Kgp, *RgpA*, and *IL-4* DNA Vaccines Induce Antibody Responses in Experimental Peri-implantitis

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Abstract

Background: Peri-implantitis is one of the most common complications of implant failure. Our previous experiments have confirmed that *kgp* and *rgpA* DNA vaccines can inhibit peri-implantitis bone loss. This study aimed to evaluate pVAX1-IL-4 (Interleukin-4) as the adjuvant to modulate the immune response of *kgp* and *rgpA* DNA vaccines to prevent peri-implantitis in dogs.

Methods: *Kgp*, *rgpA* and *IL-4* were cloned into the pVAX1 vector respectively. Titanium implants were placed into the mandible bone of adult male beagle dogs. Three months later, the dogs were divided into five groups (A,B,C,D and E) immunized with pVAX1-kgp, pVAX1-rgpA, pVAX1-kgp+pVAX1-IL-4, pVAX1-rgpA+pVAX1-IL-4 and pVAX1 separately. Cotton ligatures infiltrated with *Porphyromonas gingivalis (Pg)* were placed around the neck of the implants. Before and after immunization, Immunoglobulin G (IgG) and Immunoglobulin A (IgA) antibodies were detected by Enzyme-LinkedImmunosorbent Assay (ELISA).

Results: The eukaryotic expression plasmid pVAX1-kgp, pVAX1-rgpA and pVAX1-IL-4 were successfully constructed. Dogs immunized with pVAX1-kgp+pVAX1-IL-4 and pVAX1-rgpA+pVAX1-IL-4 showed higher titers of IgG and IgA antibodies compared with those before immunization ($P<0.05$) as well as higher than those that were immunized with pVAX1-kgp and pVAX1-rgpA, while there were no significant differences in the animals treated with pVAX1. Furthermore, among these, the pVAX1-kgp+pVAX1-IL-4 DNA vaccines were more effective. The bone losses of the first four groups were significantly attenuated.

Conclusion: The pVAX1-IL-4 DNA vaccine could enhance the preventive effect of the pVAX1-kgp and pVAX1-rgpA DNA vaccines to peri-implantitis in experimental peri-implantitis animal models, and the immunogenicity of dogs immunized with the pVAX1-kgp+pVAX1-IL-4 DNA vaccine was the highest.

Key Words: Vaccines DNA, Peri-implantitis, Kgp protease, RgpA protease, IL-4

Introduction

Peri-implantitis, which is a major factor leading to implant failure, is an infectious diseases caused by the microbiota such as *Porphyromonas gingivalis (Pg)*, *Aggregatibacter actinomycetemcomitans (Aa)*, *Fusobacterium species (Fs)* [1]. Among these, *Pg*, gram-negative anaerobic bacterium, is considered to be the major pathogen causing peri-implantitis. *Pg* has kinds of virulence factors, such as lipopolysaccharide, fimbriae, haemagglutinins. *Pg* goes beyond simple adhesion and actively invades and replicates within epithelial cells in vitro [2]. Arginine-specific cysteine proteinase [Arg-gingipain (Rgp), encoded by rgpA and rgpB] and lysine-specific proteinase [Lys-gingipain (Kgp), encoded by kgp] are the predominant extracellular proteolytic enzymes of *Pg* [3]. Growing evidence indicates that these 2 types of gingipains synergistically contribute to the entire virulence of the organism and increase the risk of periodontal disease by disrupting the host immune system and degrading the host tissue and plasma proteins. *Rgp* and *Kgp* have been shown to function as processing enzymes for various cell-surface proteins of *Pg*, such as fimbrial components, hemoglobin-binding protein and other proteinases [4]. *RgpA, RgpB* and *Kgp* can also be posttranslationally processed for retention on the cell surface or secretion into the extracellular milieu [5]. The cell-associated gingipains comprise the majority (~80%) of *Rgp* and *Kgp* activities, and are reported to be definitive virulence factors that degrade various host proteins, leading to impaired cellular associated integrity and function [4].

DNA vaccine is formed with a plasmid vector including the gene for the antigen of interest. After application of DNA vaccine, cells of vaccinated individual express antigen that can induce immune responses. The gingipains (*RgpA* and *Kgp*) of *Pg* are potential candidates for a vaccine that could be used for prevention of *Pg*-mediated periodontal disease [6]. Our previous experiments have confirmed that the *kgp* and *rgpA* DNA vaccines can inhibit peri-implantitis bone loss [7]. Then we hypothesized that the increase of some kind of adjuvant could enhance the efficiency of the *kgp* and *rgpA* DNA vaccines in this study.

In order to enhance the immune effect of DNA vaccine, many scholars use cytokines as an adjuvant to modulate the immune response [8]. Interleukin, refers to the lymphokine interactions among white blood cells or immune cells. It is so important in the transmission of information, the activation and regulation of immune cells and in inflammatory reaction. Moreover, T and B cells' activation, proliferation and differentiation are mediated by interleukin. Among interleukin, *IL-4* is a T-lymphocyte-associated cytokine that is involved in several cell immune responses with a principal role in ameliorating inflammation-associated diseases such as

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cancer through inhibition of Th-1 responses [9-11]. This cytokine acts as a growth factor for type 2 helper T cells (TH2) and induces immunoglobulin (Ig) class switching to IgE in immune responses [12]. It promotes the proliferation and differentiation of antigen presenting cells [13]. Meanwhile, *IL-4* is essential in antibody isotype switching and stimulates the production of IgE. However, the content of IgE was too low to detect in the serum. We did not investigate IgE antibody in this study. It has been applied in the treatment of autoimmune disorder like multiple myeloma [14], cancer [15], psoriasis [16], and arthritis [17]. *IL-4* is the pleiotropic cytokine and has been widely used as adjuvants to enhance immune responses of many vaccine antigens. *IL-4* plays a majorrole in T-cell development and is thought to promote the differentiation of T helper cells into Th2 cells during an immune response [18]. Zhang et al. [19] showed that IL-4 can promote B cell proliferation and differentiation, indirectly increase the effects of killer T cells against cysticerci, augment the production of anti-cysticercosis antibodies, increase antigen presentation, and promote the secretion of cytokines involved in immune regulation. Several studies in different animal models have also demonstrated that *IL-4* can enhance antigen-specific humoral responses [19,20]. Thus, we measured only *IL-4*. The objective of this experiment is to evaluate pVAX1-IL-4 as the adjuvant to modulate the immunogenicity of *kgp* and *rgpA* DNA vaccines in dogs with peri-implantitis.

Materials and Methods

Twenty adult male beagle dogs, weight 10-15 kg, were randomly divided into five groups (4 dogs each) in this experiment. General guidelines were approved by the Animal Ethics Committee of Shandong University. All dog experiments were performed in an ethical and humane manner under veterinary supervision.

Bacterial preparation

Pg ATCC 33277 was obtained from the American Type Culture Collection (ATCC). The bacterial cells were cultured in enriched brain heart infusion (BHI) broth supplemented with yeast extract (5 g/L), hemin (5 mg/L), vitamin K1(1 mg/L), and cysteine (1 g/L) under anaerobic conditions (10% CO_2, 10% H_2, and 80% N_2) at 37°C. The *Pg* solution was collected. Total DNA was extracted from Pg cells with a DNA extraction kit (Bio Teke, Beijing, China).

Construction of the DNA vaccines

The *kgp* gene (Genbank number: NC_010729.1) encoding the hemagglutinin domain and gingipain K catalytic domain, and *rgpA* gene (Genbank number: NC_010729) encoding the hemagglutinin domain and Arg-specific proteinases catalytic domain were amplified by polymerase chain reaction (PCR), and the plasmids pVAX1-kgp and pVAX1-rgpA were constructed as our previous experiment [8].

The IL-4 gene was amplified by PCR from total RNA of human venous blood and reverse transcription with the following primers: forward primer 5' GGGGGG AAGCTT ATG GGT CTC ACC TCC CAA CTG CT 3' and the reverse primer 5' GGGGGG CTCGAG ATT CAG CTC GAA CAC TTT GAA TAT TTC3'. PCR product was digested with Hind Ⅲ and Xho I, and the purified gene fragment was inserted into the pVAX1 expression vector to obtain the plasmid pVAX1-IL-4.

The reconstruction plasmids pVAX1-kgp, pVAX1-rgpA and pVAX1-IL-4 were identified by enzyme digest and DNA sequencing (Boshang Biological Technology Company, Shanghai, China). The plasmids for vaccination were isolated and purified with a QIAGEN Plasmid Maxi kit (Qiagen Internation Company,USA). For immunization, the DNA vaccines were prepared by adding bupivacaine hydrochloride (0.25%) to the aqueous DNA solutions. The final DNA and bupivacaine concentrations were 1 μg/μl and 0.125%, respectively.

Animal model and treatment

After the second and the third bilateral mandible premolars of beagle dogs were extracted under general anesthesia with 3% phentobarbital sodium (JiangSu HengRui Pharmacy Factory, Lianyungang, China) with a dosage of 1 mg/kg, [21] four titanium dental implants (3.5 mm in diameter & 10 mm in length) were placed immediately.

Three months later, group A was immunized with pVAX1-kgp in three ways, 200 ug intramuscularly in the foreleg, 200 ug through the salivary gland, and 100 ug intra-nasally. Groups B, C, D and E were immunized with pVAX1-rgpA, pVAX1-kgp+pVAX1-IL-4, pVAX1-rgpA+pVAX1-IL-4 or pVAX1 following the same method as group A. The immunizations were carried out at 3.0, 3.5 and 4.0 months after implantation [22].

Three and a half months after implantation, cotton ligatures infiltrated with *Pg* ATCC 33277 were placed around the necks of all implants and the ligatures were replaced once after two weeks. Both before the first vaccination and before euthanasia, blood samples were obtained from the femoral vein, placed for two hours at room temperature, and centrifuged for 20 minutes at 3000 rpm then serum was collected and stored at -20.

Five months after implantation, all animals were euthanized by an overdose injection of pentobarbital sodium.

Histological observation and analysis

After the animals were euthanized implants and surrounding tissues were retrieved and immediately fixed in 10% buffered formalin at 4°C for 7 days. The specimens were dehydrated in graded alcohols from 70% to 100%, infiltrated, and embedded in light-cured resin. The blocks were cut to 100 μm-thick along with the long axis of the implants (EXAKT International Company, Germany). Slices were dyed with alizarin red and observed under a Leica DM-RBEs microscope that was equipped with an image system (Leica, Wetzlar, Germany).

Enzyme-linked Immunosorbent Assay (ELISA)

Anti-*Pg* Immunoglobulin G (IgG) and Immunoglobulin A (IgA) in the serum of all animals were detected by ELISA. A 20 ug sonic extract of *Pg* ATCC 33277 was coated onto a flat bottomed 96-well plate, and the wells were blocked at 4°C overnight and were then blocked with 3% bovine serum

album (BSA) in phosphate-buffered saline (PBS) at 37°C for 60 min. Duplicate serial two-fold dilutions of sera within an appropriate range were applied to the well, which was sand incubated at 37°C for 60 min. Following that, peroxidase-labeled goat antibody against dogs IgG and IgA (1:1000, Sigma Inc.) were applied. After reaction, the absorbance at 450 nm was recorded with a microplate reader (Leica, Wetzlar, Germany) in 30 minutes.

Statistical analysis

All data were presented as the mean± standard deviation (SD). The mean value of each group was compared to each other by analysis of variance (ANOVA). Differences between the groups were compared with Tukey's test. Antibodies change analysis before and after immunization in each group was carried out using Student's t test. Differences were considered statistically significant when the value of $P<0.05$. All statistical analyses were performed using the SPSS 16.0 statistical software package.

Results

Construction of DNA vaccines

Recombinant plasmid pVAX1-kgp, pVAX1-rgpA and pVAX1-IL-4 were successfully constructed. The DNA fragments were confirmed by PCR and DNA sequencing, and proven to be 100% identical to the original coding sequence.

Clinical observations

Three months after implantation, the peri-implant mucosa surrounding every implant neck exhibited no signs of inflammation. There was no evidence, such as hyperplasic tissues, suppuration, or color changes for inflammation through the clinical examination. Bleeding on probing (BOP) was negative. During the immunized stage, no obvious changes around the soft and hard tissues were found in these groups.

However, two weeks after ligation, soft tissue inflammation was distinctly observed, such as hyperplasic tissues, suppuration, color changes and bleeding on probing around implants. With the passage of time, the inflammatory reactions were more severe than before *(Figure 1)*. Groups A and B showed bone loss around the implants, and it seemed less than in group E, but more than in groups C and D. Obvious pit radiolucent areas were detected around the implants in groups A and B. Some screw structures exposed in group E. Among these, group C showed the mildest symptoms with the least amount of bone loss and inflammatory cell infiltration. In contrary, some implant surface structures were exposed in the oral cavity in groups E.

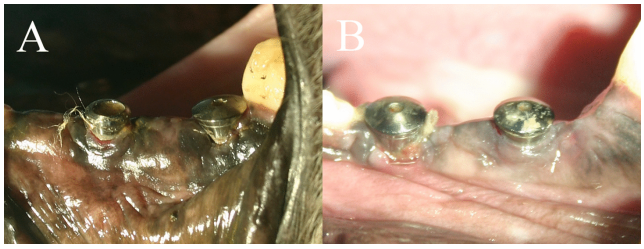

Figure 1. The pictures of the implants and peri-implantitis tissues. The pictures of A and B before euthanasia shows soft tissue inflammation including hyperplasic tissues and suppuration. There is obvious bone loss exposing the implant in the oral cavity.

Histological analysis

In this study, the bone level at the beginning of ligation was not definite in the histologic image so that we only observed qualitative assessment about peri-implantitis and bone loss instead of measuring the quantitative values of the histologic images.

Bone loss was found around the implant in all specimens *(Figure 2A-E)*. Groups A and B showed experimental peri-implantitis for example, many inflammatory cells existed around implants, and there were obvious losses in alveolar bone, leading to the exposure of some implant structures. Group E demonstrated the most severe vertical and horizontal bone loss as well as the highest number of inflammatory cells compared to the other groups. Groups C and D showed the slightest histological symptoms that the infiltration of some inflammatory cells at the neck of the implant and slight bone loss. What's more, the inflammatory areas of group D were limited around the neck of the implant. While group C showed the mildest symptoms with the least bone loss and inflammatory cells infiltration. Group C was better than group D.

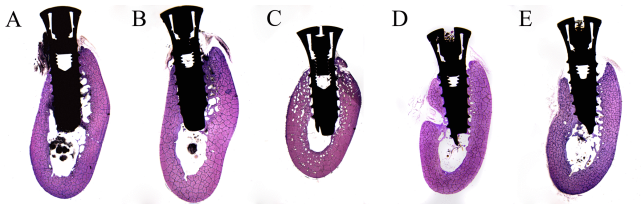

Figure 2. The histological images of the implant and peri-implantitis tissues. A: The picture of group A shows inflammatory areas reaching around the first thread. The extent of bone loss was enhanced, and inflammatory cells gathered but were not serious. Bone loss is greater than in groups C and D. B: The picture of group B shows obvious experimental peri-implantitis and inflammatory areas that invaded to the following thread. C: The picture of group C shows the slightest inflammatory symptoms and the least bone loss. D: The picture of group D shows inflammatory areas limited around the neck of the implant, with little bone loss and inflammatory cells infiltration. E: The picture of group E shows that the peri-implantitis reaction is the most severe and that inflammation is involved in a large area along the implant surface. Many inflammatory cells infiltrated, and bone loss nearly extended to the middle of the implant.

Enzyme-linked Immunosorbent Assay analysis

The IgG and IgA antibody response of five groups before and after immunization are shown in *Figures 3 and 4*. Before immunization, there were no significant differences of the IgG or IgA response in these five groups (P>0.05). After immunization, the IgG or IgA response of group E was the lowest, while groups C and D was significantly higher than that of groups A and B, as well as higher than before immunization (P<0.05). Furthermore, group C was higher than group D, and significant difference was found between these two groups (P<0.05). Whereas, groups A and B were no difference (P>0.05).

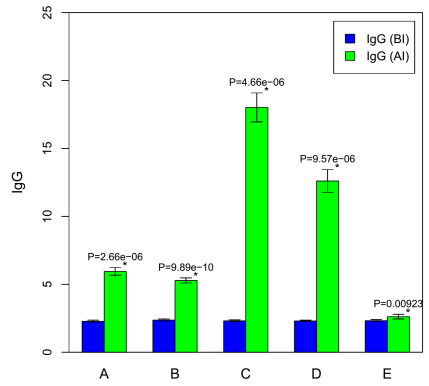

*Figure 3. Titers of the IgG antibody of in groups A, B, C, D and E before and after immunization (O.D=450 nm, Log10 antibody titer). BI: before immunization; AI: after immunization. Analysis of variance (ANOVA). Post hoc test. *P<0.05 indicated statistically a significant difference.*

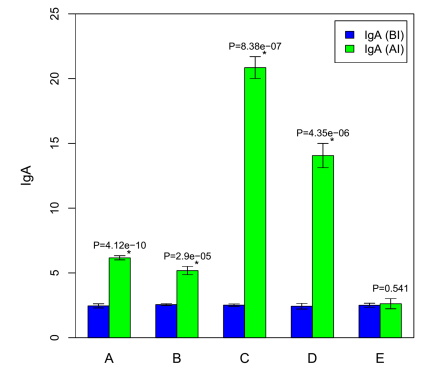

*Figure 4. Titers of the IgA antibody of groups A, B, C, D and E before and after immunization (O.D=450 nm, Log10 antibody titer). BI: before immunization; AI: after immunization. Analysis of variance (ANOVA). Post hoc test. *P<0.05 indicated statistically a significant difference.*

Discussion

Peri-implantitis was the key factor causing implant failure, which was associated with *Pg* infection from experimental and clinical reports. In this study, we constructed a peri-implantitis animal model that was immunized with pVAX1-kgp, pVAX1-rgpA, pVAX1-kgp+pVAX1-IL-4, pVAX1-rgpA +pVAX1-IL-4 and pVAX1. We have demonstrated that the pVAX1-kgp and pVAX1-rgpA DNA vaccines were effective at inducing the immune response and retarding the bone loss around the implant in the previous experiments.8 Furthermore, this experiment demonstrated that pVAX1-IL-4 as an adjuvant could enhance the immunogenicity of *kgp* and *rgpA* DNA vaccines. In this study, the qualitative assessment of bone loss was observed via histologic analysis, while quantitative assessment was not recorded.

Gingipains are essential in adhesion which is an important characteristic of *Pg* in colonizing the epithelial cells. Kuramitsu et al. [23] first demonstrated gingipains played a role in *Pg* adherence to cultured oral epithelial cells by constructing mutants lacking *RgpB* or *RgpA*. There are some differences in adhesion between *RgpA*, *RgpB*, and *Kgp*. The *RgpA* mutant displayed consistently lower binding to epithelial cells than the *RgpB* mutant. The *Kgp* mutant exhibited significantly higher levels of attachment than the *RgpA*. Those three mutant displayed lower levels of attachment than wild type. Pike et al. [24] reported that *RgpA* and *Kgp* bound to the matrix proteins fibrinogen, fibronectin and laminin. Lantz et al. [25] also shown that *RgpA* and *Kgp* bound and degraded fibrinogen and fibronectin. Baba et al. [26] reported that the degradation of the fibronectin receptor on human gingival fibroblasts by *RgpA* results in apoptosis. Moreover, many studies have showed that *kgp* is a more important virulence factor than either of gingipain R [27].

DNA vaccine, first discovered in 1990s, are plasmids constructed to express an encoded proteins which could induce both cellular and antibody immunity in vivo [28]. DNA vaccines have been shown therapeutic benefit for a wide range of disease, such as infectious diseases, cancer and autoimmune diseases [29]. DNA vaccination against HIV-1 infection was led to the first human Clinical in 1998 [30]. Since then, other DNA vaccines for infectious diseases such as HIV-1, Ebola virus and West Nile virus (WNV) have been applied to candidates [31-33]. DNA vaccines have been also applied to treat allergies [34] and shown efficacy in preclinical models of allergy. Miyachi et al. [35] found rpgA DNA vaccine, when used to *Pg* infection in mice via the nasal cavity, induced salivary IgA and serum IgG against *Pg* and prevented alveolar bone loss. We have demonstrated that the pVAX1-kgp and pVAX1-rgpA DNA vaccines were effective at inducing the immune response and retarding the bone loss around the implant in the previous experiments [7]. In this study, we constructed recombinant eukaryotic expressing plasmid pVAX1-kgp, pVAX1-rgpA and pVAX1-IL-4, and observed the prevention effect of peri-implantitis.

The additional plasmids or additional inserts in the same plasmid, encoding molecular adjuvants, have been shown to improve DNA vaccine immunogenicity. IL-12, GM-CSF and IL-15 were demonstrated augment immune responses when they were co-deliveried with DNA vaccine [36-39]. The use

of cytokines as vaccine adjuvant has been previously reviewed [40,41]. IL-4, secreted by CD4+T, is Characteristics cytokines of Th2 cells and plays important roles in the differentiation of T cells. Human Th1 cells are essential for cellular immune responses, and Th2 cells are important for immunoglobulin E synthesize by B cells and immunoregulation [42]. IL-4 has the effect of regulation the balance between Th1 cells and Th2 cells and then regulation the balance between cellular immune responses and Humoral immune responses [43]. In this study, we constructed recombinant eukaryotic expressing plasmid pVAX1-IL-4 as adjuvant to assist the *kgp* and *rgpA* DNA vaccines to prevent peri-implantitis. The results showed that the groups A, B, C and D could significantly improve organism immunity ability, especially groups C and D. Among these, the antigen-specific responses of group C was the most obvious. While group E was ineffective. K.F. Griffin also opinioned that IL-4 as adjuvants improved immunity ability of vaccine [44]. In this study, the addition of IL-4 as the immune adjuvant increased the immunogenicity of the experimental animal model effectively.However, there were not any histological measurements for the soft tissue around implants in our study. This is the defect of this experiment. Developing an effective immune adjuvant is an important concern in vaccine research. Although several works have been performed to explore and immune response characteristics of IL-4, many problems must be overcome before the vaccine can be applied to the clinical.

Conclusion

Kgp and *rgpA* DNA vaccines could significantly slow down the bone loss in experimental peri-implantitis dogs model, and the *IL-4* as the adjuvant could enhance the specific immune response.

Summary

IL-4 can enhance the effect of *kgp* and *rgpA* DNA vaccines inhibiting peri-implantitis bone loss.

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