Article

Protection of Mice from Lethal Endotoxemia by Chimeric Human BPI-Fcγ1 Gene Delivery

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To evaluate the potentiality of applying gene therapy to endotoxemia in high-risk patients, we investigated the effects of transferring an adeno-associated virus serotype 2 (AAV2)-mediated BPI-Fcγ1 gene on protecting mice from challenge of lethal endotoxin. The chimeric BPI-Fcγ1 gene consists of two parts, one encods functional N-terminus (1 to 199 amino acidic residues) of human BPI, which is a bactericidal/permeability-increasing protein, and the other encodes Fc segment of human immunoglobulin G1 (Fcγ1). Our results indicated that the target protein could be expressed and secreted into the serum of the gene-transferred mice. After lethal endotoxin challenge, the levels of endotoxin and TNF-α in the gene-transferred mice were decreased. The survival rate of the BPI-Fcγ1 gene-transferred mice was markedly increased. Our data suggest that AAV2-mediated chimeric BPI-Fcγ1 gene delivery can potentially be used clinically for the protection and treatment of endotoxemia and endotoxic shock in high-risk individuals. *Cellular & Molecular Immunology*. 2006;3(3):221-225.

Key Words: BPI-Fcγ1, AAV2, gene therapy, minimal lethal dose (MLD)

Introduction

Lipopolysaccharide (LPS), the major glycolipid component of Gram-negative bacterial (GNB) outer membrances, is a potent endotoxin responsible for many infections. The released endotoxin activates macrophages and endothelial cells to produce and release potent inflammatory mediators such as TNF-α and IL-1β. The mediators, especially TNF-α, cause endotoxic shock syndrome. Although antibiotics kill bacteria, they can not neutralize endotoxin. Drug-resistance is a severe problem in antibiotic therapy, on the other hand, GNB lysis may excessively release endotoxin and instantly strengthen endotoxemia (1, 2).

Bactericidal/permeability-increasing protein (BPI) is a 55 kD human neutrophil granule-associated molecule specific for Gram-negative bacteria (3-5). BPI has specific effect of neutralizing endotoxin and directly killing GNB, but no adverse effect on eukaryotic cells. It has been demonstrated that the N-terminus of BPI is identical to natural BPI in the effect on LPS and GNB (6-10). However, $rBPI_{21}$ has relatively low efficacy and short half-life *in vivo*, and administration of $rBPI_{21}$ in large dosage is very expensive and difficult to maintain an optimal therapeutic level (11).

In order to prolong and improve the activity of recombinant BPI_{21} for clinical therapy of GNB infection, we designed a chimeric BPI and Fcγ1 protein consisting of human BPI functional N-terminus and the human IgG1 Fc segment. It has been demonstrated that the BPI-Fcγ1 protein had the ability to neutralize endotoxin, kill GNB (including drug-resistant GNB), fix complement as well as mediate opsonization (12). Based on our preliminary work, we developed a recombinant adeno-associated virus (AAV) encoding the chimeric BPI-Fcγ1, named as rAAV2-BPI-Fcγ1, and evaluated its efficacy in protecting mice from lethal endotoxin infection.

Materials and Methods

Construction and production of rAAV2-BPI-Fcγ1

Briefly, BPI gene encoding the signal peptide and the functional N-terminus (1 to 199 amino acidic residue) of human BPI, named as BPI_{700}, was generated by RT-PCR using the primers (sense: 5'-CTG GTA CCA TGA GAG AGA ACA TGG CCA-3' and anti-sense: 5'-GCA AGC TTC TAT TTT GGT CAT TAC TGG CAG-3') from the mRNA of HL-60 cell line (ATCC CCL-240). Fcγ1 gene encoding the Fc fragment of human immunoglobulin G1, known as

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$Fc\gamma1_{700}$, was generated by RT-PCR using the primers (sense: 5'-GTA AGC TTC TAC ATG CCC ACC GTG CCC AG-3' and anti-sense: 5'-TCG TCG ACG GAT CCT TAT TTA CCC GGA GAC AGG GAG-3') from the mRNA of human peripheral blood lymphocytes of healthy volunteer. BPI and Fcγ1 were digested with KpnI/HindIII and HindIII/SalI respectively, and then the resulting fragments were inserted into the KpnI/SalI sites of the pSNAV vector containing CMV promoter and SV40 polyA by co-ligation. After screening and sequencing, the pSNAV-BPI-Fcγ1 expression vector containing the expression cassette of BPI-Fcγ1 and AAV serotype 2 inverted terminal repeats was constructed.

rAAV2-BPI-Fcγ1 viruses were prepared by the AGTC Gene Technology Co. Ltd., complying with the guideline of SFDA and GMP facility, according to the protocols as described by Wu ZJ, et al. (13, 14). Briefly, BHK-21 cells (ATCC CCL-10) were transfected with the pSNAV-BPI-Fcγ1 plasmid DNA using Metafectene (Biontex GmbH) and selected by G418. rAAV2-BPI-Fcγ1 viruses were rescued and produced by infecting the G418-resistant BHK-21 colonies containing BPI-Fcγ1 gene with recombinant HSV1-rc/ΔUL2 helper viruses. The rAAV2-BPI-Fcγ1 viruses were purified and diluted to 1×10^{12} v.g./ml for the study.

Expression of BPI-Fcγ1 chimeric gene in CHO cells

rAAV2-BPI-Fcγ1 virus with a multiplicity of infection (MOI) of 1×10^5 and 5×10^5 (v.g./cell) were used to infect CHO-K1 cells (ATCC CCL-61). The infected cells were incubated for 48-72 h in serum-free medium (DMEM/F12) at 37°C in 5% CO_2. The supernatants were analyzed for the presence of secreted BPI-Fcγ1 protein by Dot-blot and Western-blot analyses using HRP-conjugated goat anti-human IgG antibodies (Sigma) and chemiluminescent substrates (Pierce Biotech., Inc.).

Mouse models of gene transfer

BALB/c mice (5-6 weeks, female, provided by the Laboratory Animal Center of The Chinese Academy of Military Medical Sciences, China) were used for gene transferring. rAAV2-BPI-Fcγ1 or rAAV2-EGFP (provided by the AGTC Gene Technology Co. Ltd.) was administered with a 100 μl injection containing 1×10^{11} v.g. through the quadriceps muscles of right hind leg; and 100 μl injection containing PBS as control. All experiments here were performed at interval of two weeks after these injections were administered.

Minimal lethal dose (MLD) of endotoxin to BALB/c mice

We used D-galactosamine (GalN)-sensitized mice to set up model of lethal sepsis. LPS (Sigma) was diluted to 9.0 μg/ml, 7.5 μg/ml, 6.0 μg/ml and 4.5 μg/ml with PBS containing 60 mg/ml D-GalN (Sigma), then 100 μl of the selected dose of LPS was intraperitoneally injected into 4 separate groups of mice. The minimal dose that caused a mortality of 90% ~ 100% within 72 h was determined as the MLD of LPS to BALB/c mice.

RT-PCR analysis

mRNA was extracted from rAAV2-BPI-Fcγ1 injected mouse muscles by the Oligotex Direct mRNA Kit (Qiagen, Hilden, Germany). RT-PCR was performed according to the manufacturer's instruction for Access RT-PCR System (Promega, Madison, WI, USA) in order to detect BPI-Fcγ1 gene expression at mRNA level. The specific primers used in RT-PCR are P1 and P4 described above.

Enzyme linked immunosorbent assay (ELISA)

To examine the secreted BPI-Fcγ1 in mouse sera, a modified ELISA was performed as follows. The serum (100 μl) of BPI-Fcγ1-transferred mice was fully absorbed by nitrocellulose membrane for 15 min, while 100 μl serum from rAAV2-EGFP mice as control and 100 μl 10% albumin solution as blank. The nitrocellulose membranes were dried in air for 20 min and laid on naked microtiter plates, and then tested by a biotinylated antibody against human BPI according to the protocol of Human BPI ELISA Kit (HyCult Biotechnology).

Tumor necrosis factor-α (TNF-α) in serum samples was detected by ELISA accrording to the kits from R&D Systems Inc.

Limulus amebocyte lysate (LAL) assay

Serum samples were diluted (1:10) by pyrogen-free water, and then tested according to Limulus Amebocyte Lysate Kit (Shyihua Corp., Shanghai, China).

Histopathological observation

The paraffin-embedded sections of the main tissues of liver, small intestine, spleen and kidney at 6 h post lethal LPS challenge were prepared and examined by standard Haematoxylin and Eosin (H&E) staining.

Statistical analysis

Data were analyzed by SPSS10 and presented as mean ± SD. Chi-square test was performed for the survival rate comparison. Differences among groups were analyzed by independent-samples *t*-test. The *p* values less than 0.05 were considered to be statistically significant.

Results

Expression of chimeric BPI-Fcγ1 in cells and mouse tissue

About 60% confluent CHO-K1 (ATCC CCL-61) cells were infected by rAAV2-BPI-Fcγ1 at various MOI (multiplicity of infection) 48 hours after transferring. Dot-blot analysis demonstrated the presence of secreted BPI-Fcγ1 in the conditioned medium in a virus load-dependent manner (Figure 1A). The protein appeared as two expected bands at 48 kD (DTT-deoxidized or reduced) and 96 kD (non DTT-deoxidized, non-reduced) by Western blot analysis (Figure 1B).

The injected muscles were examined by RT-PCR and immunohistochemical staining to identify BPI-Fcγ1 expression in BPI-Fcγ1-transferred mice two weeks after intramuscular administration. An expected 300-bp band was found by

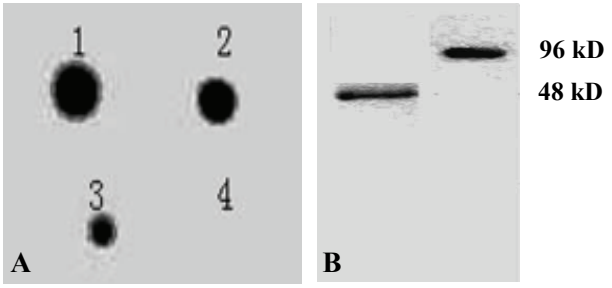

Figure 1. Expression of chimeric BPI-Fcγ1 protein in CHO cells infected with rAAV2-BPI-Fcγ1 virus. Dot & Western blot analysis of the conditioned medium of CHO-K1 infected by rAAV2-BPI-Fcγ1 virus at various MOI. (A) The supernatants of samples were dotted. The MOI was 5×10^5 (dot 2) and 1×10^5 v.g./cell (dot 3) respectively. Human IgG1 (0.1 μg) was dotted as positive control (dot 1), the supernatants of CHO-K1 cells without infection was dotted as negative control (dot 4). (B) Western blot analysis of the 10-fold concentrated medium of CHO-K1 infected with rAAV2-BPI_{23}-Fcγ1 virus at 5×10^5 v.g./cell (MOI). Left was DTT treated (reduced) and right was non-DTT treated (non-reduced).

RT-PCR (Figure 2). The results indicated that the chimeric BPI-Fcγ1 gene was successfully transcribed in the injected muscles of BPI-Fcγ1-transferred mice 2 weeks after injection.

Blood samples from orbital bulb were collected 2 weeks after administration of vector genomes. The serum was analysed by ELISA to detect the secreted BPI-Fcγ1 protein in serum. The OD_{450} value for the serum from rAAV2-BPI-Fcγ1 gene transferred mice was 0.849 ± 0.164 (n = 3), while those for the serum from rAAV2-EGFP gene transferred control mice and PBS control mice were 0.283 ± 0.026 (n = 3) and 0.290 ± 0.020 (n = 3) respectively. There was statistically significant difference between rAAV2-BPI-Fcγ1 gene transferred mice and the control mice ($p < 0.05$) but not between rAAV2-EGFP gene transferred control mice and PBS control mice ($p = 0.832$). It was proved that there was secreted BPI-Fcγ1 protein in the serum of rAAV2-BPI-Fcγ1 gene transferred mice.

Protection of mice from lethal LPS attack

To titrate the minimal lethal dose (MLD) of LPS to BALB/c mice, different doses of LPS were administered intraperitoneally to groups of 10 BALB/c mice. The mortality rate of each group injected with the different doses of LPS within 24 h were 100%, 100%, 90% and 60%, respectively. Hereby, 600 ng/mouse was determined as the MLD of LPS to BALB/c mice.

To prove protection of mice from endotoxemia, BPI-Fcγ1-transferred mice were attacked by MLD LPS intraperitoneally two weeks after transferring. Then, the survival rate was observed within 24 hours after LPS challenge (Table 1). The survival rate of BPI-Fcγ1-transferred mice (40%) was significantly higher than those of EGFP-transferred mice (5%) and PBS control mice ($p < 0.05$), while there was no statistically significant difference between that of EGFP-transferred mice and that of PBS control mice ($p = 0.916$). The results suggested that AAV2-mediated BPI-Fcγ1 gene transfer protected mice from lethal endotoxemia.

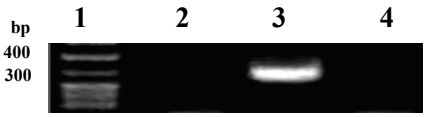

Figure 2. The injected muscles were examined by RT-PCR. An expected 300-bp band was found in BPI-Fcγ1-transferred mice (Lane 3), which were not present in the controls. Lane 1, pBR322 DNA/Msp I marker; Lane 2, PBS; Lane 4, rAAV2-EGFP.

Biological functions of secreted BPI-Fcγ1

To further evaluate the biological activity of secreted BPI-Fcγ1 protein in rAAV2-BPI-Fcγ1 gene transferred mice, the levels of endotoxin and TNF-α *in vivo* were measured after MLD LPS attack. Blood samples were collected from orbital bulb at 30 min, 60 min and 120 min after attack. Each 100 μl of the mixed serum sample was detected by LAL assay for endotoxin and by ELISA for TNF-α. The level of endotoxin in the serum of BPI-Fcγ1-transferred mice reached its peak at 60 min and was significantly lower than that of

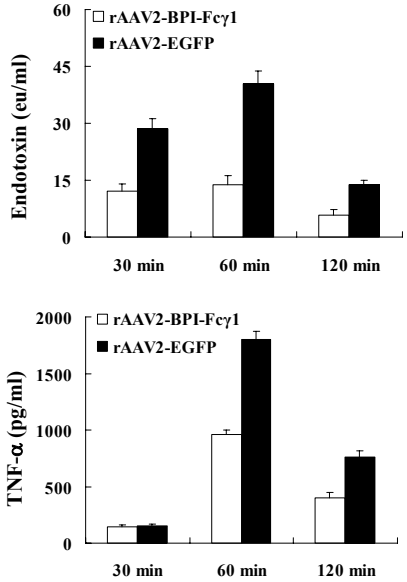

Figure 3. The level of endotoxin and TNF-α in serum after lethal endotoxemia challenge. Mice injected intraperitoneally with either rAAV2-BPI-Fcγ1 or rAAV2-EGFP were attacked by MLD LPS. Blood samples were collected from orbital bulb at 30 min, 60 min and 120 min after attack. Each 100 μl of the mixed serum sample was detected by LAL assay for endotoxin and by ELISA for TNF-α.

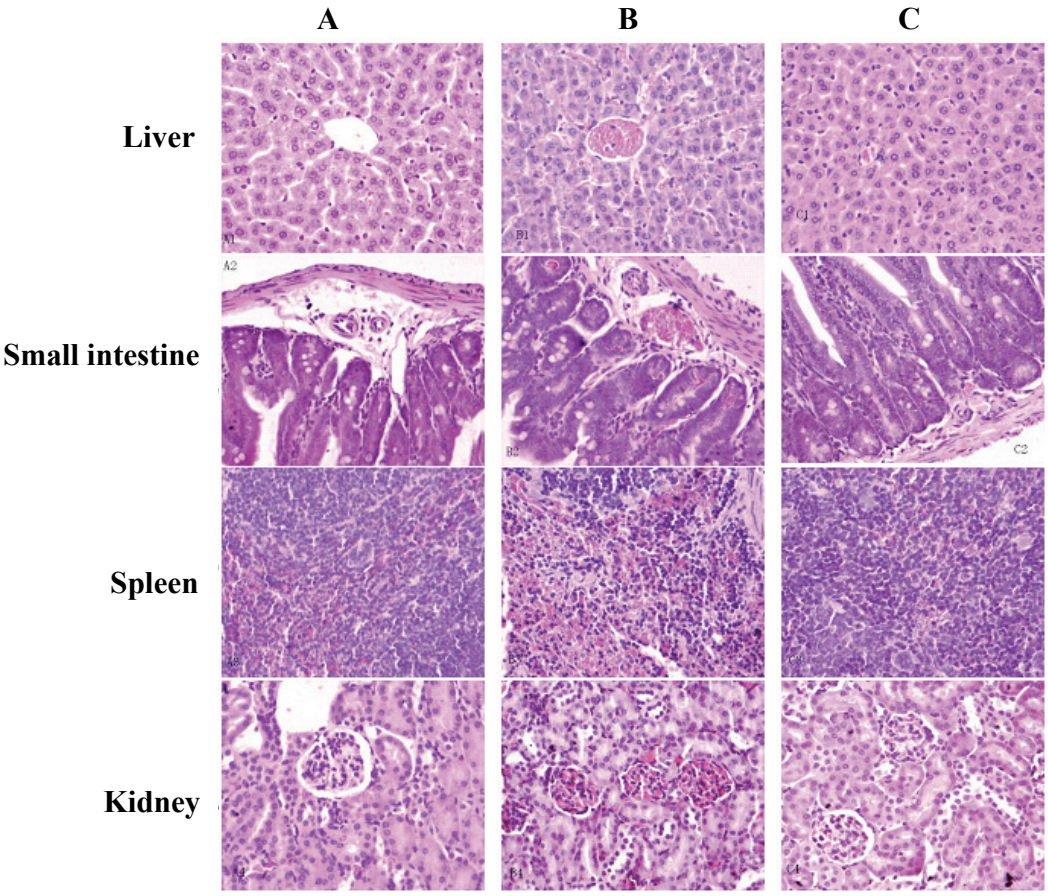

Figure 4. Pathological alterations of the main viscera from rAAV2-BPI-Fcγ1 and rAAV2-EGFP gene transferred mice after lethal LPS attack. Mouse tissues were collected at 6 hours after lethal LPS attack and examined by H&E staining. Column A was the main viscus of non-infected normal mice; Column B was the main viscus of the agonal mice from rAAV2-EGFP gene transferred mice; Column C was the main viscus of the survivals of rAAV2-BPI-Fcγ1 gene transferred mice. The four rows represent the viscera of liver, small intestine, spleen and kidney respectively (HE staining, original magnification × 200).

EGFP-transferred at 30 min, 60 min, and 120 min ($p < 0.01$) (Figure 3). Meanwhile, the level of TNF-α in the serum of BPI-Fcγ1-transferred mice was significantly lower than that of the EGFP-transferred at 60 min and 120 min ($p < 0.01$). It was obvious that the markedly increasing level of endotoxin and proinflammatory cytokines in serum of EGFP-transferred mice was responsible for the death of animals (the morality up to 97.2%) after LPS attack. The results suggested that BPI-Fcγ1 gene transfer could resist the endotoxic shock.

In addition, the main viscus of the experimental mice involving liver, small intestine, spleen and kidney were examined by standard haematoxylin and eosin (H&E) staining 6 hours after challenge of lethal LPS challenge. In comparison, the main viscus of the survival mice protected by rAAV2-BPI-Fcγ1 gene transfer showed only a slight congestion, while the main viscus of the agonal mice from rAAV2-EGFP transferred control mice showed significant pathological alterations (Figure 4), such as capillary dilatation and congestion which were consistent with what endotoxic shock should show.

Discussion

Viral delivery system has been widely used in gene therapy protocols for its high efficiency (15). Gutless viral vector is more safe because of less oncogenicity and less immunogenicity (16, 17), and it is the first choice for gene therapy of bacterial infection because it does not exaggerate the inflammatory reaction caused by infection. AAV vectors, a kind of gutless vector, are based upon a class of viruses that commonly colonize a human host without any host damage. In particular, AAV2 has been widely used as a gene delivery vehicle in preclinical studies and as well has been reported in early-phase clinical trials (18, 19).

In this article, we used AAV2 as delivery vector of BPI-Fcγ1 gene. The abundant blood stream in skeletal muscles facilitates the products of therapeutic gene to be secreted into blood circulation and to function well. After lethal LPS attack, the levels of endotoxin and proinflammatory cytokines in serum of BPI-Fcγ1-transferred mice were decreased, and the survival rate was increased

markedly.

The results of this study indicate that BPI-Fcγ1 gene delivery has potential in endotoxemia. Compared with traditional antibiotics, BPI-Fcγ1 has the advantages of neutralizing endotoxin that can protect host from endotoxemia and endotoxic shock. With the success of AAV2-BPI-Fcγ1 transgene-based modality against endotoxemia in mice model, we believe that AAV2-BPI-Fcγ1 can protect high-risk patients against endotoxemia and sepsis. We also consider that BPI-Fcγ1 gene delivery by mini-Ad vector, another kind of gutless vector developed recently from adenoviral vector, can mediate therapeutic gene to express more effectively with a relatively short-term than AAV vector (20-22), so it will achieve quick effect against GNB infection with high efficiency and rational duration and will be especially potential for treatment and prophylaxis.

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