sections. Constitutive expression of PECAM-1 is a fundamental characteristic of endothelial cells [16]. As shown in Fig. 1B, Rho-PNA was largely co-localized with PECAM staining (green), suggesting that endothelial cells are the major cell type that takes up PNA. There was hardly any accumulation in the lung when free PNA was used (data not shown).

Systemic delivery of PNA is associated with minimal CpG-related toxicities

We have previously shown that systemic administration of CpG-ODN, alone or complexed with cationic liposomes, triggered a potent proinflammatory cytokine response [15,17,18]. In this study we investigated whether a PNA of the same sequence will similarly induce a cytokine response. A PNA that has a same sequence as that of ODN 1668 was synthesized and complexed with

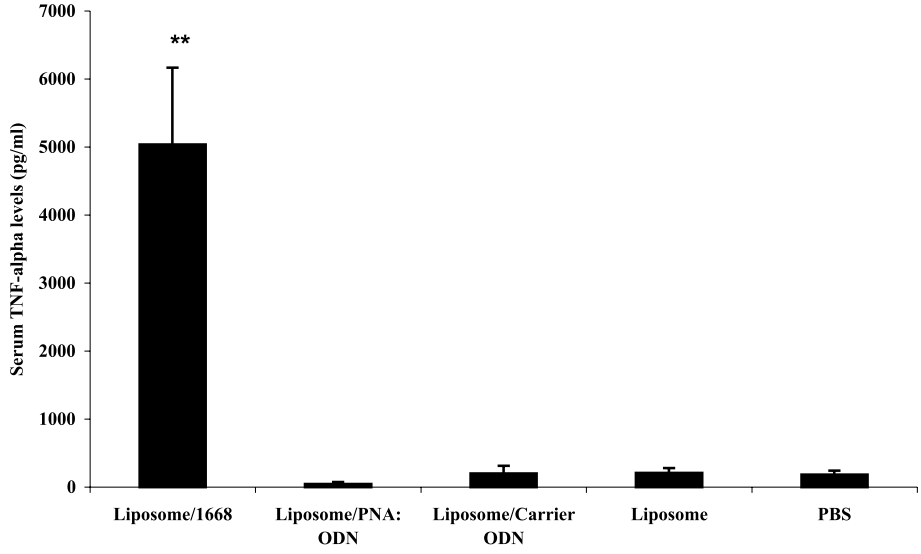

Fig. 2. Serum TNF-α levels 2 h following i.v. injection of PNA:ODN complexed with DOTAP liposomes. Groups of six mice received tail vein injection of dextrose, DOTAP liposome, or liposomes complexed with PNA:DNA, carrier ODN or ODN 1668. The doses for DOTAP, PNA, and ODN were 212, 25, and 25 μg per mouse, respectively. Two hours following injection, blood was collected and the levels of TNF-α in serum were determined using a specific ELISA kit. ** $P < 0.01$ (vs PBS).

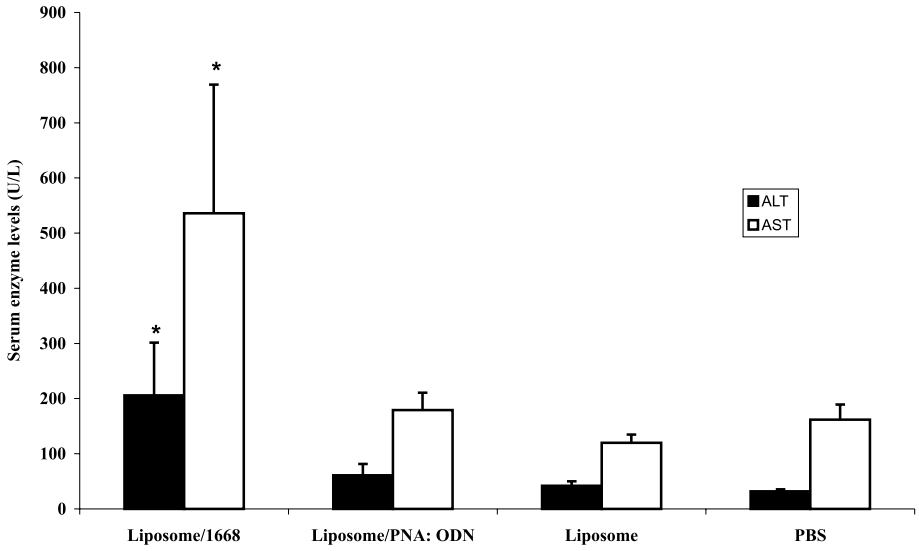

Fig. 3. Serum levels of transaminases after intravenous injection of PNA:ODN complexed with DOTAP liposomes. Mice received tail vein injection of PNA:DNA complexed with liposomes as described in the legend to Fig. 2. Twenty-four hours following injection, blood was collected and the levels of transaminases in serum were determined. *$P < 0.05$ (vs PBS).

DOTAP liposomes via a PO carrier ODN (5′-AGC ATCAGG-3′). In agreement with previous studies [15,17,18] intravenous injection of DOTAP/ODN 1668 complexes resulted in a potent TNF-α response (Fig. 2). Interestingly, dramatically different from the ODN counterpart, systemic administration of PNA of the same sequence is essentially inert in triggering the TNF-α response. Injection of the non-CpG carrier ODN complexed with DOTAP liposomes also gave a background level of TNF-α response. Fig. 3 shows the serum levels of transaminases 24 h following i.v. injection of PS ODN or PNA. Injection of 1668 ODN resulted in a significant increase in the serum level of transaminases. In contrast injection of liposome/PNA:ODN was associated with minimal changes in the serum levels of transaminases (Fig. 3). A control of free PNA could not be included in the studies (Figs. 2 and 3) due to the poor solubility of this sequence in aqueous solutions.

PNA is inert in triggering TNF-α response in cultured mouse macrophages

To further confirm that PNA is inert in CpG-associated immunotoxicity, TNF-α response was also ex-

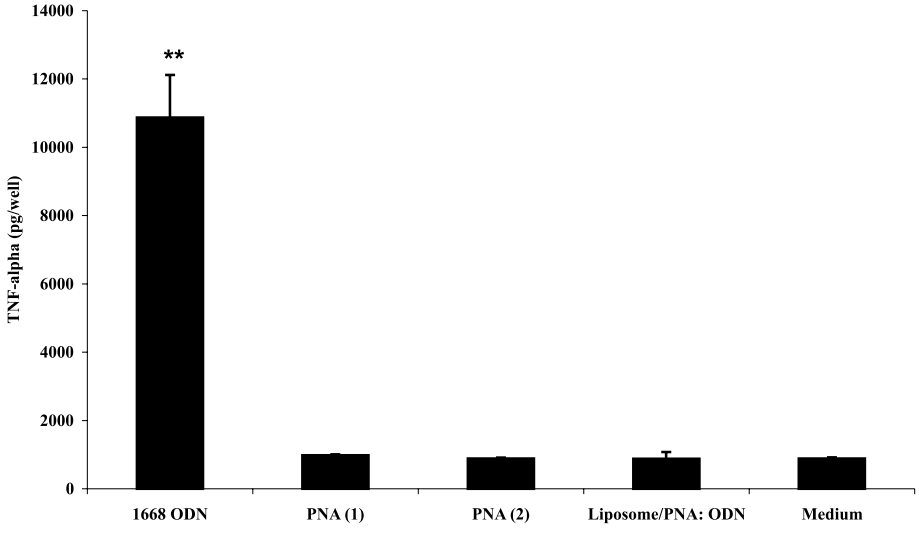

Fig. 4. TNF-α response in cultured RAW cells following exposure to PNA. RAW cells were exposed to 1668 ODN (1 μg/well), PNA (1 or 2 μg/well) or PNA:DNA (2 μg/well) complexed with DOTAP liposomes. Four hours later, the transfection medium was replaced with complete medium and cells were cultured for another 12 h. TNF-α levels in the supernatants of treated cells were determined. PNA (1): 1 μg PNA/well; PNA (2): 2 μg PNA/well. ** $P < 0.001$ (vs medium).

amined in cultured mouse macrophages following exposure to PNA, alone or complexed with DOTAP liposomes via a carrier ODN. In agreement with previous studies [13], addition of 1668 PS ODN to RAW cells triggered a strong TNF-α response (Fig. 4). In contrast, there was essentially no difference between free PNA and medium control with respect to TNF-α response. Complexation of PNA with DOTAP liposomes via a carrier ODN also had minimal effect on TNF-α response (Fig. 4), despite a significant improvement in its intracellular delivery (data not shown).

Discussion

We have recently shown that cationic lipids mediate efficient delivery of ODN to pulmonary circulation following systemic administration [15]. In the present study we showed that PNA that is annealed to a short ODN could also be efficiently delivered to pulmonary microvasculature by cationic liposomes. Co-localization with PECAM immunohistochemical staining suggests that endothelial cells are the major cell type that takes up PNA.

PNA is a DNA/RNA mimic in which the polyanionic PO linkage is replaced with a neutral peptide backbone. Thus systemic administration of PNA may avoid a number of side effects that contribute to the polyanionic nature of ODN. In the present study we have also shown for the first time that systemic administration of PNA avoids the CpG cytokine response that is associated with ODN. We and other have previously shown that systemic administration of ODN that contains a CpG motif triggers a potent proinflammatory cytokine response and that such response is augmented when the CpG-ODN is delivered by cationic lipids [17–20]. The potency of this response is also affected by the chemical structure of backbone: PS CpG-ODN is significantly more potent than a PO counterpart [18]. While this cytokine response can be beneficial for the treatment of cancers [18], it needs to be well controlled when ODN are used for the treatment of non-malignant diseases, particularly inflammatory diseases. This may render antisense development difficult. Due to the fact that there are limited available sites that can be efficiently targeted by antisense ODN due to the secondary structure of a given mRNA, an active ODN that is obtained following rigorous screening may contain a potent CpG motif. This problem might be resolved via the use of PNA: the CG-containing PNA is essentially inert in inducing TNF-α response (Figs. 2 and 4). Eventually the molecule can be designed such that the CpG motif is replaced with PNA to reduce the CpG-related immunotoxicity while the remaining non-CpG portion of the ODN can efficiently interact with cationic liposome to achieve intracellular delivery. In addition to the simplicity of delivery, PNA/DNA chimera might be more efficient than PNA in antisense activity. Recent studies have shown that unlike ODN, PNA/RNA dose not serve as a substrate for RNaseH and PNA exhibits its antisense activity solely via blocking the translation [21]. In contrast, PNA/DNA chimera can achieve its antisense activity via both mechanisms [21,22]. We are currently examining the feasibility of delivering PNA/DNA chimera to pulmonary endothelium via cationic liposomes.

It is not clear at present why ODN and PNA differ so dramatically with respect to their CpG-immune response. Hemmi et al. [23] reported TLR9 as a receptor of bacterial DNA containing stimulatory CpG motifs. However it is not known whether TLR9 is involved in the initial interaction with the CpG-containing DNA (ODN) and the precise cellular localization of TLR9 is not known either. Internalization of the CpG DNA appears to be necessary for activation to occur. The endosomal acidification also appears to be necessary for CpG induced activation [24]. Immune responses can be inhibited by drugs that block endosomal acidification or maturation [25]. It appears that lysosomal processing by acidic nucleases is necessary, especially in the case of double stranded DNA (dsDNA), to initiate a biological effect [26]. The fact that CG-PNA is inactive in triggering an immune response might be due to its resistance to acidic nucleases. It is also possible that CG-PNA is poorly recognized by TLR9. More studies are required to better understand the structure–function relationship.

In summary, we have developed a strategy for efficient delivery of PNA to pulmonary endothelium. We have also shown for the first time that unlike the CpG-ODN while is highly pro-inflammatory, CG-containing PNA is essentially inert in TNF-α response in cultured macrophages and in intact mice. Delivery of PNA to pulmonary endothelium may prove to be a therapeutically useful for the treatment of pulmonary diseases. It may also be used as a research tool to study the function of novel genes in pulmonary endothelium.

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