Published in final edited form as:

Methods Mol Biol. 2014 ; 1139: 337–344. doi:10.1007/978-1-4939-0345-0_27.

TLR-9 Agonist Immunostimulatory Sequence Adjuvants Linked to Cancer Antigens

Hidekazu Shiota¹ and Dennis M. Klinman²

¹Department of Clinical Oncology, Tohoku University, Sendai, Japan

²Cancer and Inflammation Program, Frederick National Laboratory for Cancer Research, NCI, Frederick, MD, USA

Abstract

The primary goal of cancer vaccines is to elicit tumor-specific cytotoxic T lymphocytes (CTL) capable of eradicating established tumors and preventing/eradicating their metastatic spread. CpG oligonucleotides (CpG ODN) activate and support the maturation of immune cells, including plasmacytoid dendritic cells and B lymphocytes, that express Toll-like receptor 9 (TLR9) and are capable of presenting tumor antigens to T cells. Thus, CpG ODN are effective vaccine adjuvants. The adjuvant activity of CpG ODN is improved by maintaining them in close physical and temporal proximity to the co-administered vaccine antigen. This work describes a method of chemically conjugating CpG ODN to antigens and/or cancer cells that improve the resulting CTL response.

Keywords

CpG oligonucleotide; Vaccine; Adjuvant; Tumor

1 Introduction

Improving the host's ability to recognize and reject tumor cells is a key goal of cancer immunotherapy. Animal studies and clinical trials utilizing tumor-associated antigens (TAA) and whole non-viable cancer cells as vaccines demonstrate the feasibility of inducing an adaptive immune response capable of eradicating established tumors and tumor metastasis [1–3]. Key to the success of such immunotherapy is the ability to magnify the immunogenicity of TAA by inclusion of adjuvants capable of inducing a strong CTL response [4, 5]. In this context, whole killed tumor cells represent an excellent source of diverse TAA and also eliminate the need to identify individual antigens on tumors from different individuals [6].

Non-methylated CpG motifs are present at high frequency in bacterial, but not vertebrate DNA [7]. These motifs are sensed by immune cells which express the Toll-like receptor 9 (TLR9). The resulting interaction triggers an innate immune response that facilitates the elimination of the infectious pathogen. Characteristic of CpG-mediated immune responses is the activation of antigen-presenting cells (APC), Th1-cytokine-producing cells, antibody-secreting B cells, and CTL, all of which support the development of adaptive immunity [8,

9]. From these reported studies, CpG ODN are findings used as adjuvants to enhance the adaptive immune response.

We and others previously documented that the efficacy of CpG-based adjuvants is critically dependent on maintaining close physical and temporal proximity between ODN and Ag, as free ODN diffuse from the site of Ag delivery [10–12]. Physically cross-linking ODN to the target can improve the resulting immune response by up to a 100-fold over that induced by simply mixing ODN with the immunogen [10, 13, 14]. We also have shown that chemically cross-linking CpG ODN to the surface of apoptotic tumor cells generates a highly immunogenic vaccine capable of accelerating tumor rejection in murine models [15]. CpG ODN conjugation improves the uptake of apoptotic tumor cells by host APC and promotes the functional maturation of dendritic cells thereby facilitating the induction of tumor-specific responses. These findings suggest that CpG-conjugated TAA or autologous cancer cells may be useful in cancer immunotherapy.

2 Materials

A number of different methods have been evaluated for their ability to induce antitumor immunity. Below is a description of how protein-based TAA and whole apoptotic tumor cell vaccines can be prepared in conjunction with CpG ODN.

2.1 Oligodeoxynucleotides (ODNs)

The following ODNs (MW 6,500) were used:

CpG ODN 1826 (TCCATGACGTTTCCTGACGTT).

Control ODN 1745 (TCCATGAGCTTCCTGAGTCT).

Both of these ODNs can be purchased as thiolated or NH₂-modified phosphorothioate from multiple sources. The ODN should be free of detectable protein or endotoxin contamination.

2.2 Chemical Conjugation of ODN to TAA

1. Lyophilized thiolated phosphorothioate ODN.
2. Sterile endotoxin-free water.
3. Sulfo-SMCC (sulfosuccinimidyl 4-[N-maleimidomethyl] cyclohexane-1-carboxylate) (Thermo Scientific, Rockford, IL, USA) MW 436 (*see* Notes 1 and 2).
4. Conjugation Buffer, phosphate-buffered saline (PBS): 100 mM sodium phosphate, 150 mM sodium chloride, and pH 7.2 (*see* Note 3).
5. Amine-modified recombinant tumor antigen (Protein-NH₂).
6. Desalting column to separate modified protein from excess cross-linker.
7. Dialysis equipment, MW cut-off 10,000.
8. BCA protein assay reagent (Thermo Scientific).

2.3 Conjugation of ODN to Apoptotic Tumor Cells

1. Lyophilized NH₂ modified phosphorothioate ODN.
2. Sterile endotoxin-free water.
3. BS³ (bis[sulfosuccinimidyl] suberate). Prepare this cross-linker immediately before use by dissolving BS³ in water or 50 mM PBS. BS³ may be added directly to cells to decrease the extent of hydrolysis (*see* Notes 4 and 5).
4. Conjugation Buffer: Use a non-amine-containing buffer at pH 8.0, such as NaOH-adjusted PBS.
5. Apoptotic tumor cells. Induce apoptosis by irradiation (15,000 rad) or chemical treatment, e.g., incubation with 10 µg/mL of mitomycin C for 8–12 h.

3 Methods

3.1 Chemical Conjugation of CpG ODN to TAA Protein (Reaction Scheme, Fig. 1)

1. Dissolve lyophilized thiolated phosphorothioate ODN in sterile endotoxin-free water at a concentration of 5 mg/mL. This material can be stored at –20 °C.
2. Dissolve amine-modified recombinant tumor antigen in conjugation buffer.
3. Add the appropriate amount of cross-linker to the protein solution. The concentration of the protein-NH₂ determines the amount of cross-linker to use. Suggested cross-linker molar excesses are shown in Table 1.
4. Incubate reaction mixture for 30 min at room temperature or 2 h at 4 °C.
5. Remove excess cross-linker by passing the mixture through a desalting column equilibrated with Conjugation Buffer.
6. Combine and mix CpG ODN-SH and desalted cross-linker-activated protein in a molar ratio corresponding to that desired for the final conjugate and consistent with the relative number of sulfhydryl and activated amines that exist on the protein and ODN (*see* Note 6 and Table 2).
7. Incubate the reaction mixture overnight at 4 °C.
8. Remove unconjugated ODN by dialysis against 20 mL of PBS overnight at 4 °C.
9. Measure the unconjugated ODN in dialysis buffer by NanoDrop and calculate/estimate protein-bound CpG ODN (*see* Note 7).
10. Change the dialysis buffer and dialyze two more times at 4 °C.
11. Measure protein concentration using a protein assay kit.

In the process of protein-ODN conjugation, the protein may aggregate. An example of this effect is seen in Fig. 2 where CpG ODN was conjugated to ovalbumin. We recommend separating the unconjugated material by gel filtration chromatography and visualizing the outcome using SDS-PAGE stained for protein (A) and ODN (B). In the example provided, the lane furthest on the right represents crude freshly synthesized OVA-ODN. The output of

the gel filtration shows that some fractions stain for protein, but not DNA. These are unconjugated protein and they lack immunostimulatory activity. Other fractions stain for both protein and DNA. These correspond to stimulatory CpG-OVA conjugates and strongly induce Th1 differentiation. In our experience, both aggregates and monomers containing CpG ODN and protein are immunostimulatory.

3.2 Chemical Conjugation of CpG ODN to Cells

1. Induce apoptosis by irradiation (15,000 rad) or chemical treatment of target cells using 10 µg/mL of mitomycin C for 8–12 h.
2. Wash cells extensively with ice-cold PBS, pH 8.0, to remove amine-containing culture medium and proteins released by apoptotic cells.
3. Suspend 5×10^7 cells in 1 mL of PBS, pH 8.0, and add NH₂-modified ODN at a final concentration of 500 µg/mL, i.e., 10 µg/ 1×10^6 cells. Incubate for 1 h at 4 °C.
4. Add BS³ solution to a final concentration of 1–5 mM and incubate for 30 min at 4 °C.
5. Wash cells 1× by centrifugation to remove unbound ODN.
6. Measure the unconjugated ODN in the supernatant and use this to calculate the amount of ODN bound to the cells (*see Note 8*).
7. Count the cells (*see Note 9*).

4 Notes

1. Sulfo-SMCC is moisture-sensitive. Therefore, you should store the material frozen in an airtight container that includes a desiccant. Allow the vial containing the sulfo-SMCC to reach room temperature before opening it to avoid any water condensing inside the container. Dissolve only the amount of reagent needed for use and add it immediately (before hydrolysis occurs) to the protein. Discard any excess dissolved sulfo-SMCC as it will be inactive.
2. We recommend the use of “No-Weigh Microtubes” for sulfo-SMCC. Immediately before use, puncture the microtube foil with a pipette tip, add 200 µL of ultrapure water, and pipette up and down to mix. Store unused microtubes frozen in the presence of a desiccant.
3. Avoid buffers containing primary amines, e.g., Tris or glycine, or sulfhydryls during conjugation because they will compete with the intended reaction. If necessary, dialyze or desalt samples into an appropriate buffer such as PBS.
4. BS³ is moisture-sensitive. To avoid moisture condensation in the storage vial, it must be equilibrated to room temperature before opening.
5. The NHS-ester moiety readily hydrolyzes and becomes nonreactive. Therefore, prepare the cross-linking reagent immediately prior to use. Discard any unused reconstituted cross-linker.

6. The typical molar ratio of CpG ODN to protein in the conjugate is 5–10:1.
7. Subtract total free CpG ODN (ODN concentration in dialysate $\times$ 20 mL) from total added-CpG ODN.
8. Subtract total free CpG ODN (ODN concentration in supernatant $\times$ volume) from total added-CpG ODN.
9. The typical number of CpG ODN-conjugated cells used as a vaccine in mice is 2×10^6 .
10. We found that 80–90 % of ODN bind to cells using this technique. This can be visualized directly by using FITC-modified ODN and studying the cells using confocal microscopy (E.G7 cells) (Fig. 3).

Acknowledgement

This research was supported by the Intramural Research Program of the NIH, National Cancer Institute.

References

1. Greten TF, Jaffee EM (1999) Cancer vaccines. *J Clin Oncol* 17:1047–1060 [PubMed: 10071300]
2. Lollini PL et al. (2006) Vaccines for tumour prevention. *Nat Res Cancer* 6:204–216
3. Gilboa E (2004) The promise of cancer vaccines. *Nat Rev Cancer* 4:401–411 [PubMed: 15122211]
4. Schijns VE, Lavelle EC (2011) Trends in vaccine adjuvants. *Expert Rev Vaccines* 10:539–550 [PubMed: 21506650]
5. Chiang CL et al. (2011) Adjuvants for enhancing the immunogenicity of whole tumor cell vaccines. *Int Rev Immunol* 30:150–182 [PubMed: 21557641]
6. Klebanoff CA et al. (2011) Therapeutic cancer vaccines: are we there yet? *Immunol Rev* 239:27–44 [PubMed: 21198663]
7. Krieg AM et al. (1995) CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 374:546–548 [PubMed: 7700380]
8. Hemmi H et al. (2000) A Toll-like receptor recognizes bacterial DNA. *Nature* 408:740–745 [PubMed: 11130078]
9. Takeshita F et al. (2001) Cutting Edge: role of toll-like receptor 9 in CpG DNA-induced activation of human cells. *J Immunol* 167: 3555–3558 [PubMed: 11564765]
10. Shirota H et al. (2000) Regulation of murine airway eosinophilia and Th2 cells by antigen-conjugated CpG oligodeoxy-nucleotides as a novel antigen-specific immunomodulator. *J Immunol* 164:5575–5582 [PubMed: 10820231]
11. Tighe H et al. (2000) Conjugation of protein to immunostimulatory DNA results in a rapid, long-lasting and potent induction of cell-mediated and humoral immunity. *Eur J Immunol* 30:1939–1947 [PubMed: 10940883]
12. Heit A et al. (2005) Protective CD8 T cell immunity triggered by CpG-protein conjugates competes with the efficacy of live vaccines. *J Immunol* 174:4373–4380 [PubMed: 15778402]
13. Shirota H et al. (2001) Novel roles of CpG oligodeoxynucleotides as a leader for the sampling and presentation of CpG-tagged antigen by dendritic cells. *J Immunol* 167:66–74 [PubMed: 11418633]
14. Sano K et al. (2003) Oligodeoxy-nucleotides without CpG motifs work as adjuvant for the induction of Th2 differentiation in a sequence-independent manner. *J Immunol* 170: 2367–2373 [PubMed: 12594259]
15. Shirota H, Klinman DM (2011) CpG-conjugated apoptotic tumor cells elicit potent tumor-specific immunity. *Cancer Immunol Immunother* 60:659–669 [PubMed: 21318638]

Fig. 1.
Schema for conjugation of protein to CpG ODN

Fig. 2. Synthesis of protein—CpG ODN conjugate. The molar ratio of CpG ODN:protein in this conjugation was 5:1. CpG ODN was bound to ovalbumin protein. The lane on the far right shows the crude, unseparated conjugate. This was separated over a Sephacryl S-200 HR column and the fractions collected analyzed on SDS-PAGE gel stained for protein (*A*) or CpG (*B*). Note that fractions 24–26 were strongly stimulatory

Fig. 3. Binding of CpG ODN to the surface of apoptotic tumor cells. E.G7 cells were treated with mitomycin C for 24 h to induce apoptosis. The cells were then conjugated to FITC-labeled CpG ODN and binding examined by confocal microscopy (*see* Note 10)

Table 1

Cross-linker preparation and molar excess to use for 1 mL of sample

NH ₂ protein concentration (based on a 50 kDa protein)	10 mg/mL	1 mg/mL	0.5 mg/mL
Cross-linker molar excess	5×	20×	50×
Sulfo-SMCC (50 mM, 4.8 mg/mL)	100 μ L	40 μ L	50 μ L

Table 2

Preparation of cross-linker-activated protein and ODN-SH for 1 mL of sample

Cross-linker-activated protein (based on a 50 kDa protein, 1 mg/mL)			
Molar ratio of protein:CpG ODN	20:1	5:1	2:1
ODN-SH			
(5 mg/mL)	520 μ L	130 μ L	52 μ L