Preparation of Biodegradable PLGA-Nanoparticles Used for pH-Sensitive Intracellular Delivery of an Anti-inflammatory Bacterial Toxin to Macrophages

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Regular Article

Poly(ε-caprolactone-co-glycolic) acid (PLGA) is a synthetic copolymer that has been used to design micro/nanoparticles as a carrier for macromolecules, such as protein and nucleic acids, that can be internalized by the endocytosis pathway. However, it is difficult to control the intracellular delivery to target organelles. Here we report an intracellular delivery system of nanoparticles modified with bacterial cytoxins to the endoplasmic reticulum (ER) and anti-inflammatory activity of the nanoparticles. Subtilase cytotoxin (SubAB) is a bacterial toxin in certain enterohemorrhagic Escherichia coli (EHEC) strains that cleaves the host ER chaperon protein BiP and suppresses nuclear factor-kappaB (NF-κB) activation and nitric oxide (NO) generation in macrophages at sub-lethal concentration. PLGA-nanoparticles were modified with oligo histidine-tagged (6×His-tagged) recombinant SubAB (SubAB-PLGA) through a pH-sensitive linkage, and their translocation to the ER in macrophage cell line J774.1 cells, effects on inducible NO synthase (iNOS), and levels of tumor necrosis factor (TNF)-α cytokine induced by lipopolysaccharide (LPS) were examined. Compared with free SubAB, SubAB-PLGA was significantly effective in BiP cleavage and the induction of the ER stress marker network (TGN), SubAB cleaves BiP, resulting in ER stress-induced cytotoxicity through activation of ER stress sensors, PERK, IRE1α and ATF6 signaling, which mediate a unique signaling pathway including phosphorylation of eIF2α and expression of C/EBP homologous protein (CHOP). To date, several studies have demonstrated that SubAB induces cytotoxicity in vitro and lethal severe hemorrhagic inflammation in mice, inhibition of autophagy and stress granule formation. Our previous study demonstrated that SubAB inhibits LPS-induced nitric oxide (NO) production in macrophages through the suppression of nuclear factor-kappaB (NF-κB) activation and subsequent inducible NO synthase (iNOS) expression. Since SubAB inhibits activation of NF-κB, a master regulator of LPS-induced gene expression and inflammation, SubAB may have therapeutic potential for immunosuppression in macrophages.

Poly(ε-caprolactone-co-glycolic) acid (PLGA) is a synthetic copolymer composed of lactic acids and glycolic acids. PLGA exhibits complementary properties due to the lactic acid-derived hydrophobic crystalline character and glycolic acid-derived hydrophilic amorphous character. PLGA degrades by cellular hydrolysis of its ester linkages, resulting in degradation to two monomers, lactic and glycolic acid, both familiar cell metabolites. These compounds are metabolized via the tricarboxylic acid (TCA) cycle and are subsequently eliminated from the body as carbon dioxide and water, leading...

Introduction

An innate immune system is essential for host defense against bacterial pathogens. Phagocytic cells, including macrophages and neutrophils, produce pro-inflammatory cytokines and reactive oxygen/nitrogen species (ROS/RNS) as an inflammatory mediator in response to a bacterial cell wall component, such as lipopolysaccharide (LPS), during a Gram-negative bacteria infection. These inflammatory mediators are important for a systemic host response to eliminate invading pathogens. However, in the case of autoimmune diseases or chronic inflammatory diseases, excessive and prolonged production of cytokines is a major causative agent, which leads to tissue damage, hemodynamic changes, and multiple organ failure with a wide range of clinical manifestations and complications.

Subtilase cytotoxin (SubAB), a member of the AB 5 toxin family, was identified by Paton et al. in an enterohemorrhagic Escherichia coli (EHEC) O113:H21 that was associated with an outbreak in Australia. SubAB is formed by an enzymatically active A subunit with a molecular weight of 35 kDa; it cleaves host endoplasmic reticulum (ER) chaperon protein BiP and pentameric B subunit (each monomer 13 kDa) that binds to the cell surface receptor, and mediates uptake into a target cell. After translocation into ER via the trans-Golgi network (TGN), SubAB cleaves BiP, resulting in ER stress-induced cytotoxicity through activation of ER stress sensors, PERK, IRE1α and ATF6 signaling, which mediate a unique signaling pathway including phosphorylation of eIF2α and expression of C/EBP homologous protein (CHOP).

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to minimal systemic toxicity.\textsuperscript{22} PLGA membranes and PLGA nanoparticles (PLGA NPs) have been widely studied in the pharmaceutical and medical fields as particulate materials.\textsuperscript{13–15} PLGA NPs is one of the best defined biomaterials available for drug delivery with respect to design and performance as a biodegradable micro/nano-device.\textsuperscript{16,17} A recent study demonstrated that the combined use of an anti-tumor molecule and anti-inflammatory molecule showed inhibition of tumor growth.\textsuperscript{19} PLGA NPs are also expected as a carrier for macromolecules, such as proteins\textsuperscript{19,20} and nucleic acids,\textsuperscript{21,22} into cells because micro/nanoparticles loaded with macromolecules can be internalized by the endocytosis pathway. However, it is difficult to control the intracellular delivery to target organelles.

In this study, we modified surface of PLGA NPs with SubAB via a pH responsive linker. We then examined the intracellular pH-dependent release of SubAB in macrophage cells and their translocation into ER by detecting BiP cleavage and anti-inflammatory effects. We provide a novel strategy to develop a functional nano-material for targeting macrophages as a potential therapeutic intervention.

**Experimental**

**Reagents** Poly(D,L-lactide-co-glycolic) acid (PLGA, 85:15, MW 190000–240000) was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). N-Hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (water soluble carbodiimide, WSC) and N-(5-amino-1-carboxypropyl) iminodiacetic acid (AB-NTA) were purchased from DOJINDO (Kumamoto, Japan). Purified mouse anti-BiP/GRP78 antibody (Cat#610979) was purchased from Cell Signaling Technology (Danvers, MA, U.S.A.). Anti-beta-actin mouse monoclonal antibody was purchased from Wako Pure Chemical Corporation (Osaka, Japan). Anti-CHOP mouse monoclonal antibody (#2895), anti-mouse immunoglobulin G (IgG), HRP-linked antibody (#7076) and anti-rabbit IgG, HRP-linked antibody (#7047) were purchased from Cell Signaling Technology (Danvers, MA, U.S.A.). Anti-iNOS mouse monoclonal antibodies were prepared as previously reported.\textsuperscript{23}

**Preparation of the PLGA Nanoparticles (PLGA NPs)**

PLGA NPs were prepared according to an emulsion/evaporation method described in a previous report with minor modification.\textsuperscript{24,25} Briefly, 17 mL of the PLGA solution (60 mg/mL in chloroform) and 10 mL of a stearic acid solution (4.5 mg/mL in chloroform) were added to 423 mL of a solution containing 7.7 mM NHS and 7.7 mM WSC in 20 mM phosphate buffer (pH 8.2). The mixture was shaken for 2 min at 37°C followed by washing with a 20 mM phosphate buffer (pH 8.2). After adding 100 mL of 7.62 mM AB-NTA into the NHS-activated PLGA NPs, the mixture was shaken for 2 min at 37°C. After washing, the resultant NTA-modified PLGA NPs was mixed with 200 mL of a 15.4 mM NiCl\textsubscript{2} aqueous solution and shaken for 30 min at 37°C. After washing with 20 mM phosphate buffer (pH 8.2), the mixture of Ni-NTA-modified PLGA NPs was re-dispersed in 95 mL of 20 mM phosphate buffer (pH 8.2), and 5 mL of 347 mg/mL His-tagged SubAB was added, followed by shaking for 1 h at room temperature. After washing with 20 mM phosphate buffer (pH 8.2), the resultant His-tagged SubAB-modified PLGA NPs (SubAB-PLGA NPs) were stored at 4°C.

**Evaluation of the pH-Dependent Release of His-Tagged SubAB**

100 mL of the His-tagged SubAB-modified PLGA NPs (SubAB-PLGA NPs) was centrifuged (12000 × g, 5 min) and the supernatant was removed. The NPs were re-dispersed in 100 mL of ultrapure water. This solution was again centrifuged (12000 × g, 5 min) and the supernatant removed; the NPs were re-dispersed by 75 mL of 8 mM phosphate buffer (pH 8.2), 2.5 mL of 347 mg/mL His-tagged SubAB and SubAB-PLGA NPs into J774.1 cells were determined by BiP cleavage and anti-inflammatory effects. Western Blotting After treatment, cells were lysed in SDS-sample buffer (62.5 mM Tris–HCl pH 6.8, 2% SDS, 6% glycerol, 0.005% bromophenol blue and 2.5% 2-mercaptoethanol) and then boiled for 3 min. Proteins were separated by SDS-PAGE and transferred in a polyvinylidene difluoride (PVDF) membrane (Merck Millipore; Darmstadt, Germany)
Membranes were blocked with 5% non-fat milk in TBS-T (20 mM Tris–HCl pH 7.5, 137 mM NaCl and 0.1% Tween 20) for 60 min and then incubated for 60 min at room temperature or overnight at 4°C with the primary antibodies as indicated. After washing with TBS-T, membranes were incubated with HRP-conjugated secondary antibodies at room temperature for 60 min. After washing with TBS-T, protein bands were detected using Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore) with a luminous image analyzer ChemiDoc™ XRS system (Bio-Rad, Hercules, CA, U.S.A.)

**Determination of the Tumor Necrosis Factor (TNF-α) Production in J774.1 Cells**

Cytokine TNF-α in culture supernatants were measured using a mouse TNF-α enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, U.S.A.) according to the manufacturer’s instruction. Briefly, diluted culture supernatants or standards were incubated with antibody pre-coated microplates and then reacted with the detection antibody. After incubation and washing, the reaction was detected by hydrogen peroxidase conjugated with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate and then the absorbance was measured at 490 nm using an iMark™ microplate reader (Bio-Rad). The amount of TNF-α was calculated from a 4-parameter logistic curve of standard.

**Statistical Analysis**

All data from densitometric analysis of the Western blotting and ELISA are expressed as means ± standard deviation (S.D.) Data for each experiment were acquired from three experiments. Statistical analyses were performed by using Student’s t-test, with significance set at \( p < 0.05 \).

**Results and Discussion**

**Characterization of PLGA NPs**

PLGA NPs were synthesized by an emulsion method using stearic acid. TEM observation was used to confirm the microstructure of the PLGA NPs. The PLGA NPs were spherically shaped and dispersed individually (Fig. 1). Based on dynamic light scattering (DLS) analysis, the particle size of the PLGA NPs was 253.1 ± 1.6 nm and the average zeta potential was −37.6 ± 1.2 mV (Figs. 1c,d and Table 1). The negative zeta potential indicates that the stearic acids were successfully anchored in the NPs, and their carboxyl groups were exposed to the surface of the NPs.

**Surface Modification of PLGA NPs with His-Tagged SubAB and Evaluation of Its pH-Responsive Release**

The surface of the PLGA NPs was modified with His-tagged SubAB through a Ni\(^{2+}\) chelate complex. The oligo histidine-tagged (His-tagged) recombinant protein binds strongly to a divalent metal chelate such as the Ni(II) nitrilotriacetate (NiNTA) complex at pH 8.0. In NiNTA resin chromatography, a low pH buffer can be used to elute the His-tagged protein of interest. Reducing the pH to 5.3–4.5 protonates the imidazole nitrogen atom of the histidine residue (\( pK_{a3} = 6.04 \)) and disrupts the coordination bond between the histidine and transition metal ions including Ni\(^{2+}\).\(^{27,28}\) We usually purify His-tagged SubAB by NiNTA agarose chromatography without a loss of catalytic activity.\(^{26}\) Therefore, we modified PLGA NPs through the NiNTA complex with the His-tagged SubAB (SubAB-PLGA NPs) and evaluated the properties of SubAB-PLGA NPs. After centrifuging the reaction mixture, two bands of A subunit (35 kDa) and B subunit (13 kDa) were observed in the precipitate fraction, while no band was observed in the supernatant fraction (Fig. 2a), indicating that most of the added His-tagged SubAB modified the PLGA NPs. As shown in Table 1, the average size of the SubAB-PLGA NPs was 353.5 ± 16.4 nm, and the average zeta potential was

|                | Size (nm) | Polydispersity index | Zeta potential (mV) |
|----------------|-----------|----------------------|---------------------|
| PLGA NPs       | 253.1 ± 1.6 | 0.241 ± 0.025        | −37.6 ± 1.2         |
| SubAB-PLGA NPs | 353.5 ± 16.4 | 0.425 ± 0.060       | 8.89 ± 0.18         |

Data are expressed as means ± S.D. (\( n = 3 \)).
8.89 ± 0.18 mV in water. We calculated theoretical isoelectric point (pI) of SubAB subunits using a Compute pI/\(M_w\) tool, ExPaSy (https://web.expasy.org/compute/pi/). Analysis of the deduced amino acid sequence indicated that theoretical pI value of A and B subunits were 9.28 and 8.54, respectively. Therefore, the positive zeta potential of SubAB-PLGA NPs was reasonable.

To evaluate the pH responsive dissociation of His-tagged SubAB from PLGA, we treated the SubAB-PLGA NPs with buffer solutions at different pH values as indicated in Fig. 2b. Most of the His-tagged SubAB dissociated at pH 5.0 and 5.5, but not at values greater than pH 6.0. Although it did not exactly match the \(pK_{a3}\) of histidine, the linkage of His-tagged SubAB and PLGA NPs was pH dependent. The difference of the \(pK_{a3}\) of histidine and actual dissociation pH might be due to lowered \(pK_{a3}\) of hexameric histidine residues of the His-tag, which observed in cluster of histidines. 29)

**Evaluation of the ER Stress in Macrophages** In mammalian culture cell lines, such as HeLa cells, SubAB binds to sialoglycan-modified cell surface proteins,30,31) using the B subunit and enters via actin/lipid-raft-dependent macropinocytosis-like uptake,7) followed by translocating to ER via the COPI-dependent Golgi retrograde trafficking pathway.33) In ER, SubAB cleaves BiP to 44kDa and 28kDa of polypeptides and induces ER stress response, leading to expression of ER stress-related protein including CHOP. To investigate the effect of PLGA on His-tagged SubAB entry into macrophages, we treated J774.1 cells with SubAB-PLGA NPs or the free SubAB, and the cleavage of BiP and expression of CHOP indicating translocation of the His-tagged SubAB into the ER were evaluated by Western blot analysis.

As shown in Fig. 3, more cleavage of BiP was observed in the case of SubAB-PLGA NPs compared with the free SubAB (Fig. 3a). SubAB-PLGA NPs induced higher CHOP expression than the free SubAB. Densitometric analysis for cleaved BiP and CHOP expression indicated that SubAB-PLGA NPs cleaves BiP and induced more than twice as much CHOP expression as the free SubAB (Figs. 3b and 3c). These results indicated that SubAB-PLGA NPs were internalized in J774.1 cells by macropinocytosis or phagocytosis, and His-tagged SubAB was released from PLGA NPs at lower pH compartments, such as endocytotic vesicles, which induced ER stress without catalytic activity loss. Since the free SubAB did not show efficient BiP cleavage or CHOP induction, PLGA NPs...
had an important role of delivery of His-tagged SubAB into the ER of macrophages.

**Inhibitory Effects of SubAB-PLGA NPs on iNOS Expression and Cytokine Production**

Previously, we reported that SubAB inhibits LPS-induced iNOS expression through activation and nuclear translocation of NF-κB.11) Our previous finding suggested that SubAB induces ER stress and thus inhibits LPS-induced cytokine production. Based on the results in the previous section, we expected that SubAB-PLGA NPs can exhibit an anti-inflammatory effect more than the free SubAB. To confirm the inhibitory effects of SubAB-PLGA NPs on LPS-induced inflammation, we examined the effect of SubAB-PLGA NPs on iNOS expression by Western blotting analysis, and found that the free SubAB slightly inhibited LPS-induced iNOS expression in J774.1 cells (Fig. 4a). In contrast, SubAB-PLGA NPs significantly suppressed iNOS expression more than free SubAB (Fig. 4b).

TNF-α is a major pro-inflammatory cytokine, which is also downstream of LPS/TLR4/NF-κB signaling in macrophages. We next examined the effect of SubAB-PLGA NPs on the TNF-α production in the culture supernatant. As shown in Fig. 4c, SubAB-PLGA NPs significantly suppressed TNF-α in dose-dependent manner compared with PLGA NPs alone. Alternatively, in the case of free SubAB, no significant difference was observed (Fig. 4c). These results indicate that conjugation of His-tagged SubAB on PLGA NPs reinforced delivery of His-tagged SubAB into the cells and the His-tagged SubAB-mediated anti-inflammatory effect. A previous study reported that LPS-induced TNF-α production was suppressed by pretreatment with SubAB.31) In this study, we successfully prevented TNF-α production by co-treatment with LPS and a low concentration of His-tagged SubAB by conjugating with PLGA agreement with the previous report. Phagocytes such as neutrophils and macrophages use phagocytosis to initiate the innate immune response, and acidification of their phagosomal lumen appears to be required for effective bacterial killing.35,36) which has been reported to reach pH 5.5.37–39) Our data suggested that the acidification of the phagosome pH in J774.1 cells was sufficient for the pH-sensitive His-tagged SubAB release from PLGA NPs.

**Conclusion**

In this study, we designed PLGA nanoparticles loaded with His-tagged SubAB via a Ni-NTA complex that act as pH responsive linker to deliver anti-inflammatory activity to macrophages. PLGA nanoparticles were prepared by an emulsion method in the presence of stearic acid. SDS-PAGE confirmed the His-tagged SubAB bound on the surface of PLGA-NPs at pH 8.2 and dissociated from PLGA-NPs by reducing the pH. The His-tagged SubAB-modified PLGA nanoparticles (SubAB-PLGA NPs) were internalized by the endocytosis pathway, translocated to ER, and then, induced BiP cleavage and CHOP expression. Significant suppression of LPS-induced iNOS expression and TNF-α production were observed in the presence of SubAB-PLGA NPs. In contrast, free SubAB did not show these effects. These results suggest that the modification of anti-inflammatory bacterial toxin on PLGA nanoparticles is a potent strategy for inhibiting the inflammatory response of macrophages. However, it is still unclear how SubAB translocated to the ER and interacts with BiP in macrophages and whether His-tagged SubAB-PLGA exhibit an anti-inflammatory effect in a mouse animal models with inflammation-associated diseases such as endotoxin shock or rheumatoid arthritis. SubAB-PLGA NPs may be a powerful tool for the treatment of inflammatory diseases with further studies into the mechanism. Further modification of the SubAB-PLGA NPs system with active pharmaceutical...
drugs or proteins will enable an intracellular drug delivery system specific to ER.

**Acknowledgments** This work was supported in part by Grants-in-Aid for Scientific Research [(B), (C), Innovative Areas (Research in a Proposed Area), and Challenging Exploratory Research] from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan, to H.T. (17K10019) and T.S. (17K19205, 18H02098); a Grant from Japan Agency for Medical Research and Development (AMED) to T.S. (JP18fm0208029); a Grant from Takeda Culture, Sports, Science and Technology Foundation to H.T.; a grant from Japan Science and Technology Agency (JST), Core Research for Evolutionary Science Foundation to H.T.; a grant from Japan Science and Technology Agency (JST), Core Research for Evolutionary Science and Technology (CREST) to T.N. (JPMJCR18H5).

**Conflict of Interest** The authors declare no conflict of interest.

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