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The use of calcium phosphate nanoparticles encapsulating Toll-like receptor ligands and the antigen hemagglutinin to induce dendritic cell maturation and T cell activation

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ABSTRACT

Dendritic cells (DCs) are potent antigen-presenting cells that possess the ability to stimulate naïve T cells. Antigen presentation by mature (activated) DCs is a prerequisite for the stimulation of antigen-specific T cells, whereas antigen presentation by immature DCs results in the generation of specific tolerance. Our aim was to develop calcium phosphate nanoparticles which can serve as carriers of immunoactive oligonucleotides into dendritic cells for their activation. We analyzed size, surface charge, and morphology of calcium phosphate nanoparticles loaded with the TLR ligands CpG and poly(I:C) and also with the antigen hemagglutinin (HA) by scanning electron microscopy, dynamic light scattering, Brownian motion analysis and ultracentrifugation. The uptake of fluorescence-labeled nanoparticles into dendritic cells was illustrated by confocal laser scanning microscopy. Immunostimulatory effects of these nanoparticles on DCs were studied, i.e., cytokine production and activation of the cells in terms of upregulation of surface molecules. We show that functionalized calcium phosphate nanoparticles are capable to induce both innate and adaptive immunity by activation of DCs.

1. Introduction

Unlike most bacterial infections, many viral infections do not result in pathogen clearance but rather in viral persistence and the development of a chronic state of infection (e.g., HIV, Hepatitis B and C). In general, the development of a chronic disease is associated with impaired antigen presentation, either due to the virus itself or in the context of the chronic inflammatory process. For most of these diseases, an effective prophylactic vaccine is not available. Traditional vaccination strategies utilizing live attenuated viruses or inactivated (killed) pathogens have been employed widely for the treatment and prevention of diseases. Although these approaches have generated successful results for a large number of diseases, safety concerns have led to the development of vaccines based on pathogen-derived protein antigens [1]. An ideal protein- or peptide-based vaccine should target and activate DCs *in vivo* and effectively generate protective immunity while limiting the use of potentially hazardous immunostimulatory agents. To achieve these demands, antigen delivery vehicles have been explored for use in vaccination [2]. For optimal performance, antigen delivery vehicles should closely mimic the composition and immunological processing of actual pathogens; they should actively or passively target professional antigen-presenting cells (APCs) such as DCs, protect the antigenic protein from degradation until reaching the target cells, direct the nature of resulting immune responses, and finally, induce APC maturation by interacting with elements of the innate immune system such as Toll-like receptors (TLRs). To address some of these issues, several strategies have been reported in the literature such as directly conjugating TLR ligands to proteins [3] or co-encapsulating immunostimulatory agents and proteins in liposomes [4] or hydrophobic polymeric particles [5].

In our previous work we have established the generation of calcium phosphate nanoparticles and their application as biomedical carriers [6]. Calcium phosphate has a good biocompatibility as it is the inorganic component of biological hard tissues, such as bone, teeth, and tendons [7]. In nanoparticulate form, it is easily taken up by cells and subsequently dissolved in lysosomes. The calcium ions are rapidly pumped out of the cell, and therefore the intracellular calcium level does not exceed a harmless level [8].

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Calcium phosphate nanoparticles can be prepared by rapid precipitation, followed by an immediate surface functionalization with DNA, RNA or oligonucleotides [9,10]. These particles typically have a size around 100 nm and form stable colloidal solutions. We applied these nanoparticles both for transfection (expression of certain proteins) [9] and for gene silencing/antisense experiments (selective inhibition of protein expression) [10]. To protect biomolecules from premature degradation by enzymes, we have prepared multi-shell nanoparticles in which DNA, RNA or oligonucleotides were incorporated into the particle [9]. In the case of such multi-shell nanoparticles, the transfection efficiency was much higher than that of single-shell nanoparticles [11].

The most important TLRs for viral components are TLR3 and TLR9. TLR3 recognizes viral double-stranded RNA (dsRNA) and its synthetic analogue polyriboinosinic:polyribocytidylic acid (poly(I:C)) and induces IFN-α, inflammatory cytokine/chemokine production and DC maturation [12,13]. The innate immune system appears to use TLR9 for the detection of non-methylated CpG dinucleotides that are relatively common in bacterial and viral genomes. TLR9 activation by CpG, DNA or synthetic oligodeoxynucleotides (ODNs) induces DCs to mature to highly effective APCs, characterized by the expression of costimulatory molecules, resistance to apoptosis, and secretion of Th1-promoting chemokines and cytokines such as IFN-α, IFN-γ, IP10 and other IFN-inducible genes. Because microbes express different sets of TLR ligands, it is not surprising that some TLRs cooperate for increased DC activation, thereby ensuring the generation of protective immunity. For example, DCs stimulated by TLR3/TLR4, TLR7/TLR8 or TLR9 act synergistically on DCs to induce the production of IL-12p70 and trigger a very potent Th1 development [14].

Therefore, in this study, we prepared calcium phosphate nanoparticles functionalized with CpG, poly(I:C), and a combination of both. In addition, a viral peptide of the influenza A virus hemagglutinin (HA) was encapsulated into the particles. By laser confocal microscopy we observed the uptake of fluorescence-marked nanoparticles by DCs.

2. Materials and methods

2.1. Preparation of functionalized nanoparticles

The preparation of single-shell calcium phosphate nanoparticles was performed as described previously [9]. The nanoparticles were prepared by fast mixing of an aqueous solution of calcium nitrate (6.25 mM) with an aqueous solution of diammonium hydrogen phosphate (3.74 mM). The pH of both solutions was adjusted beforehand to 9 with NaOH (0.1 M). Mixing was achieved by rapidly pumping both solutions into a glass vessel. Immediately after mixing (after a few seconds), 1 mL of the calcium phosphate was mixed with an aqueous solution of oligonucleotides in a polyethylene vessel. In the cases where either CpG or poly(I:C) was used, 0.2 mL solution was added. In the case of the mixture of CpG and poly(I:C), 0.2 mL of each oligonucleotide was added.

Two different types of dissolved oligonucleotides were used: CpG (63 μM = 400 μg mL^{-1}) and poly(I:C) (1 mg mL^{-1}). The final concentrations of oligonucleotides in the dispersion of single-shell calcium phosphate nanoparticles were 10.5 μM = 66.7 μg mL^{-1} for CpG only and 167 μg mL^{-1} for poly(I:C) only, and 9 μM = 57.1 μg mL^{-1} for CpG plus 143 μg mL^{-1} for poly(I:C) in the mixture. CpG-1826 with the following sequence: 5′-TCCATGACGTTCCTGACGTT-3′ (20; $M = 6364$ g mol^{-1}) was obtained from Eurofins MWG Operon (Ebersberg, Germany). Polyinosinic-polycytidylic acid potassium salt, poly(I:C), was obtained from Sigma–Aldrich, Germany (200–1000 bp).

Multi-shell nanoparticles were prepared as follows: First, oligonucleotide-coated calcium phosphate nanoparticles were prepared as described above. To this dispersion, we added 50 μL of the model-antigen HA (1 mg mL^{-1}) and then 0.5 mL of calcium nitrate (6.25 mM) and 0.5 mL of diammonium hydrogen phosphate (3.74 mM). This resulted in a crystallisation of calcium phosphate on the surface of the particles. The final colloidal stabilization (to prevent aggregation of the calcium phosphate surfaces) was then accomplished by adding either 0.2 mL of oligonucleotide solution (CpG, poly(I:C)) or a mixture of 0.2 mL of each oligonucleotide in the above given concentrations. This leads to the following final concentrations: CpG-loaded triple-shell calcium phosphate nanoparticles: 10.3 μM CpG and 20.4 μg mL^{-1} HA; poly(I:C)-loaded triple-shell calcium phosphate nanoparticles: 163 μg mL^{-1} poly(I:C) and 20.4 μg mL^{-1} HA; CpG/poly(I:C)-loaded triple-shell calcium phosphate nanoparticles: 8.8 μM CpG; 140 μg mL^{-1} poly(I:C), and 17.5 μg mL^{-1} HA. All cell experiments, except those with fluorescing nanoparticles (see below), were carried out with triple-shell nanoparticles.

For fluorescing calcium phosphate nanoparticles functionalized with both CpG and red-fluorescing TRITC-BSA ("CaP/CpG/TRITC-BSA"), [15] 1 mL of the dispersion of calcium phosphate before functionalization was taken immediately after mixing with a syringe and rapidly mixed with 0.2 mL of CpG (63 μM), followed immediately by 1 mL TRITC-BSA (1 mg mL^{-1}; Sigma, Steinheim, Germany).

All inorganic salts were of p.a. quality. Ultrapure water (Purelab ultra instrument from ELGA) was used for all preparations. All formulations were prepared and analyzed at room temperature. For dynamic light scattering, the samples were filtered through a sterile syringe filter with a 0.45 μm cellulose acetate membrane (Schleicher and Schuell) to remove larger aggregates.

The particles were characterised by scanning electron microscopy (ESEM Quanta 400) with palladium-sputtered samples. Dynamic light scattering and zeta potential determinations were performed with a Zetasizer nanoseries instrument (Malvern Nano-ZS, $\lambda = 532$ nm). The particle size data refer to scattering intensity distributions (z-average). For particle size determination, the Brownian motion of the particles was followed with a NanoSight LM10 instrument equipped with the NTA 2.0 Analytical Software.

2.2. Mice

BALB/c mice were obtained from Harlan Laboratories (Harlan Winkelmann GmbH, Borchen, Germany). TCR-HA transgenic (6.5) mice expressing an α/β-TCR recognising the MHC class II (H-$2E^d$:HA110-120)-restricted epitope of the HA protein have been described previously [16]. Mice aged 8–10 weeks were used.

2.3. Isolation of dendritic cells

For the isolation of DCs, splenic (SP) tissue was first cut into small pieces and then treated with 1 mg mL^{-1} collagenase type D (Roche Diagnostics GmbH, Mannheim, Germany) and 10 μg mL^{-1} deoxyribonuclease (DNase) I type II (Sigma–Aldrich Chemie GmbH, St. Louis, MO) diluted in phosphate buffered saline (PBS) with 2% FCS and 2 mM EDTA, incubated for 45 min at 37 °C, and mechanically minced and filtered through a 100 μm cell strainer. Cells were washed with PBS containing 2% FCS and 2 mM EDTA. $CD11c^+$ cells were positively selected on MACS columns according to the manufacturer's instructions (Miltenyi Biotec).

2.4. Visualization of the uptake of nanoparticles by dendritic cells by confocal laser scanning microscopy

DCs were isolated and subcultivated according to standard cell culture protocols. The cells were seeded in cell culture dishes with 2×10^5 cells per well in a 48-well dish and incubated for 24 h in IMDM complemented medium containing 10% FCS, 25 μmol 2-β-mercaptoethanol and 100 μg mL^{-1} penicillin/streptomycin.

The transfection with nanoparticles was carried out as follows: 40 μL of the particle dispersion were thoroughly mixed with 0.5 mL cell medium with DCs. The cells were incubated with nanoparticles for either 3, 5, or 7 h, followed by removal of the medium with the nanoparticles, twice washing with PBS and microscopic inspection. In the long-term experiment, the duration of incubation with nanoparticles was 7 h, and then the colloidal dispersion was removed and replaced by 0.5 mL of fresh medium without nanoparticles. The cells were then incubated for another 41 h. Then, the cells were washed twice with PBS to remove all dispersed nanoparticles, stained with DAPI and studied by confocal laser scanning microscopy (Zeiss LSM 510, Axiovert 200).

2.5. Stimulation of dendritic cells with functionalized nanoparticles

DCs were isolated as described in Section 2.3. For stimulation, 5×10^5 cells per well were incubated with the soluble TLR ligands (CpG, poly(I:C) or a combination of both) or calcium phosphate nanoparticles containing these TLR ligands in 400 μl of medium in a 48-well plate at 37 °C. After 24 h, supernatants and cells were harvested and used for further analysis.

2.6. Antibodies and flow cytometry

The monoclonal antibodies α-MHC II (2G9), α-CD80 (16–10A1), α-CD86 (GL1) and α-CD4 (RM4-5) were obtained from BD Biosciences Pharmingen (Heidelberg, Germany). Flow cytometry was performed with a LSR II instrument using the DIVA software (BD Biosciences).

2.7. Cytokine profile of stimulated DCs

DCs were isolated (Section 2.3) and 2.5×10^5 cells per well were stimulated as described in Section 2.5. After 24 h the culture supernatants were taken and the quantification of cytokines was performed with a Procarta Cytokine assay kit