Original Article

Cellular Morphology and Immunologic Properties of *Escherichia coli* Treated with Antimicrobial Antisense Peptide Nucleic Acid

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ABSTRACT

Background & Objectives: **Antisense peptide nucleic acids (PNA) that target growth essential genes show potent bactericidal properties without cell lysis. We considered the possibility that whether PNA treatment influence the bacteria total nucleic acids content and apply approach to develop a new delivery system to Dendritic cells (DCs). DCs are the most potent antigen presenting cells in the immune systems. Since the uptake of bacteria by DC is a necessary step for generation of effective DNA vaccine, we studied the uptake efficiency of PNA treated bacteria by DCs.**

Material & Methods: **Total nucleic acids of anti- *acyl carrier protein* (*acpP*) peptide-PNA treated Escherichia coli Hb101 containing plasmid have been isolated. In addition, peripheral blood monocytes have been purified using mouse anti- cluster of differentiated (CD14) coated magnetic beads and then culture in presence of growth factors. Generated DCs have been assessed for their ability of uptake of flurescein isothiocyanate (FITC) - labeled peptide-PNA treated bacteria and heat inactivated by flow cytometry.**

Results: **The preparation of total nucleic acid from peptide-PNA treated showed five distinct bands which correspond to chromosomal DNA, plasmid DNA, 23S ribosomal RNA (rRNA), 16S rRNA and mixture of small RNA. DCs incubated with FITC- labeled peptide-PNA treated and heat inactivated bacteria showed almost equal fluoresence intensity.**

Conclusion: **Peptide–PNA treatment bacteria are intact and do not appear to alter cell barriers and nucleic acid content. They can be taken up by DCs efficiently. These finding may confirm new application for peptide-PNA in immunology and DNA vaccine.**

Keywords: Peptide nucleic acids, Dendritic cell, Ghost cell, Flow cytometry

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Introduction

Conventional antibiotics target bacterial enzymes in one of the following processes; cell wall biosynthesis, fatty acid biosynthesis, folic acid biosynthesis, DNA/RNA synthesis and translation. When sufficient inhibition or damage to one or more cell pathways or structure occurs, the bactericidal effect appears and this is often associated with cell lysis due to the extensive damage content (1, 2). Bacterial growth essential pathways are most commonly targeted at the protein level. Also there are possibilities to target it at other steps such as mRNA level.

There are several different procedures available to silence bacterial gene expression. One of these procedures is antisense technology. This approach typically involves antisense oligonucleotides, which can hybridize to a specific complementary mRNA, and then the function of mRNA is blocked.

Peptide nucleic acid (PNA) is a stable mimic of DNA. Due to its unnatural backbone, it is very stable against nucleases and peptidases. This molecule can hybridize to DNA or RNA strongly. The potency of PNA to inhibit the translation and decrease the protein synthesis specifically in a number of different organisms has been reported (3).

Theoretically the PNA antisense targets the mRNA of every gene in bacteria. It has been shown that when the essential gene such as *acyl carrier protein (acpP)* in *Escherichia coli* is targeted, the bactericidal effect will appear (4). The anti-*acpP* peptide–PNA is more potent than some conventional antibacterial such as ampicillin in *in vivo* infection models and also in *in vitro* (5-7). It has been reported that PNA accumulates in bacteria and shows a long retention period which appear to explain the bactericidal effects observed (8).

If PNA can kill bacteria without lysis, it may also be possible to use the approach to develop a new vaccine candidate and delivery system like as bacterial ghost. With novel development in science and technology, vaccinology has been enlarging its area to cover both prophylactic and therapeutic vaccines against cancer, allergy, autoimmune diseases as well as infectious diseases including bacterial and viral diseases (9).

Vaccines are traditionally prepared with either live attenuated or killed bacteria or viruses. They can induce all aspects of immune responses and in many cases; these approaches have proven to be successful. However, safety concern may limit the use of these approaches. New generation vaccines, such as recombinant protein-based vaccines and DNA vaccine are thought to be potentially safer than traditional vaccines. These new generation vaccines are often very poorly immunogenic, partially because the component having adjuvant activities were discarded in purification or synthetics process (10).

To take advantage the immunestimulatory effects of whole cell-based vaccines and avoid its disadvantages new approaches are needed. Some attentions are focused on alteration approach such as bacterial ghosts. Bacterial ghosts are empty non-denaturated envelopes derived from bacteria by protein E-mediated lysis, which retain all morphological and structural features of the natural cell. They can be used as vaccine candidates with intrinsic adjuvant properties and as a delivery system for proteins/antigens, nucleic acids, drugs and soluble compounds (11, 12).

In this study, we wanted to know more about the characters of *E.coli* killed using PNA antisense and possible applications to develop a new vaccine candidate and delivery system.

Material and Methods

Bacteria, growth medium, and antimicrobials.

This work was experimental study on *E. coli* strains as a model organism. *E. coli* strains were obtained from the Coli Genetic Stock Center (http://cgsc.biology.yale.edu). Mueller– Hinton (MH) and Luria Bertani (LB) growth mediums, SYBR Green I stain were purchased from Sigma-Aldrich (St. Louis, MO). The anti-*acpP* ((KFF) 3K-eg1-ctcatactct) complements to the start codon region of the *acpP* was obtained fro Panagene (Daejeon, Korea). Cells were grown in 9 well low attachment plates (COSTAR 3474, Corning Lowell, MA).

Treatment of *E. coli* Hb101/pGL3 with anti-*acp* peptide-PNA.

E. coli Hb101/pGL3 cultures were grown overnigl and diluted to 5×10^5 cells/ml in MH broth, and 8 µl aliquots were added to wells of a low attachme 96-well plate. Anti-*acpP* peptide-PNA aliquots (20 µl) of 2 µmol/l concentration were added to give a final volume of 100 µl. Plates were incubated for 20 h at 37 °C in a VersaMax spectrophotometer with shaking, and optical density (OD_{550}) measurements were taken every 5 min. The killing effect on bacteria was assessed by preventing the measurable growth

after 20 hours and plating the samples on LB agar plates for overnight at 37 °C.

Total nucleic acids extraction from anti-*acpP* peptide-PNA treated *E. coli* Hb101/pGL3.

Total nucleic acids were extracted essentially as described (13). First the eukaryotic expression pGL3 vector was transformed to *E. coli* Hb101. The *E. coli* K12 wild type and some engineering strains with different plasmids have been checked; finally we found that *E. coli* Hb101 containing pGL3 plasmid is an established strain to continue. The transformed strain along with *E. coli* Hb101 was treated with final concentration of 2 µmol/l for 20 hours as described above. The harvested cells were resuspended in 50 µl STE buffer containing 100 mM NaCl, 10 mM Tris buffer, pH 7.0, 1 mM EDTA followed by the addition 50 µl phenol to release the nucleic acids from the cells. After 30 second of vigorous vortex, the mixture was centrifuged for 5 min at 13000g. A normalized volume of supernatant was loaded on 1% agarose gel. The gel was stained with SYBR Green I and scanned using a Typhoon 9400 Scanner (Amersham Biosciences, Piscataway, NJ) using green excitation (532 nm) and emission (526 nm, short pass) filters and the photomultiplier tube set at 550 V. Images were processed using ImageQuant software version 5 (Amersham Biosciences).

Blood cell separation

The blood Buffy coat of healthy individuals was obtained from Karolinska Institutet Blood Center. Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation at 2000 rpm for 20 min on a ficoll-paque gradiant (Ficoll- Pagued / Amersham-Pharmacia).

Generation of dendritic cells (DC)

DC was isolated from $CD14^+$ precursors as previously described (14). Briefly, $CD14^+$ monocytes were immunomagnetically isolated from PBMC by passing through Mini MACS column (Miltenyi Biotech, Bergisch Gladbach, Germany) following incubation with magnetic particles covered with Mouse Anti cluster of differentiated 14 (CD14) based on company protocol for 15 min at 4ºC. one million/ml $CD14^+$ monocytes were cultured in RPMI medium containing (100U/ml) ampicillin, (100 µg/ml) streptomycin, (500 U/ml) recombinant human interleukin – 4 (rhIL-4) and (1000 U/ml) recombinant human granulocyte macrophage colony- stimulating factor (rhGM-CSF) (Schering-Plaugh Research Institutet, Kenilworth, Ng/ USA). In day three the culture medium was changed and in day five the immature DCs was applied to study the phagocytosi efficiency (15).

Labeling peptide- PNA treated and heat kille bacteria with FITC.

E. coli Hb101/pGL3 was treated with 2 µm/l anti *acpP* peptide-PNA for 20 hours as described abov section. Also, *E. coli* Hb101 was killed by incubatio for 10 min at 70ºC on hot block. Full killing wa examined with plating on LB plate. 1mg/ml solutio of FITC in phosphate buffered saline (PBS) was filtered through a 0.45 µm membrane filter. A heavy suspension of bacteria in 1ml PBS buffer was prepared in 14 ml tube and then an equal volume of FITC (Flurescein isothiocyanate) solution was added. The tube was rotated gently at 37º C for 30 min. The bacteria were rinsed twice with PBS buffer by centrifugation at 2500 rpm for 5 min (16). Finally, FITC labeled bacteria was resuspended in 1 ml PBS and adjusted to 1 at OD_{550} (approximately 10^9 CFU/ml)

Fluorescence microscopy.

Peptide-PNA treated FITC labeled bacteria were incubated with immature DCs at a ratio 100:1 for four hours at 37º C and 5% CO_2 and then observed with a Leica DMRA2 microscope (Leica Mikroskopie and Systeme GmbH, Wetzlar, Germany) at ×1000 and the images were processed using Openlab software version 3.1.4 (Improvision, Coventry, England).

Uptake of FITC labeled *E. coli* treated with peptide-PNA or inactivated with heat by dendritic cells

Immature DCs were incubated with FITC labeled bacteria at a ratio of 1:100 for four hours at 37ºC and 5% CO_2. DCs were rinsed twice with PBS and stained with antibodies against HLA-DR and CD_{1a} markers. The cells posses these two markers were considered as DC. The fluorescence intensity was measured by FACS (Fluorescent activated cell sorting) instrument. Fluorescence of FITC labeled bacteria bound to the DC surface was quenched with trypan blue (TB) (0.5 µg/ml in tris Hanks buffer) added to the cell suspension prior to flow cytometry. Unpulsed DC was used as negative control.

Results

Peptide- PNA treated bacteria contain nucleic acids

The previous experiment shows that the peptide-PNA treatment does not lyse the bacteria. To test

whether this treatment affects on bacteria nucleic acids content, total nucleic acids were isolated from the untreated and treated *E. coli* Hb101 containing pGL3 plasmid using STE/ phenol method. The preparation of total nucleic acid from these cells was done and five distinct bands which correspond to chromosomal DNA, plasmid DNA, 23S rRNA, 16S rRNA and mixture of small RNA were observed when analyzing the extracted nucleic acids on agarose gel. These results are similar to untreated bacteria (Fig. 1). Therefore peptide–PNA treatment did not appear to alter nucleic acid content.

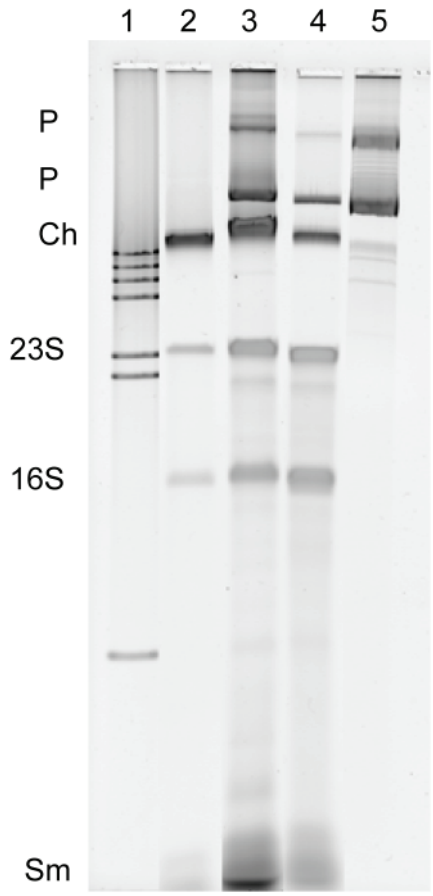

Fig. 1: Effect of PNA treatment on cellular nucleic acid content

Dendritic cells (DCs) take-up anti- *acpP* peptide-PNA treated *E. coli* as efficiently as heat treated *E. coli*

We presented FITC conjugated bacteria to dendritic cells (Fig. 2) and the uptake efficiency has been assayed by flow cytometric techniques. Fluoresence intensity in DCs pulsed with FITC- labeled peptide-PNA treated and heat inactivated bacteria were almost equal and were clearly more than unpulsed controls. Trypan blue (TB) was used to quench the surface flurosent due to uninternalized bacteria on DCs (Table 1).

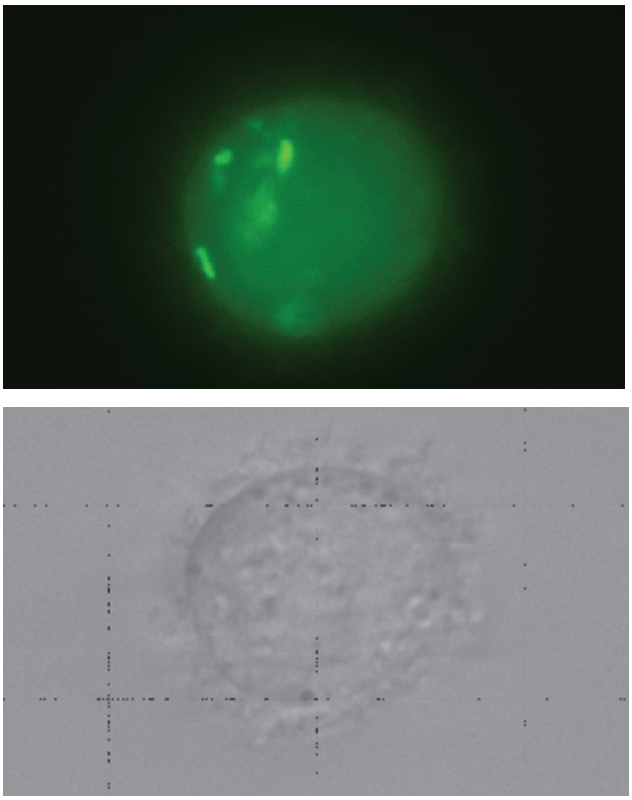

Fig. 2: Denderetic cell (DC) with peptide-PNA treated FITC labeled *E. coli*

Table 1: The average of fluorescence intensity of FITC measured with FACS analysis

	PNA treated	Heat treated	Unpulsed
DC	363	324	10
DC/ TB	124	139	2

DC = Dendritic cell
T.B. =Trypan blue

Discussion

Anti-*acpP* peptide–PNA treatment did not lyse cells or alter gross cellular integrity or nucleic acids content (8). Although the bactericidal activity of conventional antimicrobials are often associated with cell lysis (17). *AcpP* is an essential gene in fatty acid biosynthesis (FAB) pathway which possess critical role in bacterial survival. There is difference between bacterial and mammalian FAB pathways which make it good target for novel antibacterial agents. In particular, the FAB protein ACP encoded by the *acpP* gene has been shown to be a valid target for

developing novel antibacterial agents (4, 18).

Antisense anti-*acpP* peptide–PNAs kill cells in the absence of lysis. This kind of dead cell can be applied in biotechnology. We think that the outer barriers of PNA treated bacteria are intact and contain immune- stimulating compounds, such as LPS and peptidoglycan and therefore have the potential to enhance the immune response to the same extent as bacterial ghosts. Also, they can be used as delivery systems like as bacterial ghost for recombinant proteins, foreign DNA or other pharmacological relevant drugs. The bacterial ghosts evoke the immune system in animal model and have been proposed as an alternative to conventional vaccines (19) and can be applied for delivery drugs and biological material to DCs (20) and macrophages (21).

We have shown here that the anti-*acpP* peptide-PNA treated bacteria keep their nucleic acids which is distinguished to bacterial ghost so this system might be used to deliver genetic information to phagocytes such as DCs and macrophages in immune system and also they could be used to develop DNA vaccine.

In recent years, the DC ability as cellular adjuvant to stimulate antitumor immune responses has been studied (22). Almost all of these studies were promising. Antigen uptake and presentation to immune system by DC is very critical step in this procedure. We reported here for the first time that DCs derived from health individual monocyte take up peptide-PNA killed bacteria as efficient as heat inactivated ones as a control. To show the uptake efficiency, we labeled heat and peptide-PNA treated bacteria with FITC which bind covalently to lysine amino acid residue in surface proteins. There is possibility that free FITC molecule or labeled bacteria attached to DC outer membranes interfere with experiment, so to prevent this interference, the DC cells have been washed with Trypan blue buffer which quenches the surface fluorescence before flow cytometry.

In vitro studies showed that bacterial ghosts filled with plasmid DNA encoding the green fluorescent protein (GFP) were taken up very efficiently by DCs (20) and macrophages (21), and marker protein GFP was expressed at high levels. Also, DNA- loaded bacterial ghost have been tested successfully *in vivo* for their potential as vaccine candidates (23). As we showed here, peptide-PNA killed bacteria are taken up efficiently like as reported for bacterial ghosts. We might be able to extend this similarity to more potential in vaccine candidate and delivery system issues. Although, PNA killed bacteria have some characters which are different from bacterial ghost. Peptide-PNA killed bacteria keep their cytoplamic components such as nucleic acids while they have been lost during bacterial ghost preparation. It has been shown that bacterial DNA but not vertebrate DNA has a direct immunestimulatory effect on immune cells *in vitro* (24). This effect is due to the presence of unmethylated CpG dinucleotide (25). We assume that peptide-PNA killed bacteria will active cells of the innate immune system including macrophages and DCs efficiently.

Conclusion

Our findings open a new field for peptide-PNA technology application in immunology and DNA vaccines. Based on these promising results, we will treat immature DC cells derived from Peripheral blood mononuclear cells (PBMC) with anti- *acpP* peptide-PNA treated *E. coli* containing pGL3 plasmid and assay the expression of luciferase reporter gene.

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