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Use of Nanoparticles to Deliver Immunomodulatory Oligonucleotides

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Abstract

Synthetic oligonucleotides (ODN) containing unmethylated 'CpG motifs' stimulate the innate immune system to produce cytokines, chemokines and polyreactive antibodies. CpG ODN have shown promise as vaccine adjuvants and for the treatment of infectious diseases and cancer. The immunostimulatory activity of CpG ODN is inhibited by DNA containing "suppressive" motifs. ODN expressing suppressive motifs (Sup ODN) reduce ongoing immune reactions and show promise in the treatment of autoimmune and inflammatory diseases. This work reviews recent progress in the use of nanoparticles as carriers of CpG and Sup ODN to target their delivery to the GI tract and lungs.

Introduction

DNA has multiple and complex effects on the immune system. Bacterial DNA contains CpG motifs that stimulate cells expressing Toll-like receptor 9 to mount a protective innate immune response (1–3). Synthetic ODN containing CpG motifs mimic this immunostimulatory activity (4,5) and have shown efficacy in mouse models of infection and cancer. These ODN are the subject of several dozen clinical trials.

Immune responses induced to protect the host can have serious deleterious consequences if inadequately regulated, causing or exacerbating inflammatory and autoimmune diseases (6–9) reviewed in (10). Over exuberant immune activation can be inhibited by mammalian DNA, an effect mediated by the TTAGGG motifs present at high frequency in telomeric DNA (11,12). Suppressive oligonucleotides (Sup ODN) disigned to express these telomeric TTAGGG motifs have shown utility in preventing or reducing toxic shock, autoimmune arthritis, iritis and lupus, and inflammation driven cancers (13,14)(9,15,16).

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Conflict of Interest

Members of Dr. Klinman's lab have patents related to the use of CpG and suppressive oligonucleotides. All rights to such patents have been assigned to the Federal Government.

ODNs used to stimulate or suppress the immune system are typically fabricated from phosphorothioate nucleotides (PS-ODN). These confer resistance to serum DNases which improves their *in vivo* half-life. Immunomodulatory ODN have historically been delivered by i.v., i.m., i.p. or s.c. injection. Yet recent reports suggest that their therapeutic activity could be improved by more selective delivery. In murine models of arthritis, iritis, and silicosis, Sup ODNs were maximally effective when delivered directly to the joints, eyes, and lungs, respectively (16–18). Similarly, the anti-tumor activity of CpG ODNs was maximized by direct injection into the tumor bed (19). These observations fuelled interest in methods that could deliver ODNs to target organs and insure that they remain at the site of delivery (rather than diffusing into serum and re-distributing throughout the body). For the treatment of inflammatory diseases and cancer of the lungs and Gi tract there could be considerable benefit to administering ODN directly to those sites, including increased efficacy, reduced dose, lower cost and fewer side effects.

Use of microparticle-formulated CpG ODN for the treatment of lung cancer

Lung cancer is the leading cause of cancer-related death in the United States. One strategy for improving the host's anti-tumor response relies on stimulating the innate immune system. As described above, triggering cells that express Toll-like receptor 9 by administering CpG ODN can substantially improve anti-tumor activity. This effect can be optimized by insuring that the ODN reach and persist at the tumor site (20,21). While both local and systemic administration of CpG ODN stimulates the generation of tumor-specific CTL, the cytotoxicity of such cells is reduced by the immunosuppressive microenvironment surrounding lung tumors. Local but not systemic delivery of CpG ODN inhibits the activity of regulatory T cells and myeloid-derived suppressor cells (MDSC) in the tumor milieu and triggers the latter to mature into tumoricidal macrophages (20–22). Local delivery also reduces the accumulation of regulatory T cells that protect the tumor from immune elimination (22).

The lungs are an inviting target for therapeutic intervention as they have a large surface area that provides ready access to the blood stream. The lung also lacks digestive enzymes that might break down or otherwise impede the activity of ODN delivered by that route. In studies involving the therapeutic administration of ODN, systemic delivery (the route used in most clinical trials) resulted in rapid clearance of the ODN with a median t_{max} of 2 h and mean $t_{1/2}$ of $\approx$ 12 h (23). This contrasts to intrapulmonary administration where free ODN was found to persist up to 48 h in murine models (22). To further improve local uptake and persistence, the possiblity of formulating ODN conjugated to nanoparticles (NP) has been evaluated.

Early efforts examined the physical characteristics (size, morphology, charge and other properties) that affected the intra-pulmonary delivery of NP. Results showed that particles under 1 micron in size 1) readily reach the distal alveoli, 2) gain access to the bloodstream through the alveolar epithelium and 3) escape clearance by alveolar macrophages (24,25). Unfortunately, small NP can also be exhaled prior to being adsorbed into pulmonary tissue (26). By comparison, microparticles 1–5 microns in diameter are effectively deposited and retained in the bronchiolar region of the lungs.

To improve their utility as inhalational agents, ODN were adsorbed onto particles designed to meet the following requirements: 1) capable of slowly releasing the ODN to prolong their duration of activity, 2) biodegradability for safe for repeated administration and 3) physical properties optimized for delivery via inhalation. Many types of engineered carriers were evaluated including polymeric, liposomal, carbon nanotube based, apatite, mesoporous silica, and micelles (22,27–37).

As a general rule, ODN conjugated to NP induced stronger Th1 responses than free ODN. Co-encapsulation with Ag yielded vaccines that were generally superior at generating protective CTL and/or Ab responses. In vivo studies showed that multiple types of NP could be delivered and had activity tumor treatment using murine challenge models.

Among these, polymeric NP had advantages when compared to other types of polymer based particles. Polymeric NP encapsulate ODN with high efficiency, release the ODN at a defined rate depending on the pH and can be formulated into microparticles (MP) of optimal size for reaching the distal bronchi (38,39). Unlike PLGA based NP, polyketal NP do not induce inflammation upon degradation in the lungs. Polyketal nanoparticles were first developed by Heffernan et al. and used as an acid-sensitive delivery system for drugs to tumors and sites of inflammation (40). They were subsequently administered by the intratracheal route and shown to effectively treat bleomycin-induced fibrosis without serious adverse events (39). Thus, polyketal based particles may be optimal for controlled delivery of ODN to the lungs. They are also considerable less expensive than lipid-based NP (27,28).

Effect of CpG ODN on the growth of pulmonary tumors

The experimental system used to study CpG ODN on polyketal MP (hereafter CpG-MP) involved the intra-tracheal delivery of 10^6 Lewis lung cancer cells (LLC) into syngeneic mice (41,42). This caused animals to develop peri-bronchial tumors resembling those present in humans with non-small cell lung cancer (41,43). Survival time among these animals in the absence of therapy was ≈ 22 days. Systemic treatment with free CpG ODN had little effect on survival whereas delivery into the lungs increased survival time to 38 days (22). This effect was sequence specific as control ODN had no effect.

While local delivery of CpG ODN was superior to systemic administration, most mice still succumbed to cancer. As free ODN rapidly diffuse from the lungs into the blood stream, the study was repeated using CpG-MP. Mice were challenged with 10^6 LLC and treated weekly for one month with CpG-MP starting on day 7. When delivered systemically (the route by which free CpG ODN was ineffective in phase III human trials) (44,45), CpG-MP nearly doubled median survival time. Far better results were achieved when CpG-MP was instilled directly into the lungs: 82% of mice survived indefinitely (animals were followed for up to 1 year)(22). Moreover, the surviving mice developed a protective memory response and remained tumor free when re-challenged with LLC months after cessation of CpG-MP therapy. Delivering control ODN adsorbed onto polyketal microparticles had no significant effect on survival.

Effect of CpG-MP on immune cells *in vivo*

To assess the effect of CpG-MP in the lungs, bronchioalveolar lavage fluid (BAL) was collected. When compared to saline treated controls, cellularity rose by ≈ 25% in mice treated with free CpG ODN and by nearly 4-fold in the BAL of mice treated with CpG-MP (22). This cellular infiltrate consisted primarily of macrophages and lymphocytes. Consistent with previous studies, free CpG ODN induced a significant increase in pulmonary IL-12 levels, an effect magnified >10-fold by delivery of CpG-MP (22). No such changes occurred in mice treated with control ODN-MP or saline.

To clarify the basis of the anti-tumoral immunity elicited by local delivery of CpG-MP, serial sections were taken through tumor beds and examined histologically. The tumor burden of mice treated with CpG-MP fell by >90% (22). The frequency of apoptotic cells in these tumors rose by 3-fold while the number of $CD8^+$ T cells infiltrating the tumor site increased by nearly 8-fold. Immunohistologic analysis of serial lung sections showed that the frequency of immunosuppressive cells in the tumor microenvironment (T_{regs} plus M2 macrophages) declined by over half while the number of M1 macrophages doubled (22). The magnitude of these effects increased over time and with repeated therapy culminating in >80% long term survival of animals that would otherwise succumb to cancer in 22 days.

Development of nanoparticles for oral delivery of ODN

The ability of immunomodulatory ODN to treat inflammation, autoimmunity and cancer is optimized by early and repeated delivery. For example, studies show that CpG ODN protect against lethal bacterial challenge when administered shortly after exposure but not when treatment is delayed (46). In cancer models CpG ODN were maximally effective when administered shortly after tumor challenge (19). The benefit of early treatment with Sup ODN was established in studies of autoimmune disease, toxic shock and inflammatory lung disease (9,13,16,47). The therapeutic efficacy of immunomodulatory ODN declined as administration was delayed. Yet physicians are hesitant to recommend agents requiring long-term parenteral administration to patients with mild or recent onset disease. This contrasts to agents that are taken orally (or by inhaler) which are prescribed quite commonly to reduce the risk of heart disease, stroke, asthma and diabetes. An important limitation to the use of ODN-based therapies has been their reliance on parenteral routes of administration, with virtually all clinical trials delivering ODN by injection (48,49). One strategy to circumvent this limitation involves formulating ODN for delivery by inhaler (described above). Another involves formulating ODN for oral use.

A number of criteria must be met for oral delivery to be effective. The ODN 1) needs to be protected from DNases, digestive acids and enzymes in the GI tract, 2) must be in a form that can be adsorbed from the gut while 3) retaining its immunomodulatory properties. It would also be of benefit if the orally delivered ODN were of low cost, reached specific target cells, and remained active over a prolonged period. In this context, although PS modification reduces ODN sensitivity to DNase digestion it does little to alter susceptible to the harsh environment of the GI tract. In 2012, Zhu et al. described the delivery of ODN to the large intestine using pH-dependent PLGA (poly-D,L-lactide-co-glycolide) NP (50).

These NP remained intact when passing through the upper GI tract but were triggered to release their cargo by the pH change encountered in the large intestine. More recently, Yamamoto et al showed that complexes consisting of carbonate apatite microparticles formed in a high glucose environment were protected against the low pH and DNA degrading enzymes found in the GI tract (29). ODN are composed of negatively charged nucleotides, they readily bind to positively charged calcium ions and grow to form nanoparticles 50–200 micron in size that contain approximately 1% DNA by weight. Recent studies show that these “edible” ODN retain there immunomodulatory activity when taken up by cells in the intestinal mucosal (see below).

Activity of orally delivered CpG-CaNP

Preliminary *in vitro* studies showed that immune cells could recognize and respond to CpG ODN adsorbed onto calcium carbonate NP (CaNP) (51). CpG-CaNP resisted degradation by gastric acids. When fed to mice, they were taken up by cells in the Peyer’s patches. Macrophages located in these PP were stimulated by CpG-CaNP, a response characterized by locally increased production of IFNγ (control CaNP had no such effect). After 4 weeks of daily oral CpG-CaNP treatment, systemic effects became evident including enlargement of the spleen (which is observed after repeated parenteral injection) and increased expression of IL-33 mRNA (51). Orally delivered CpG-CaNP were found to mediate cytotoxicity against colon cancer cells (29).

Activity of orally delivered Sup ODN

Atopic dermatitis (AD) is a type 1 allergic condition. Therapy generally involves topical application of steroids, skin softeners and/or oral anti-histamines. A murine model of chemically induced AD was used to evaluate whether orally administered Sup-CaNP could alter systemic immunity and susceptibility to AD. Dermatitis was induced by repeatedly applying a contact sensitizing agent to the backs and ears of normal mice. Inflammation developed after the third weekly application and worsened over time. Treatment with control-, CpG- or Sup-CaNP was initiated concurrent with the first application of the sensitizing agent and continued daily for 70 days.

Spleen cells from mice fed Sup-CaNP produced less IL-4 and IL-33 when stimulated *ex vivo* with allergen than splenocytes from controls. The mechanism by which Sup-CaNP reduced systemic immunity is still under investigation but include inhibiting STAT phosphorylation which prevents the differentiation of allergen-activated Th2 cells into IL-4 producers (30) by blocking the signal transduction cascade needed to maintain inflammatory and autoimmune conditions (30,33). In contrast, spleen cells from mice fed CpG-CaNP produced more IFNγ and IL-33 after re-stimulation, consistent with the amplification of Th1-biased immunity observed by other groups after parenteral CpG administration (52,53). Consistent with their ability to activate inflammatory cells, the magnitude of AD was significantly worsened by administration of CpG-CaNP. In contrast, the severity of disease in mice treated with Sup-CaNP was significantly reduced while CaNP alone or containing control ODN had no effect on disease.

Implications

Clinical trials demonstrate that systemic treatment with CpG ODN is of limited benefit to patients with cancer (45,54–57). Animal models suggest that ODN therapy is optimized by local rather than systemic delivery (58,59). To improve the uptake and persistence of ODN for the treatment of lung cancer, CpG ODN were adsorbed onto biodegradable polyketal microparticles whose size was optimized for delivery throughout the bronchial tree (22,60,61)in tumor nests.

To enable oral delivery, ODN were adsorbed onto calcium-based NP. This formulation protected the ODN from degradation while enhancing their uptake by cells in the GI tract. The ODN retained their immunomodulatory activity, leading to changes in both local and systemic immune function that impacted disease susceptibility. The ability to delivery biologically active ODN by the oral or inhalational routes considerably broadens their potential therapeutic utility. If clinical efficacy can be achieved in the absence of parenteral injection, the cost, complexity and inconvenience of immunomodulatory therapy could be dramatically reduced.

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