Szent István University

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Development of porcine circovirus vaccines

Brief Summary of PhD Theses

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INTRODUCTION

The most effective means of controlling infectious disease is by implementing biosafety/epidemiological regulations and vaccination. Due to increase in multi-drug resistant bacteria, as well as the re-emerging or emerging of viral diseases, a wide range of vaccines for medical and veterinary use are available, and these are constantly improved through new formulations to allow, for example the differentiation of vaccinated and infected animals. Vaccines can be produced conventionally, or in case of new generation vaccines, by taking advantage of recombinant DNA technology, with this technology mainly being used to generate subunit vaccines. Subunit vaccines, containing only an immunodominant part of a pathogen are becoming more and more prevalent. Instead of the full antigen repertoire of a microbe, this type of vaccines comprises only of the very parts essential to elicit protection and to induce specific neutralising antibody or to activate production of a cytotoxic T cell response. Generation of subunit vaccines is mostly based on heterologous gene expression. Multiple expression systems are available, each having their own benefits and disadvantages. These have to be taken into account when deciding on using a platform.

Plants are suitable hosts for gene expression, having several advantages; such as post-translational modifications similar to those of mammalian cells, inexpensive production, stability and long storability without the need of a cold-chain and,

most importantly, the possibility of gene expression in edible parts. By targeting protein production towards edible plant parts (fruits, seeds, tubers), the expression host can be utilized as a vaccine delivery method, resulting in oral vaccination and induction of mucosal immune responses.

Although parenterally administered vaccines elicit systemic immune responses, they normally only induce limited protection on mucosal surfaces, which in case of most pathogens are the primary sites of entrance and infection. In contrast, antigens applied on a mucosal membrane (orally, intra-nasally, on the conjunctivae) prime local mucosal as well as systemic immune responses. In the development of subunit mucosal vaccines, the objective is that the antigens should reach sites of specific immunological priming in adequate quantities. This can be achieved by increasing the amount of the administered antigen significantly, or by the protective encapsulation of the vaccine subunit. Physical protection of antigens is extremely important in case of oral vaccines, which are exposed to effects of digestion. Proteins produced in plant cells are naturally well-protected against the physico-chemical milieu in the gastrointestinal system.

Protein production in plants is a rapidly developing field and several methods are already available. Genetic modification can be achieved by creating transgenic plants using direct methods, e.g. biolistic transformation and electroporation; or using indirect, *Agrobacterium tumefaciens*-mediated transgene introduction. In

this case, every single cell of the transgenic plant contains the transgene (stable transformation). Plant virus vectors can be used to achieve transient gene expression. In this case, the protein to be produced is encoded by a virus, and the transgene is only present as long as the virus is replicating in the host.

Porcine circovirus type 2 (PCV2) is a ubiquitious and economically very damaging pathogen of swine herds. Commercially available inactivated whole virus and subunit vaccines are effectively decreasing these damages in subclinical infections and also in clinical disease. These vaccines, only used parenterally, are not able to prevent PCV2 spreading and shedding, nor do they induce sterile immunity.

The objective of the present studies was to create orally administrable PCV2 antigens in plants by using two different approaches: firstly transient expression in Tobacco (*Nicotiana* spp.) by cucumber mosaic virus (CMV) vector; followed by stable transformation of the unicellular, photosynthesizing microalga *Thalassiosira pseudonana*.

MATERIALS AND METHODS

Development of a recombinant CMV expressing a PCV2 epitope and evaluation of its immunogenicity

We assessed gastrointestinal stability of CMV particles *in vivo* in mice. CMV was fed to mice in different doses and serum

or intestinal fluid samples were collected and analysed for the presence of CMV specific IgG or IgA antibodies using enzyme-linked immunosorbent assay (ELISA). As positive control, animals in a different group were immunised with CMV virions intra-peritoneally, whereas negative control mice were left untreated.

For the generation of a recombinant CMV expressing a PCV2 derived epitope on its surface, we first determined the immune dominant regions of the PCV2 capsid visually by creating the 3D virion model and also based on published data. We chose two epitopes (no. 3 and no. 5) for insertion into the CMV capsid gene, which was achieved by PCR-based mutagenesis. We then inoculated tobacco plants with the infectious RNA clones of modified CMV genomes. Recombinant CMV (RCMV) containing epitope no. 5 from PCV2 was able to replicate in infected plants, and this construct was used in our further studies. To assess correct representation of the PCV2 epitope on the CMV surface, we used Western blot and ELISA.

PCV2 specific immunogenicity of RCMV particles was first examined *in vivo* in mice. We immunised the animals using CMV (n = 5), RCMV (n = 5) or PBS (n = 5) intra-peritoneally twice in 2 weeks intervals. Indirect immune fluorescence (iIF) was performed for PCV2 specific IgG detection in serum samples.

We also assessed PCV2 specific immunogenicity of RCMV in pigs. We used conventional piglets weaned at 2 weeks of age, from a PCV2-free farm. One group (n = 5) was intramuscularly

injected with 2 mg RCMV, one group (n = 5) was vaccinated using commercial inactivated vaccine. Negative control animals (n = 3) were mock immunised with PBS. Incomplete Freund`s adjuvant was used in RCMV and PBS formulations. Treatments were repeated after 10 days, followed by challenge infection with live PCV2 strain after another 10 days. Piglets were euthanized after 60 days, tissue samples collected, viral DNA extracted and presence of PCV2 DNA assessed by real-time PCR. Furthermore, faecal samples were collected for PCV2 DNA detection and serum samples for anti-PCV2 IgG titration by iIF weekly throughout the experiment.

We assessed immunogenic properties of RCMV after oral administration. Piglets, weaned at 4 weeks of age (n = 5) were fed 50 mg RCMV four times. Another group (n = 5) was left untreated. We collected serum samples weekly from all animals and tested them for the presence of PCV2 specific antibodies using iIF.

Generation of recombinant *Thalassiosira pseudonana* microalgae expressing PCV2 capsid protein

We attempted the production of the whole PCV2 capsid in the diatom *T. pseudonana*. We used an alignment of PCV2 ORF2 sequences from the PCV2b 1A cluster prevalent in Europe to generate a consensus ORF2 sequence. This sequence was optimised according to codon preferences of *T. pseudonana*, and

the gene was synthesized. We used the Gateway method to clone the gene into an algae specific expression plasmid.

The plasmid vector contained the *fcp* regulatory regions, enabling strong gene expression in diatoms, and also an enhanced green fluorescent protein (eGFP) gene in fusion with the PCV2 ORF2. We used a separate vector to introduce antibiotic (nourseothricin, NAT) resistance. A diatom expression vector containing only eGFP gene was used as positive transformation control.

We coated tungsten (W) microcarrier particles with the expression plasmid together with antibiotic-resistance vector. Positive control particles were coated with eGFP and NAT-resistance coding plasmids. Negative control carriers were coated with water. We prepared microalga cells on artificial seawater- (ASW-) agar plates and used microprojectile bombardment (biolistic method) to introduce the particles. After overnight recovery, the diatom cells were transferred into selective agar plates and liquid media.

We used PCR for transgene detection and microscopy, together with Imagestream imaging flow cytometry for fluorescence analyses in growing cells.

RESULTS

Development of a recombinant CMV expressing a PCV2 epitope and evaluation of its immunogenic properties

Wild type cucumber mosaic virus virions induced CMV specific antibody production in mice immunized orally and in the positive control group. In animals receiving higher doses of the virus, development of measurable antibody response was faster than when lower doses were used. In case of IgA response, but not case of IgG, higher antigen doses also correlated with higher antibody levels by the end of the experiment.

We have created a 3D model of the PCV2 virion and identified 5 epitopes on its surface. Two of these (no. 3 and 5) were inserted into the CMV capsid gene. Only the recombinant CMV (RCMV) containing PCV2 epitope no. 5 could be propagated in tobacco.

We could not confirm the expression of the PCV2 derived epitope on the RCMV extracted from leaf tissue using Western blot. We confirmed correct presentation of the epitope on the recombinant virions using ELISA, however, the hyperimmune serum used for detection had to be pre-absorbed first by incubation on CMV antigen alone.

After immunising mice with the recombinant and wild type CMV or PBS, PCV2 specific antibodies were only detectable in

sera of animals injected with the RCMV. In these samples, final antibody titres were between 1:80 and 1:320.

In the immunisation and challenge experiment using piglets, animals were vaccinated twice with RCMV, inactivated vaccine or PBS, followed by challenge with live PCV2. None of the animals were seropositive at the start of the trial. In the positive control group, anti PCV2 IgGs were already detectable at the time of the second vaccination. In the group immunised with RCMV, seroconversion was delayed but already evident at the time of infection. In samples from negative control piglets, PCV2 specific antibodies were only detected as a result of infection. Real-time PCRs were negative without exception in tissue samples from positive control piglets. In animals immunised using the RCMV antigen, PCV2 DNA was detected in three samples of two piglets. All mock immunised animals were positive. We didn't find any PCV2 positive faecal samples in this experiment.

In the oral immunisation trial, all piglets were seronegative for PCV2 on the first day and negative control animals remained negative throughout the experiment. Two of the piglets fed with RCMV produced PCV2 specific serum IgG by the second week and all piglets in the immunised group responded by the third week.

Expression of PCV2 capsid gene in recombinant *Thalassiosira pseudonana*

We determined the nucleotide sequence of the ORF2 coding the PCV2 capsid protein by creating a consensus of the predominant European strains in group PCV2b 1A and codon optimisation. Codes of 160 amino acids out of the total 233 found in the capsid were modified as a result. The synthesized gene was cloned into algal expression vector and its sequence was confirmed.

We coated W particles with the expression vector and antibiotic resistance plasmid and transformed *T. pseudonana* cells. GFP expressing vector and water were used as controls. After transformation, the cells were spread on selective agar and inoculated into selective liquid ASW.

All negative control cells died within a week. Positive control cultures started growing and had to be subcultured weekly. Alga cells transformed with the PCV2 capsid gene started slow growth after delayed recovery and were subcultured after two weeks. We only observed colony formation on the positive control agar plates.

We screened for GFP fluorescence using microscopy and imaging flow cytometry. Only the positive control cells showed positivity. These were also the only cultures positive for plasmid DNA in PCR.

DISCUSSION

Immunogenic properties of recombinant CMV

CMV survived in the gastrointestinal tract *in vivo* and proved to be a suitable carrier for oral immunisation processes, confirming *in vitro* results available in literature. We determined the 3D structural model of the PCV2 virions and identified 5 potential antigenic epitopes on its surface, which were later confirmed experimentally by other groups. We have successfully expressed one of these epitopes (No. 5) in CMV and the resulting RCMV was infectious and was able to replicate in tobacco plants. The RCMV elicited PCV2 specific antibody response in mice. When administered parenterally to piglets, RCMV induced the production of PCV2 antibodies and the resulting serum IgG titres were comparable to those achieved by immunisation with a commercial inactivated PCV2 vaccine, even though RCMV only contained 10 out of the 233 amino acids found in the complete PCV2 capsid. Although there was an amino acid difference between the epitope expressed in RCMV and the one found in the PCV2 strain used for challenge infection, immunisations with RCMV were able to decrease PCV2 replication and conferred partial protection. The antigen candidate was unable to fully prevent PCV2 replication. PCV2 specific serum IgG production was also achieved after oral administration of RCMV.

Production of PCV2 capsid protein in recombinant *Thalassiosira pseudonana* microalga

We confirmed the nucleotide sequence of the diatom plasmid vector expressing the PCV2 capsid in fusion with eGFP and coated W particles with the DNA. We used these and control microcarriers to transform *T. pseudonana* cells. Following transformation, growth of cultures kept in selective media was delayed compared to positive control. We only observed green fluorescence in positive control cells; and the expression vector DNA could only be detected by PCR in these cultures. Among cells that were transformed with the PCV2 ORF2 plasmid, only the ones containing the NAT resistance gene survived. During the coating of microcarriers, the vast majority of particles bind both kinds of plasmids; therefore the likelihood of creating cells that are transformed with only one is very low. However, aside from the positive control, these were the only cells that grew in our experiment. These results suggest that the produced PCV capsid-eGFP fusion protein was toxic to cells containing both expression plasmids The N-terminal 41 amino acids (nuclear localisation signal) is known to be toxic to prokaryotic cells, resulting in low protein expression, but not in the destruction of the cells. Toxicity to algal cells could be confirmed by attempting PCV2 capsid protein expression without the first 41 amino acids.

Development of vaccine antigen production methods using plants as expression hosts is still in its early stage and our work joined this decades-long process at its beginning. After determining and successfully producing an epitope, followed by assessing its immunogenic properties *in vitro* and *in vivo*, some aspects remain to be clarified. The range of promising antigen candidates is narrowed by further experiments, such as statistically significant field trials resembling farm conditions and using larger sample number, long-term application, analyses of safety and economic feasibility. Furthermore, antigen expression models used in this study might be more suitable to produce vaccines against different pathogens.

In case of PCV2, it is not evident that a plant based oral vaccine would be superior to commercially available products. However, we confirmed that the antigen expression methods used in our work are relevant platforms in veterinary vaccine production.

NEW SCIENTIFIC RESULTS

1. We confirmed *in vivo* that the vector CMV is suitable for oral immunisation and induces local IgA secretion in the gut.

2. We generated a structural model of PCV2, identified potential epitopes and determined their locations on the virion surface.

3. We were the firsts to insert a PCV2 capsid epitope into CMV coat protein and we established that the C-terminal 10 amino acid long epitope of the PCV2 capsid was represented with the same antigenic properties as on PCV2.

4. Immunising mice, we have shown that the CMV vector expressing the PCV2 epitope is suitable to induce PCV2 specific antibody response.

5. In pigs, the recombinant nanoparticles were able to elicit PCV2 specific immune response after parenteral and oral administration as well.

6. We demonstrated that although parenteral vaccination of piglets with the recombinant virus did not prevent PCV2 infection, it did decrease virus replication

7. We utilised microalga expression system for the production of PCV2 capsid protein and found that similarly to bacterial expression, this protein toxic to microalgae, possibly as a result of the nuclear localisation signal.

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