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The suppression of IgE-mediated histamine release from mast cells following exocytic exclusion of biodegradable polymeric nanoparticles

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ABSTRACT

The objective of this study is to evaluate the effect of polymeric nanoparticles (NPs) on the allergic response of mast cells that release inflammatory mediators such as histamine through exocytosis. Submicron-sized biodegradable poly(DL-lactide-co-glycolide) (PLGA) NPs were prepared by the emulsion solvent diffusion method. Here, we examined the interactions of the mast cells with two types of PLGA NPs, unmodified NPs and NPs modified with chitosan (CS), a biodegradable cationic polymer. The cellular uptake of NPs increased by CS modification due to electrostatic interactions with the plasma membrane. NPs were taken up by mast cells through an endocytic pathway (endocytic phase) and then the cellular uptake was saturated and maintained plateau level by the exclusion of NPs through exocytosis (exocytic phase). Antigen-induced histamine release from mast cells was inhibited during the exocytic phase. The extent of histamine release inhibition was related to the amount of excluded NPs. Exocytic exclusion of NPs competitively antagonize the antigen-induced exocytotic release of histamine by highjacking exocytosis machinery such as SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins, since histamine release was recovered in mast cells that overexpress SNAP-23. The inhibitory effect of the allergic response by PLGA NPs was also evaluated *in vivo* using the mouse model for systemic anaphylaxis. The administration of NPs suppressed the antigen-induced systemic allergic response *in vivo*. In conclusion, PLGA NP itself has actions to inhibit the allergic responses mediated by mast cells.

1. Introduction

Biodegradable polymeric nanoparticles (NPs) have been developed as unique drug carrier systems because of their characteristic colloid-like behavior. In general, polymeric NPs can be used to deliver medicinal compounds because of their high stability and ease of cellular uptake through endocytosis [1,2]. We have successfully developed submicron-sized poly(DL-lactide-co-glycolide) (PLGA) NPs as cellular drug delivery systems for use in delivering anticancer drugs and genes [3–5]. PLGA has been widely accepted as a biocompatible and biodegradable material for drug delivery systems and has been approved by the US Food and Drug Administration for certain clinical uses in humans [6]. We have previously reported that the cellular uptake of PLGA NPs increases as the diameter decreases to the submicron level and when used in coating with chitosan (CS), which is a biodegradable cationic polymer that mediates surface modification [7]. CS modified-PLGA NPs (CS-PLGA NPs) have a positive zeta potential, and thus, could be associated with negatively charged cell membranes through electrostatic interactions. The intracellular trafficking of PLGA NPs after their uptake into cells has been considered as a typical endocytic–exocytic route in previously published studies [8–12]. Furthermore, the cellular uptake and intracellular distribution of NPs depends on the surface properties of NPs [8].

Mast cells play significant roles in allergic diseases secreting various mediators. In mast cells, the cross-linking of high-affinity IgE (Fcε-RI) receptors by multivalent antigens causes the activation of an intracellular signaling cascade that leads to the exocytic release of inflammatory mediators in secretory granules (degranulation), which results in allergic responses [13–15]. Several groups including us have shown the involvement of soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins during the degranulation process [16–23]. Recent studies have suggested that the vesicle-associated membrane proteins

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VAMP-2, VAMP-7, and VAMP-8 are possible v-SNAREs (vesicle SNARE) proteins that are responsible for degranulation of mast cells [16–21]. As for t-SNARE (target SNARE) proteins that reside on the plasma membrane, syntaxin-3, -4 and synaptosomal-associated protein of 23 kDa (SNAP-23) are thought to be involved in degranulation [16,17,22,23].

The aim of this study is to examine the effect of PLGA NP itself on the allergic response mediated by mast cells without loading drug. There are some reports on the interactions between mast cells and nanomaterials, such as fullerene and gold, suggesting their negative and positive effects on the activation of mast cells [24,25]. Furthermore it has been reported that diesel exhaust particles (DEP) deteriorate allergic responses [26,27]. Therefore it is likely that the allergic responses mediated by mast cells could be altered through the interactions with PLGA NPs. Considering the clinical application of PLGA NPs, therefore, it is important to examine their effects on mast cells. In this study, the rat basophilic leukemia cell line, RBL-2H3, which possesses the phenotypic properties of mucosal mast cells, was used [28]. To evaluate the effects of PLGA NPs uptake on the antigen-induced activation of mast cells, the antigen-induced release of β-hexosaminidase and histamine were examined in mast cells. Furthermore, the effect of PLGA NPs on allergic responses *in vivo* was investigated using a mouse model of systemic anaphylaxis.

2. Materials and methods

2.1. Materials

PLGA (lactide:glycolide = 75:25; MW = 20 000) was purchased from Wako (Osaka, Japan). Polyvinyl alcohol (PVA; MW = 25 000; hydrolyzation degree = 88.0%; polymerization degree = 500) was purchased from Kuraray (Osaka, Japan). Hydroxypropyltrimonium CS was obtained from Katakurachikkarin (Tokyo, Japan). The laser grade fluorescent dye 6-coumarin, [3-(2-benzothiazolyl)-7-(diethylamine) coumarin], was purchased from MP Biomedicals (Solon, OH, USA). All other chemicals were of the highest grade commercially available.

2.2. Preparation of PLGA NPs by the emulsion solvent diffusion (ESD) method

PLGA NPs were loaded with 6-coumarin as the fluorescent label and prepared using the previously reported ESD method in an aqueous solution [29]. PLGA (100 mg) and 6-coumarin (1 mg) were dissolved in 3 mL of a mixture of acetone and ethanol (acetone:ethanol = 2:1). The resulting organic solution was poured into 25 mL of an aqueous PVA solution (2% w/v, in distilled water) and stirred at 400 rpm at room temperature using a propeller-type agitator with three blades. The entire dispersed system was then centrifuged (43,400 ×*g* for 10 min), and the sediment was resuspended in distilled water. This process was repeated and the resulting dispersion was freeze-dried. To prepare CS-modified PLGA NPs, CS (0.25% w/v, in distilled water)-PVA (1% w/v) was used during the dispersing phase of the ESD process.

2.3. Analysis of the physicochemical properties of NPs

The particle size and zeta potential were determined using a Zetasizer Nano ZS90 (Malvern Instruments Ltd., Malvern, UK). Particle size was measured using photon correlation spectroscopy. The zeta potential was determined based on the electrophoretic mobility of NPs in an aqueous medium.

2.4. Cell culture

The RBL-2H3 cells were cultured in RPMI 1640 medium (Invitrogen, Grand Island, NY, USA) containing 10% FBS (Invitrogen) at 37 °C in an atmosphere of 5% CO_2.

2.5. Cytotoxicity assay

Cytotoxicity was assessed using the CellTiter 96® Aqueous One Solution assay (Promega, Madison, WI, USA). The solution reagent contained 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate, an electron coupling reagent. The RBL-2H3 cells were seeded in a 96-well plate (2.0×10^4 cells/well). After confluent growth, different concentrations of NPs in RPMI 1640 were added to the wells. After 2 or 20 h of incubation, the cells were washed three times with RPMI 1640. Twenty microliters of the Aqueous One Solution reagent and 100 μL of the culture medium were added directly to the culture wells. After 1 h of incubation, absorbance was measured using a Model DTX880 Multimode Detector (Beckman Coulter, Fullerton, CA, USA) at a test wavelength of 490 nm and a reference wavelength of 660 nm. The quantity of the formazan product formed, as determined by absorbance at 490 nm, is directly proportional to the number of viable cells. The cell viability (%) relative to the control wells that contained the cell culture medium without any test samples was calculated using the following equation: $[A]_{test}/[A]_{control} \times 100$, where $[A]_{test}$ is the absorbance of the test sample and $[A]_{control}$ is the absorbance of the control sample.

2.6. Cellular uptake of PLGA NPs by the RBL-2H3 cells

The RBL-2H3 cells were seeded in 24-well plates (1.0×10^5 cells/well). After confluent growth, the medium was replaced with the 6-coumarin-labeled NPs suspension in RPMI 1640 and incubated for 0.5–20 h at 37 °C. For the uptake experiments that were performed at 4 °C, the cells were preincubated with serum-free RPMI 1640 for 30 min before incubation at the respective NPs dispersion temperatures. Uptake was terminated by washing the cells three times with ice-cold phosphate-buffered saline (PBS) and solubilizing the cells with 0.1 mL of the reporter cell lysis reagent (Promega). The 6-coumarin fluorescent dye was extracted from NPs by mixing each sample with 3 mL of a methanol/chloroform mixture (1:1). The samples were centrifuged at 1400 ×*g* for 10 min and the 6-coumarin concentration was determined by fluorescent spectrophotometry (Hitachi F-2500, 490 nm excitation wavelength, 520 nm emission wavelength; Hitachi Instruments, Tokyo, Japan). The protein content of the cell lysates was measured using the Pierce BCA protein assay kit (Rockford, IL). Uptake was expressed as the amount (μg) of NPs associated with each unit weight (mg) of cellular protein.

2.7. Observation of cells exposed to PLGA NPs using confocal laser scanning microscopy

The RBL-2H3 cells were seeded in a Lab-Tek® II Chamber #1.5 German Coverglass System (Nalge Nunc International, Naperville, IL, USA) at a concentration of 1.0×10^5 cells/well. After 1 day, in order to stain the endolysosomal compartments, the cells were incubated for 30 min in NPs suspension (1 mg/mL) of RPMI 1640 that contained 75 nM LysoTracker Red DND-99 (Molecular Probes, Eugene, OR, USA). Cellular uptake was terminated by washing the cells three times with ice-cold PBS and the cell monolayers were fixed using 0.5 mL of a 4% paraformaldehyde solution. After washing with PBS, the fixed cells were observed using an LSM 510 META microscope (Carl Zeiss, Gottingen, Germany) that was equipped with a Zeiss Plan-Apochromat 63×/1.4 oil objective lens.

2.8. Assays for β-hexosaminidase and histamine secretions

The RBL-2H3 cells were seeded in 24-well plates (1.0×10^5 cells/well). After confluent growth, the medium was replaced with the NPs suspension in RPMI 1640 (2.5 mg/mL) and incubated for 2 or 20 h at 37 °C. Uptake was terminated by washing the cells three times with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered saline (140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.6 mM $MgCl_2$, 0.1% glucose, 0.1% BSA, and 10 mM HEPES; pH 7.4). Cells were sensitized by incubation with anti-dinitrophenyl (DNP)-IgE (200 ng/mL) for 20 min, and then further incubated with DNP groups that had been conjugated with bovine serum albumin (DNP-BSA) in 200 μL of HEPES-buffered saline for 30 min at 37 °C [30]. Degranulation of the RBL-2H3 cells was monitored by measuring the activity of β-hexosaminidase, a granule-stored enzyme that was secreted into the cell supernatant. Aliquots of the supernatant were incubated with a substrate solution (2 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide in 100 mM citrate; pH 4.5) for 45 min at 37 °C. After terminating the reaction with a Na_2CO_3–$NaHCO_3$ buffer, we measured the absorbance at 405 nm using a Model DTX880 Multimode Detector (Beckman Coulter). The supernatant was also used to measure the release of histamine by following the manufacturer's protocol for the enzyme immunoassay (EIA) system (Oxford Biomedical Research, Oxford, MI, USA).

2.9. Plasmid construction and generation of stable cell lines

Poly(A)$^+$ RNA was obtained using the QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech) from 1×10^7 RBL-2H3 cells; the poly(A)$^+$ RNA obtained served as the template for cDNA synthesis with SuperScript II RT (Gibco RBL), as reported previously [17]. The primer pair for rat SNAP-23 was 5′-GAATTCAATGGATGATCTATCACCA-3′ (sense; the *EcoRI* site is underlined) and 5′-GTCGACTTAGCTGTCAATGAGTTTC-3′ (antisense; the *Sal I* site is underlined). The PCR products were subcloned into pCR2.1-TOPO and confirmed by sequencing. Verified cDNA was ligated with pEYFP-C1 (CLONTECH, Palo Alto, CA, USA). To verify that the RBL-2H3 cells were able to stably express the yellow fluorescent protein (YFP)-tagged SNAP-23 protein, the RBL-2H3 cells were transfected with the plasmid DNA encoding YFP-SNAP-23. The RBL-2H3 cells were then electroporated in cold PBS with 40 μg of plasmid DNA at 250 V and 950 μF using the Gene Pulser II (Bio-Rad, Hercules, CA, USA). Stable transfectants were selected using G418 (Geneticin, Sigma, St. Louis, MO, USA). The localization of YFP-SNAP-23 into the RBL-2H3 cells was observed using fluorescence microscopy (BZ-9000, Keyence, Osaka, Japan).