Anti-radiation damage effect of polyethylenimine as a toll-like receptor 5 targeted agonist

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A number of agents are now available for use in protecting against ionizing radiation. These radiation-protective agents, however, have many adverse effects. Efforts have been made to develop new radiation-protective agents for medical application. Here, we investigated whether a compound, polyethylenimine (PEI), which activates Toll-like receptor 5 (TLR5)-mediated NF-κB signaling pathways, could have an anti-radiation effect on a mouse model. First, a cell-based screening model for an agonist of TLR5-mediated NF-κB pathway was established and then validated by activation of TLR5-mediated NF-κB luciferase reporter activity with a known TLR5 agonist, flagellin. We found that PEI induced dose-dependent activation of the TLR5-mediated NF-κB pathway, indicating that PEI is indeed a TLR5 agonist. Furthermore, the anti-radiation effect of polyethylenimine was assessed using a γ-ray total body irradiation (TBI) mouse model. Compared with the irradiation control, both survival time and survival rate were significantly improved in mice that received either a low dose of polyethylenimine (P = 0.019) or a high dose of polyethylenimine (P < 0.001). We also observed a positive correlation between animal body weight and survival time in mice that received a low dose of polyethylenimine, a high dose of polyethylenimine and amifostine, over a period of 30 days, r = 0.42 (P < 0.02), 0.72 (P < 0.0001) and 0.95 (P < 0.0001), respectively, while a negative correlation between animal body weight and survival time was observed in the irradiation control (r = −0.89; P < 0.0001). These results indicate that polyethylenimine is a new TLR5 agonist with potential application in offering protection for patients receiving radiotherapy or in radiation-related accidents.

Keywords: Polyethylenimine (PEI); NF-κB; radiation protection; Toll-like receptor 5 (TLR5); total body irradiation (TBI)

INTRODUCTION

Exposure to ionizing irradiation may result in tissue damage, and have negative impacts on our health and safety. Nuclear accidents can lead to serious social economic issues [1]. The toxicity of ionizing irradiation, described as acute radiation syndrome (ARS) or radiation sickness is caused by total- or partial-body exposure to a high dose of penetrating ionizing radiation over a short period of time. Even at low doses, ionizing irradiation is capable of causing illness, and ARS can occur within hours to days [2]. In clinical medicine, ARS often exists as the adverse side-effect of radiation therapy for cancer and other diseases. Administration of medicine for radiation damage...
Burdelya and colleagues [10] have successfully produced plants and mammals. Based on the structure of flagellin, recognized by the natural immune system of fruit flies, drugs, which may be introduced prior to or immediately after irradiation exposure. Biological drugs have poor specificity. Most radio-protective agents have severe toxicity [3, 4]. Therefore, there is an urgent need to develop effective compounds that offer radiation protection.

The human immune system is one of the most vulnerable systems targeted by radiation and has been a key target for research in radio-protective strategies [5]. Toll-like receptors (TLRs) are an important family of immune receptors. To date, at least 13 human TLRs have been identified and extensively investigated in the immune system of invertebrates. TLRs are important cell surface receptors in the innate immune system and play a vital role in the natural immune system at the initiation stage of multiple immune signaling pathways. It has been reported that TLRs can induce antigen-presenting cell maturation and secretion of inflammatory chemokines and cytokines, resulting in a connection between innate immunity and acquired immunity. TLRs can induce strong antigen-specific immune responses [6, 7].

Toll-like receptor 5 (TLR5) belongs to the TLR family and plays a pivotal role in the natural immune system. TLR5 is mainly expressed in epithelial cells, mononuclear cells, macrophages and dendritic cells. It mediates immune response and inflammation formation, and activates downstream NF-κB signaling pathways. To our knowledge, the only natural agonist available is a bacterial flagellum protein, flagellin [8, 9]. Flagellin is present as a soluble protein in certain bacteria, such as Salmonella, and can be recognized by the natural immune system of fruit flies, plants and mammals. Based on the structure of flagellin, Burdelya and colleagues [10] have successfully produced CBLB502 peptide by using genetic engineering technique. CBLB502 peptide offers protection against radiation injury both in vitro and in vivo, and can inhibit the process of normal cell apoptosis [10]. Therefore, it can prevent normal tissues from receiving radiation damage while maintaining the sensitivity of tumors to radiation. This function mainly occurs through the NF-κB signaling pathway [11]. Polyethyleneimine (PEI) is a compound that is derived mainly from industrial sewage. It has linear, branching and extra-branching types. The application of PEI in biological medicine has attracted considerable attention, since PEI can be used as a carrier in gene therapy [12, 13], and has shown anti-fungal effect in in vitro experiments [14]. Recently, it has been reported that PEI has a high-density ionic charge that can selectively activate the intracellular TLR5 receptor, promoting generation and secretion of downstream TLR5 signaling pathway factors, such as keratinocyte chemoattractant (KC) and IFN-inducible protein 10 (IP-10) in mice [13]. However, there have been no reports published investigating the protective effects of PEI against radiation injury. We hypothesize that the high-density ionic charge of PEI activates the hTLR5 receptor-mediated NF-κB pathway, and provides an anti-radiation effect. This paper aimed to investigate whether polyethyleneimine could activate the hTLR5 mediated NF-κB pathway and if so, whether polyethyleneimine provides an anti-radiation effect as a TLR5 targeted agonist in a mouse model.

MATERIALS AND METHODS

Reagents
HEK 293T cells were kindly provided by Dr Qinong Ye, Beijing Institute of Biotechnology. CaCo-2 cells were kindly provided by Dr Guangxing Bian, Beijing Institute of Radiation Medicine. Branched PEI with a molecular weight of ~25 000 by LS, Cat No. 408727 and Flag antibody were purchased from Sigma-Aldrich (St. Louis, MO, USA). The positive control drug, amifostine (AMI), was purchased from Merro Pharmaceutical Company (Dalian, China). Bacterial flagellar protein (flagellin) was a gift from Dr Changhui Ge, Beijing Institute of Radiation Medicine, China. Dulbecco’s modified Eagle medium (DMEM), TRIZOL reagent and Lipofectamine™ 2000 transfection reagent were purchased from Invitrogen Corporation (Carlsbad, CA, USA). Protease inhibitor cocktail was purchased from Sigma. ECL chemiluminescence reagents were purchased from Biomed, China. V5 antibody was purchased from MBL Company (Tokyo, Japan). Restrictive endonucleases were purchased from New England Biolabs (Ipswich, MA, USA). The anti-β actin antibody, horseradish peroxidase-conjugated goat anti-mouse IgG, goat anti-rabbit IgG, MTT cell proliferation and cytotoxicity detection kit, and dual luciferase assay kit were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). Restriction enzymes, DNA ligase and Taq enzyme were purchased from TaKaRa Bio INC (Shiga, Japan). DNA purification kit was purchased from Bio-med Company (Beijing, China).

Plasmids
pcDNA3.1-V5/HisB vector was purchased from Invitrogen. NF-κB luciferase reporter plasmid (NF-κB-Luc) was kindly donated by Huazhong Agricultural University, Wuhan, China. Renilla-luciferase (TK-PRL), used as an internal control, was purchased from Promega.

Cell culture
HEK-293 T cells were cultured in DMEM medium containing 10% fetal bovine serum (FBS) in air and 5% CO2 atmosphere at 37°C. CaCo-2 cells were cultured in DMEM medium containing 10% FBS, 4.5 g/l glucose, 2.292 mg/ml
glutamine, 10 nmol/l pyruvic acid sodium, = and 1% unsaturated amino acid in air and 5% CO₂ atmosphere at 37°C.

**Human TLR5 DNA construct cloning**

Total RNA was extracted from CaCo-2 cells using TRIZOL reagent. DNA sub-cloning technique was utilized to clone the hTLR5 gene (Fig. 1A). The F0 fragment (254 bp) was synthesized. Fragments of the F1 (1126 bp), F2 (869 bp) and F3 (633 bp) were all obtained using the reverse transcriptase polymerase chain reaction (RT-PCR) method. F0 sequence: 5′GGA TCC ACC ATG GGA GAC CAC CTG GAC CTT CTC CTA GGA GTG GTG CTC ATG GCC GGT CCT GTG TTT GGA ATT CCT TCC TGC TTT GAT GGC CGA ATA GCC TTT TAT CGT TTC TGC AAC CTC ACC CAG GTC CCC CAG GTG CTC A AC ACC ACT GAG AGG CTC TCT AGC TTC AAC TAT ATC AGG ACA GTC ACT GCT TCA TCC TTC CTT CGA CAG CTG CAG CTG CTG GAG CTC GGG AGC CAG TAT AC 3′. F1 upstream and downstream primers were P9 and P3, respectively, and their sequences were detailed as the following: P9: 5′CAT CCT TCC CCT TTC TGG AAC AGC 3′ (upstream), and P3: 5′GCT CTA GAT TTT CTA GCC TGT TTT CTG ATA AGT GG3′ (downstream). F2 upstream and downstream primers were P4 and P5.1, respectively, and their sequences are the following: P4: 5′CTC CGA GAC AAT GCT CTT ACA ACC 3′ (upstream), P5.1: 5′TTG AAC ACC AGT CTC TGG GGT TGC TAC AGT TTG CAA C 3′ (downstream). The fragment F0 was fused with the fragments of F1, F2 and F3 using PCR methods, respectively, resulting in Fa and Fb fragments. These two fragments were then ligased with pcDNA3.1-V5/HisB vector. The sequence of hTLR5 in pcDNA3.1-V5/HisB recombinant was confirmed by restriction digestion and sequencing (Fig. 1B). The pcDNA3.1-V5/HisB-hTLR5 construct was transfected into HEK293T cells using Lipofectamine™ 2000 reagent, and the expressed target protein of hTLR5 was verified by western blotting.

**Western blotting assay**

HEK 293T cells were seeded onto 24-well plates and incubated at 37°C for 18 h before transfection. The cells were subsequently transfected with pcDNA3.1-V5/HisB-hTLR5 or empty vector using Lipofectamine™ 2000 reagent.
according to manufacturer’s instructions. Forty-eight hours after transfection, cells were lysed in buffer containing 0.5% TritonX-100, 150 mM NaCl, 12.5 mM β-glycerophosphate, 1.5 mM MgCl2, 2 mM EGTA, 10 mM NaF, 1 mM Na3VO4 and 2 mM DTT plus protease inhibitor cocktail (Sigma). The cell extracts were spun down at 5000 × g at 4°C for 10 min. Protein concentration of lysate was determined using BCA Protein Assay kit (Bio-Rad). Protein extracts in 30 µl of 2× sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Biomed, China) were separated on SDS-PAGE gel and transferred to a polyvinylidene difluoride (PVDF) membrane. Blots were incubated with V5 antibody (MBL, Japan). After washing three times in 1× TBS-T buffer, blots were subsequently incubated with goat-anti-rabbit-HRP (Beyotime, China). Antibody-antigen reactions were detected using Western Lighting Plus-ECL chemiluminescence reagents (Biomed, China).

Luciferase reporter gene assay

Luciferase reporter gene assay was performed as previously described [15]. Briefly, HEK 293T cells were placed in a 96-well plate at a density of 8 × 10³ for 24 h. The pcDNA3.1-V5/HisB-hTLR5 was transiently co-transfected with the NF-κB-Luc and TK-PRL (an internal control for luciferase gene expression) by using Lipofectamine™ 2000. Twenty-four hours after transfection, the cells were treated with PEI or flagellin (a known TLR5 agonist), as a positive control, for 6 h, then washed and lysed. Luciferase activity was measured using a dual-luciferase assay kit according to the manufacturer’s instructions. The increased amount of promoter activity was expressed as the ratio of induction, which was the relative luciferase activity of the stimulated cells divided by the relative luciferase activity of the mock cells. The assays were repeated three times independently.

Assessment of cell viability by MTT assay

To evaluate PEI cytotoxicity, cell viability was examined by MTT assay. Briefly, HEK 293T cells were seeded in 96-well plates at a density of 8 × 10³ for 24 h. When cells were 40–60% confluent, the cells were incubated at various concentrations of PEI in DMEM for 48 h. Ten microliters of MTT was added to each well and incubated for 4 h at 37°C. One hundred microliters of formazan lysis was then added to each well and incubated for another 4 h at 37°C. The absorbance of the solubilized formazan was measured at 570 nm. The viability of cells incubated in control medium was considered as 100%.

Anti-radiation effect in vivo

Following animal ethics approval from the Experimental Animal Care and Ethics Committee of Beijing Institute of Radiation Medicine, 41 male C57BL/6J mice (7–8 weeks old, 19.86 ± 0.98 g) were purchased from the Experimental Animal Center, Beijing Institute of Radiation Medicine. Animals were handled in accordance with Experimental Animal Care and Ethics Committee guidelines and the Code of Practice for the Care and use of Animals for Scientific Purposes. Animals were housed in a parasite-free environment. Bedding was changed twice a week, and food and water were provided ad libitum. Animals were allowed to acclimatize to new surroundings before the experiment began. According to our experimental design, the minimal number of animals required was used. Mice were randomly divided into four groups: IR mock group (saline 20 ml/kg, irradiation, n = 10), low dose PEI group (5 mg/kg of PEI, irradiation, n = 10), radiation protection positive control group (AMI, 150 mg/kg, irradiation, n = 10) and high dose PEI group (10 mg/kg of PEI, irradiation, n = 11).

Animals were intra-peritoneally injected with different agents 30 min before an 8-Gy ⁶⁰Co γ-ray total body irradiation (TBI). Daily weights of survival mouse and observations were performed over a period of 30 days. Animal survival rate was calculated.

Statistical analysis

Data are expressed as mean ± SD (number of mice). Statistical difference between groups was determined using the unpaired two-tailed t test. When there were more than two groups, differences were analyzed using analysis of variance if the variances were equal, and the Mann–Whitney nonparametric test if variances were unequal [16]. Linear regressions were calculated using a statistical computer package, Number Cruncher Statistical Systems [16]. A value of P < 0.05 was considered statistically significant.

RESULTS

A cell-based model for screening TLR5 agonists targeting TLR5-mediated NF-κB pathway

Following the cloning of human TLR5 gene into pcDNA3.1-V5/HisB (BamHI/ApaI site) (Fig. 1A and B), the stable expression of pcDNA3.1-V5/HisB-hTLR5 was confirmed in HEK 293T cell by western blot analysis, indicating that the molecular weight of the expressed protein was 96 kD (Fig. 1C). Twenty-four hours after transfection of pcDNA3.1-V5/HisB-hTLR5 and NF-κB-luciferase into HEK 293T cells, these cells were treated with flagellin ranging from 0.1 to 10 µg/ml for 6 h to examine hTLR5 gene activation. Our results showed that NF-κB-luciferase activity was significantly activated by flagellin, especially at concentrations ranging from 1.0 to 10.0 µg/ml (Fig. 1D), indicating that a cell-based model was established with the potential for screening of TLR5 agonists targeting the TLR5-mediated NF-κB pathway. Our data demonstrated that the TLR5 receptor-mediated NF-κB signaling pathway was specifically activated with the positive TLR5 ligand of...
flagellin in a dose-dependent manner, and the cell-based system has potential application for screening of new TLR5 agonists that target the TLR5-mediated NF-κB pathway.

**Polyethylenimine (PEI) is a novel TLR5 agonist activating the hTLR5-mediated NF-κB pathway**

First, the cytotoxicity of PEI was assessed at concentrations ranging from 0.01 to 5 μmol/l by MTT assay. We found that there was no significant toxicity observed on HEK 293T cells as the concentrations of PEI were below 0.5 μmol/l (Fig. 2A). Then the above cell-based model was used to assess whether PEI is a novel TLR5 agonist that targets the hTLR5-mediated NF-κB pathway. Twenty-four hours after co-transfection of pcDNA3.1-V5/HisB-hTLR5 and NF-κB-luciferase into HEK 293T cells, these cells were treated with PEI at concentrations ranging from 0.1 to 1.0 μmol/l or flagellin at a concentration of 1 μg/ml for 6 h. We found that TLR5 was significantly activated by PEI in a dose-dependent manner as luciferase activity of the downstream NF-κB reporter was increased just like the TLR5 agonist, flagellin (Fig. 2B). These results suggested that, similar to flagellin, PEI is a novel agonist of TLR5 that activates the hTLR5-mediated NF-κB pathway.

**Fig. 2.** PEI is a novel TLR5 agonist that activates the NF-κB intracellular signaling pathway mediated by TLR5. (A) The cytotoxicity of PEI was measured by MTT assay. There was no significant cytotoxicity when PEI concentration was below 0.5 μmol/l. (B) PEI is a novel TLR5 agonist that activates the TLR5-mediated NF-κB intracellular signaling pathway. Dual-luciferase reporter gene expression, showing PEI (0.2–1.0 μmol/l) can activate the TLR5-mediated NF-κB intracellular signaling pathway. *P < 0.05, vs. control.

**Anti-radiation damage effect of PEI in vivo**

The maximum non-toxic dose of PEI was first determined *in vivo* to be 70 mg/kg. Without γ radiation, C57BL/6J mice received an intra-peritoneal injection of 70 mg/kg PEI causing no sign of stress or body weight loss, and zero mortality (data not shown). The potential *in vivo* effect of PEI against ionizing radiation damage was assessed in C57BL/6J mice at a single dose of 8-Gy 60Co γ-ray total body irradiation (TBI). The animal survival rate and survival time over a period of 30 days are shown in Fig. 3A. No mice in the irradiation control (IR mock) survived beyond 17 days post-irradiation. The final survival rates of mice that received a low dose of PEI, a high dose of PEI or AMI (the radiation protection positive control), on Day 30 were 50%, 64% and 80%, respectively. Compared with the irradiation control (IR mock), both animal survival time and survival rate significantly improved in mice that received a low dose of PEI (P = 0.019), or high dose of PEI (P < 0.001). Compared with the radiation protection positive control (150 mg/kg of AMI, 8.8 ± 0.9), the animal survival rate was higher in mice that received a high dose of PEI (9.1 ± 1.8), but lower for the low dose PEI group (6.7 ± 2.2, P < 0.001).

The body weight of irradiated animals that received a low dose of PEI, or high dose of PEI, or AMI (the radiation protection positive control), were found to increase with time over a period of 30 days (Fig. 2B). We observed a positive correlation between animal body weight and time in mice that received low dose PEI, high dose PEI and AMI over a period of 30 days, with the correlation coefficient (r) being 0.42 (P < 0.02), 0.72 (P < 0.0001) and 0.95 (P < 0.0001), respectively. In contrast, animal body weight in the irradiation control (IR mock) declined with time over a period of 30 days (Fig. 3B), with a negative correlation (r) of −0.89 (P < 0.0001). These results demonstrate that PEI has radiation protective activity in an animal model when administered prophylactically.

**DISCUSSION**

In this study, we have achieved the following outcomes: (1) developed a model for screening agonists of the TLR5-mediated NF-κB pathway and validated its efficacy; (2) demonstrated that PEI can activate the innate immune receptor-TLR5 mediated NF-κB signaling pathway in a dose-dependent manner *in vitro*; and (3) shown that PEI has a significant protective effect against radiation injury in an *in vivo* animal model.

Activation of the TLR5-NF-κB signaling pathway has emerged as an effective mechanism in developing radiation protection compounds [17–20]. NF-κB is an important nuclear transcriptional regulation factor, which participates in the transcription regulation of diverse genes, and actively...
contributes to immune response and cell cycle activity. Investigation of the NF-κB pathway has unveiled its involvement in certain medical conditions, such as cardiovascular diseases, neurological disorders, immune dysfunctions, tumors and radiation-induced injury [17–20]. Establishment of an effective cell-based model for screening agonists of the human TLR5-mediated NF-κB pathway will help to identify compounds with greater specific action on the pathway, without side-effects on other signaling networks, to achieve pharmacological effects. For this purpose, we cloned the human TLR5 gene and created pcDNA3.1-V5/HisB-hTLR5 plasmid for use in the present study. Our human TLR5 cell model is more suitable for screening the human type TLR5-mediated NF-κB activity aiming for human therapeutic application than the early commercial murine TLR5 plasmid model used in previous studies [13].

Our previous works revealed the regulation of antiviral innate immune response through TLR signal transduction [15, 21]. We have also observed TLR5 receptor activated by a linear PEI in vitro in a separate study. This study found that branched PEI can activate the hTLR5-mediated NF-κB signaling pathway in a dose-dependent manner in a cell model. This finding is in agreement with a previous study using linear PEI that activated murine TLR5-mediated NF-κB signaling pathway [13]. The current study provides evidence that branched PEI offers radiation protection in addition to a previous report that a combination of linear PEI and siRNA produces immunostimulation in ovarian carcinoma mice [13]. The mechanism and effect of PEI are very similar to flagellin protein. It is interesting to note that the PEI used in our experiments is a chemical compound with a molecular weight of ~25 kDa, and the molecular weight of flagellin protein ranges from 30 kDa to 60 kDa. Therefore, PEI may offer the following advantages over flagellin protein: (1) higher pharmacological specificity than flagellin protein [22, 23]; (2) requires a lower dose than flagellin protein to achieve the same effect [11]; (3) easier to...
be delivered to its targets [24, 25]; and (4) stable in storage at room temperature [26, 27].

Similar to the function of flagellin protein, PEI provides a radio-protective effect. Our data obtained from in vivo experiments provide strong evidence that PEI plays a significant role in radiation protection. PEI can not only prevent animal weight loss caused by γ-ray irradiation, but also significantly increases animal body weight over a period of 30 days. In addition, PEI improves animal quality of life, prolongs animal survival time and reduces mortality. The 50% and 36% mortality rates in low- and high-dose PEI groups, respectively, are most likely due to insufficient radiation protection provided by the dose of PEI administrated. The dose might have been too low to provide complete radiation protection. If higher mortality resulted from high doses of PEI, the mortality observed in the high-dose (10 mg/kg) group should have been greater than that of the low-dose (5 mg/kg) group, but the pattern observed was in fact the opposite.

In conclusion, polyethylenimine is a new human TLR5 agonist with potential application in radiation protection, including, but not limited to, offering protection for cancer patients who receive radiotherapy or radiosurgery, and could serve as a new compound for protecting the health and safety of people in radiation-related accidents.

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