Surface modification of poly(D,L-lactic-co-glycolic acid) nanoparticles with protamine enhanced cross-presentation of encapsulated ovalbumin by bone marrow-derived dendritic cells

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Abstract: Cross-presentation is the key process in stimulation of cytotoxic T lymphocyte (CTL) immune response in eliminating many infectious diseases and tumors. Previous studies have shown that surface modification of poly(D,L-lactic-co-glycolic acid) (PLGA) particles with polycations enhanced their adjuvant ability resulting in a strong antibody response to the encapsulated antigen. However, the in vitro cross-presentation by protamine-coated PLGA nanoparticles (NPs) has not been addressed yet. In this study, a model antigen ovalbumin (OVA) was encapsulated into PLGA nanoparticles, with (OVA-NPs/protamine) or without protamine coating (OVA-NPs). These nanoparticles were then used to stimulate murine bone marrow-derived dendritic cells (BMDCs). Flow cytometry analysis revealed an increase in endocytosis of protamine-coated PLGA nanoparticles by BMDCs at 37°C. Compared with OVA-NPs-treated BMDCs, stimulation with OVA-NPs/protamine led to significantly upregulation of CD80, CD86, and CD83, increased secretion of IL-12p70, and decreased production of IL-4 by BMDCs. Furthermore, OVA-NPs/protamine-treated BMDCs also showed an enhanced cross-presentation to B32 T cell hybridoma in vitro. Transmission electron microscopy (TEM) study showed that protamine-coated PLGA nanoparticles escaped from lysosomes through the interaction with lysosomal membrane. These results demonstrated that protamine-coated PLGA nanoparticles could enhance the cross-presentation of encapsulated exogenous antigen by facilitating antigen uptake and lysosomal escape, suggesting the feasibility to be a potent adjuvant for cellular vaccines. © 2010 Wiley Periodicals, Inc.

Key Words: PLGA nanoparticles, protamine, cross-presentation, antigen delivery system, dendritic cells

INTRODUCTION

Cellular vaccine may elicit cellular (Th1 and/or CTL) immune responses sufficient to eliminate intracellular bacteria, virus infected cells, and tumor invasive cells.1,2 Cross-presentation is the ability of antigen presenting cells (APCs) to present exogenous antigenic epitope via MHC class I molecules (MHC I) to cytotoxic CD8+ T cells to stimulate CTL immune response. The efficient cross-presentation of soluble antigens is restricted in the MHC I processing pathway.3 In contrast, immunization by PLGA particulate systems can gain more efficiency through the vacuolar mechanisms4 or endolysosomal escape.5,6 Biodegradable polymer poly(D,L-lactic-co-glycolic acid) (PLGA) particles function as antigen delivery system.7,8 With a size similar to pathogens,9 PLGA particles can be rapidly and efficiently phagocytosed by APCs, including macrophages and dendritic cells (DCs),10,11 and elicit potent humoral and cellular immune responses to the incorporated or adsorbed antigen,12,13 both in vitro and in vivo.14,15 Moreover, the adjuvant effect of PLGA particles closely correlates with the efficiency of antigen uptake and presentation.16,17 A desirable immune response can be achieved by proper modification of PLGA particles, such as size14 and surface charge.18-20 The fact that PLGA particles coated with multilayer polyelectrolyte could elicit potent CTL responses18 suggested that cellular immune responses can be elicited by surface manipulation of antigen carriers.

Protamine, an arginine-rich peptide (MW 4000–4250 Da) with strong basic charge (isoelectric point 11–12), is a FDA-approved nontoxic cationic peptide for use in humans as a heparin antagonist and as a long-acting delivery system for insulin. Protamine is also an excellent DNA condenser for in vitro cationic lipid-mediated gene transfer.21 In addition, previous studies have shown that protamine-coated PLGA microparticles could induce strong IgG1/Th2 response and efficient stimulation of CD4+ T cells.19,22 However, protamine-coated PLGA microparticles had little effect on IgG2a/Th1 response unless coencapsulation with immunostimulatory CpG.19,23

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In this study, we investigated the effects of protamine-coated PLGA nanoparticles and found enhanced uptake and cross-presentation of encapsulated antigens. This work will provide insights into design of future cellular vaccines against cancer and infectious diseases.

**MATERIALS AND METHODS**

**Reagents**

Poly(ε-lactic-co-glycolic acid) at monomer ratio 50:50, MW 17 kDa, and intrinsic viscosity 0.18 dl/g, with free carboxyl groups at the polymer terminus, was purchased from Dai-gang (Jinan, Shandong, China). Chicken egg ovalbumin (OVA; grade V), lipopolysaccharide (LPS) from *Escherichia coli* 0127:B8, trypan blue, nonidet P 40 substitute solution (NP40), osmium tetroxide (OsO4), and protamine sulfate from salmon were purchased from Sigma-Aldrich (Saint Louis, MO). Fluorescein isothiocyanate (FITC)-labeled OVA were purchased from Molecular Probes (Carlsbad, CA). Poly(vinyl alcohol) (PVA; MW 23–50 kDa, 89% hydrolyzed) was purchased from Keda (Shanghai, China). Chloroform (HPLC grade) was purchased from Fisher (Nepean, ON, Canada). Recombinant murine granulocyte-macrophage colony stimulating factor (GM-CSF) and murine interleukin (IL)-4 were purchased from Peprotech (Rocky Hill, NJ). Murine cytokine ELISA kits for IL-4, IL-10, and IL-12p70 were purchased from Molecular Probes (Carlsbad, CA). Poly(vinyl alcohol) (PVA; MW 23–50 kDa, 89% hydrolyzed) was purchased from Sigma-Aldrich (Saint Louis, MO). Fluorescein isothiocyanate (FITC)-labeled OVA were purchased from Molecular Probes (Carlsbad, CA). Poly(vinyl alcohol) (PVA; MW 23–50 kDa, 89% hydrolyzed) was purchased from Sigma-Aldrich (Saint Louis, MO). Fluorescein isothiocyanate (FITC)-labeled OVA were purchased from Molecular Probes (Carlsbad, CA).

**PLGA nanoparticles preparation**

PLGA nanoparticles were prepared using the water/oil/water–solvent extraction technique with modifications described previously.24 Briefly, 200 mg of PLGA were dissolved in 4 mL chloroform as the oil phase. One milliliter of OVA (100 mg/mL), FITC-OVA (100 mg/mL), or OsO4 (10 mg/mL) in phosphate-buffered saline (PBS) was dispersed in the oil phase using a probe sonicator (JY99-2D, Xinzhi, Ningbo, China). The resulting primary emulsion was added into 20 mL of 2% w/v PVA and sonicated to form the secondary emulsion, which was added into 80 mL of 0.5% w/v PVA and stirred overnight to evaporate the chloroform. For protamine-coated nanoparticles, 0.5% (w/w) protamine PVA solution was used as external water phase. The nanoparticles were collected by centrifugation (15,000 rpm, 20 min, 4°C), washed twice with distilled water to remove residual PVA, and resuspended in 5 mL distilled water followed by lyophilization (Model 6L, Labconco, Kansas City, MO). Six types of nanoparticles were obtained: OVA-NPs, FITC-OVA-NPs, OsO4-NPs, OVA-NPs/protamine, FITC-OVA-NPs/protamine, and OsO4-NPs/protamine.

**Particle size and zeta potential measurements**

The mean size and zeta potential of nanoparticles were measured by photon correlation spectroscopy (PCS) with a Nano-ZS90 laser particle analyzer (Malvern, UK). Approximately 0.2 mg of dry nanoparticles in 1 mL distilled water was used for PCS measurement and for zeta potential measurement with 1 mM KCl (2 mL; pH = 7.6). All measurements were performed at 25°C. The mean size and zeta potential value were calculated from three independent batches.

**Determination of antigen content in the PLGA nanoparticles**

A BCA method was used to measure the amount of loaded OVA in OVA-NPs. Briefly, 10 mg of OVA containing nanoparticles were dissolved in 0.5 mL chloroform and were extracted with 0.2 mL PBS. Nanoparticles were removed by centrifugation at 3000 rpm for 5 min. The content of OVA in supernatant was analyzed by the BCA quantification kit following the manufacturer protocol. Encapsulation efficiency was calculated as the percentage of amount of OVA loaded in nanoparticles formation to the amount of OVA added in nanoparticles formation.

**Mice**

Six- to eight-week-old male C57BL/6 mice (20 ± 2 g) were obtained from the Laboratory Animal Center, Tongji Medical College, Huazhong University of Science and Technology. All experiments were performed in accordance to the Ethical Committee of Huazhong University of Science and Technology guidelines for the care and use of laboratory animals.

**Bone marrow-derived dendritic cells (BMDCs)**

Bone marrow cells were isolated from femurs and tibias of C57BL/6 mice according to the method described by Inaba et al. Approximatively 2 × 10⁷ cells were obtained per mouse. Isolated cells were plated into a 6-well plate and cultured in RPMI-1640 supplemented with 10% heat-inactivated FCS, IL-4 (2 mU), GM-CSF, and 10 ng/mL IL-4. Fresh medium containing GM-CSF and IL-4 was added to the cultures every other day. The semiadherent and nonadherent cells were harvested and reseeded into a 24-well plate at 1 × 10⁵ cells/well. The purity of the BMDCs harvested was between 85% and 90% based on the expression of CD11c at day 6.

BMDCs were then incubated with various nanoparticles (OVA-NPs, OVA-NPs/protamine, FITC-OVA-NPs, FITC-OVA-NPs/protamine, OsO4-NPs, and OsO4-NPs/protamine) at 200 μg/mL and left undisturbed for specific time points, 20 h or 8 h, separately. BMDCs exposed to OVA solution (200 μg/mL) or LPS (1 μg/mL) were included as additional control groups.

**Flow cytometry analysis**

To assess the uptake kinetics of nanoparticles,6 BMDCs (1 × 10⁶ cells/well) were incubated with FITC-OVA solution, FITC-OVA-NPs, or FITC-OVA-NPs/protamine (200 μg/mL) for various intervals at 37°C or 4°C for controls. Cells were harvested at specific time points (0, 2, 5, 10, 30, 60, 90, and 120 min), and washed twice with cold PBS to separate from particles. Extracellular FITC signal was quenched using...
0.4% trypan blue and the uptake kinetics of FITC-labeled OVA or OVA nanoparticles were determined by flow cytometry.

Following incubation with OVA-NPs or OVA-NPs/protamine for 20 h, BMDCs were collected and stained with PE anti-mouse MHC I (I-A/I-E) mAb, FITC anti-mouse MHC II (I-A/I-E) mAb, FITC anti-mouse CD80 mAb, PE anti-mouse CD86 mAb (eBioscience, San Diego, CA), or PE anti-mouse CD86 mAb (eBioscience, San Diego, CA) according to the manufacturers’ instructions. LPS (1 μg/mL) and OVA solution (200 μg/mL) were used as additional control groups. The autofluorescence was determined by staining with respective isotype control antibodies for the specific monoclonal antibody used. The acquisitions of BMDCs were resolved in a EPICS ALTRA II (Beckman, Fullerton, CA) with EXPO32 software.

Cytokine ELISA
The supernatants from BMDCs incubated with OVA-NPs or OVA-NPs/protamine for 20 h were collected for IL-4, IL-10, and IL-12p70 analysis using commercially available ELISA kits. LPS (1 μg/mL) and OVA solution (200 μg/mL) were used as additional control groups. The assays were conducted according to the manufacturers’ protocols.

Cross-presentation of exogenous OVA to B3Z T cell hybridoma
The cross-presentation of exogenous OVA by BMDCs was assessed as described before. Briefly, 1 × 10^6 BMDCs were pulsed with OVA-NPs or OVA-NPs/protamine at an equal concentration (200 μg/mL). LPS (1 μg/mL) and OVA solutions (200 μg/mL) were used as additional control groups. After 8 h incubation, BMDCs were washed twice and further cocultured with 5 × 10^5 B3Z T cell hybridoma overnight. Supernatants were then discarded and X-Gal substrate (1.5 mg/mL of X-Gal in PBS-NP40 0.25%) was added to the remaining cells for another 18 h. B3Z is a CD8+ T cell hybridoma, which is only activated by the SIINFEKL peptide, the OVA-immunodominant peptide, presented by H-2K^b MHC class I molecules. B3Z cell expresses β-galactosidase upon activation and turns to blue color following treatment with X-Gal substrate. The plate was read at 405 nm by a multilabel counter Victor^3 1420 (Perkin Elmer, USA).

Intracellular distribution study
OsO4 is a widely used electron dense agent for postfixation and staining cells for TEM. To study the intracellular distribution of protamine coating nanoparticles, BMDCs were incubated with OsO4-NPs/protamine or OsO4-NPs (200 μg/mL) for 8 h, then harvested and washed with PBS for TEM study. The harvested cells were fixed in a 2.5% glutaraldehyde solution followed by 1% osmium tetroxide. The cells were washed with PBS and dehydrated in a series of ethanol solutions (50%, 70%, 90%, 95%, 100%), and then soaked in a 1:1 ratio of 100% ethyl alcohol and Unicryl embedding resin overnight (Ted Pella, Redding, CA). The cells in fresh Unicryl resin were placed in BEEM capsules (Electron Microscopy Sciences) and were transferred to a Pelco UV-2 Cryo Chamber (Ted Pella, Redding, CA) at 4°C for 48 h for polymerization. The polymerized blocks were sectioned at 30- to 40-nm thickness (Leica Ultracut UCT ultramicrotome, Leica Microsystems, Deerfield, IL) and stained with an aqueous solution of 2% uranyl acetate, followed by Reynolds lead citrate. The slides were observed under a FEI Tecnai G² 12 microscope (FEI, Holand).

Statistical analysis
All statistical analyses were performed using one-way ANOVA in SPSS 13.0. All data were expressed as means ± SEM. Standard error of mean (SEM) was calculated based on at least triplicate experiments. A p-value less than 0.05 (p < 0.05) was considered to be statistical significance.

RESULTS
Nanoparticles characterization
OVA-NPs and OVA-NPs/protamine had a similar size distribution. The size of OVA-NPs/protamine was larger than that of uncoated nanoparticles (Table I). The mean zeta potential of OVA-NPs was negative (−7.36 ± 1.02 mV) suggesting the surface of uncoated nanoparticles bear some free carboxylic end groups of PLGA molecules. As a result of the cationic charge of protamine, the zeta potential of OVA-NPs/protamine was positive as expected. The antigen encapsulation efficiency was about 62.46% ± 0.32%.

Uptake kinetics of nanoparticles by BMDCs
The uptake kinetics of different nanoparticles by BMDCs was shown in Figure 1. Pro tamine coating notably affected
the uptake of PLGA nanoparticles at 37°C. The uptake rate of FITC-OVA-NPs/protamine was faster than that of FITC-OVA-NPs at 37°C [Fig. 1(A)]. Moreover, the total amount of FITC-OVA-NPs/protamine uptake by BMDCs as measured by geometric mean fluorescence intensity (gMFI), was 4-fold higher than that of FITC-OVA-NPs group and 7-fold higher than that of FITC-OVA group at 120 min at 37°C [Fig. 1(B)]. The positive cells and gMFI of all FITC-OVA formulations showed time independent at 4°C. Furthermore, there was a significant reduction in nanoparticles uptake kinetics at 120 min at 4°C compared with that at 37°C.

**Phenotype characterization of BMDCs following stimulation with nanoparticles**

MHC I, MHC II, CD80, CD86, and CD83 of BMDCs are important surface molecules for BMDCs maturation and antigen presentation. The surface expression of these markers on immature BMDCs (imBMDCs, negative control), mature BMDCs (mBMDCs, treated with LPS, positive control), and BMDCs treated with free or encapsulated OVA (OVA solution, OVA-NPs and OVA-NPs/protamine) were illustrated in Figure 2. Compared with imBMDCs, OVA-NPs/protamine stimulated BMDCs showed remarkable increases in gMFI of all the surface markers tested, indicating BMDCs maturation. Furthermore, the increased gMFI of CD80, CD86, and CD83 in OVA-NPs/protamine stimulated BMDCs had significant difference with OVA-NPs stimulated BMDCs, and there were 1.7-fold, 8.9-fold, and 2.4-fold increased, respectively, [Fig. 2(B)]. But the gMFI of MHC I and MHC II of OVA-NPs/protamine-treated BMDCs showed no significant difference with that of OVA-NPs-treated BMDCs.

**Cytokine secretion of BMDCs after phagocytosis of nanoparticles**

The activation of BMDCs stimulated by nanoparticles was further determined by measuring Th2-polarization cytokines IL-4, IL-10, and Th1-polarization cytokine IL-12p70 secretion. As shown in Figure 3(A), the secretion level of IL-4 in both free OVA and OVA-NPs stimulated BMDCs was significantly higher than imBMDCs. Remarkably, the amount of IL-4 produced by OVA-NPs/protamine stimulated BMDCs was 3.1-fold lower than that of OVA-NPs-treated cells. The level of IL-10 was undetectable in all test groups except mBMDCs (data not show).

Treatment of BMDCs with OVA-NPs/protamine resulted in secretion of IL-12p70, which was more than 3.4-fold higher than the cells stimulated with OVA-NPs and ~ 420-fold above the baseline (imBMDCs) [Fig. 3(B)].

**OVA-NPs/protamine facilitating cross-presentation of OVA to specific CD8+ T cells**

OVA cross-presentation by BMDCs via MHC I was quantitated based on β-galactosidase expression of B3Z cell cocultured. No β-galactosidase activity was observed in B3Z cell cocultured with free OVA solution. OVA-NPs-treated BMDCs only marginally activated B3Z cell. Compared with OVA-NPs-treated BMDCs, OVA-NPs/protamine significantly enhanced β-galactosidase expression of cocultured B3Z cell (p < 0.001) (Fig. 4).

**Intracellular localization of nanoparticles**

TEM images of intracellular localization of OsO4-NPs/protamine and OsO4-NPs were shown in Figure 5. OsO4-NPs/protamine and OsO4-NPs could be clearly identified as dark spherical structures in the cellular compartments. Some OsO4-NPs/protamine was interacting with vesicular membrane [Fig. 5(A)], whereas some OsO4-NPs/protamine successfully escaped from lysosomes and translocated into cytoplasm [Fig. 5(B)], which was the prerequisite for the cross-presentation of encapsulated antigen. In contrast to OsO4-NPs/protamine, OsO4-NPs had no interaction with vesicular membrane [Fig. 5(C)].

**DISCUSSION**

PLGA particles have been widely used as a delivery system for antigens including DNA,28,29 proteins, and peptides.15,30,31 In recent years, extensive studies have been...
carried out to ask whether modification of the particle surface can influence the immune responses \textit{in vitro} and \textit{in vivo}. In this study, we investigated the effect of protamine on the antigen uptake and cross-presentation of BMDCs induced by protamine-coated PLGA nanoparticles. The diameter of all PLGA nanoparticles used in this study were about 300 nm, the size previously reported to be phagocytosed by DCs efficiently. As shown in this study, the internalization of FITC-OVA-NPs and FITC-OVA-NPs/protamine were in a time-dependent manner by

\begin{figure}
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\caption{OVA-NPs/protamine induce increased expression of CD80, CD86, and CD83 on BMDCs.}
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BMDCs at 37°C while the internalization were significant reduced at 4°C, suggesting that all nanoparticles uptake by BMDCs could be the energy-dependent phagocytosis. Protamine coating significantly enhanced the uptake rate of FITC-OVA-NPs by BMDCs and remarkably increased the phagocytosis capacity of BMDCs since there were more internalized FITC labeled nanoparticles in the cells treated with FITC-OVA-NPs/protamine at 37°C. This suggested that coating with protamine could further improve the delivery efficiency of PLGA nanoparticles.

Our research showed that the expression level of CD80, CD86, and CD83 in OVA-NPs/protamine-treated BMDCs was significantly higher than that in OVA-NPs-treated BMDCs. CD83 is an indicator of maturation. Upregulation of CD83 indicates the transfer of BMDCs from the state of antigen-capturing to the state of antigen-presenting. The higher CD83 expression induced by OVA-NPs/protamine suggested more BMDCs were differentiated into the antigen-presenting state. MHC I (H-2Kb) is involved in cross-presentation of SIINFEKL peptide (OVA residues 257–264). No significant difference of MHC I expression between OVA-NPs/protamine stimulation and OVA-NPs stimulation suggested OVA-NPs/protamine had minor effect on the regulation of MHC I compared with OVA-NPs.

DCs secrete certain cytokine after maturation, which is crucial in determining the type of immune response. IL-12p70 is one of the cytokines that plays a crucial role in priming Th1 immune response, as well as CTL response. We found OVA-NPs/protamine-treated BMDCs induced significantly higher expression of IL-12p70 and lower expression of IL-4 than the cells stimulated with OVA-NPs, suggesting protamine coating can drive the cytokine profile of T-cell response toward Th1 and CTL responses. Similar results have been reported in a previously published study using protamine-coated PLGA microparticles. However, their effect were mild compared with our study, and this inconsistence may be due to the particles size and cell source used in both studies.

The cross-presentation was studied by B3Z cell. OVA-NPs/protamine induced higher cross-presentation of exogenous antigens than OVA-NPs in B3Z cell, suggesting that protamine facilitated cross-presentation of encapsulated OVA via MHC I. By TEM observation, we found protamine-coated nanoparticles, once internalized, were contacting with lysosomal membrane or in the cytoplasm compartiments (Fig. 5). These phenomena were different from uncoated nanoparticles that stayed within lysosomes. The reason why the two different intracellular states of OVA-NPs/protamine happened at the same time (8 h after BMDCs treatment) might due to the specific aggregated growth state of BMDCs in vitro. The outside BMDCs interacted with OVA-NPs/protamine early, whereas the inside BMDCs interacted with OVA-NPs/protamine comparatively later. The TEM phenomena were also consistent with a previous study showing that the positively charged arginine groups of protamine could bind to the acidic phospholipid groups in the membrane of the lysosomes and induced lysosomal leakage in vitro. The polycationic protamine-coated nanoparticles might exert sufficient buffering capacity in

**FIGURE 3.** OVA-NPs/protamine induce decreased secretion of IL-4 and increased secretion of IL-12p70 by BMDCs.

**FIGURE 4.** OVA-NPs/protamine induce increased cross-presentation of exogenous OVA.
lyosomes to facilitate lysosomal escape,\textsuperscript{17,22} which resulted in cytoplasm release of exogenous antigen and augmented the chance of loading on MHC I molecules rather than increase the expression of MHC I molecules directly.

Our results have provided cellular evidence for surface modified with protamine can enhance cross-presentation of exogenous antigens encapsulated PLGA nanoparticles. Further studies are required to evaluate the effect of protamine-coated PLGA nanoparticles on inducing cellular immunity against tumor and infectious agents.

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