Production of recombinant anthrax toxin receptor (ATR/CMG2) fused with human Fc in planta

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ABSTRACT

Mass vaccination against anthrax with existing vaccines is costly and unsafe due to potential side effects. For post-infection treatment, passive immunotherapy measures are currently available, most based on anthrax protective antigen (PA)-specific therapeutic antibodies. Efficient against wild-type strains, these treatment(s) might fail to protect against infections caused by genetically engineered Bacillus anthracis strains. A recent discovery revealed that the von Willebrand factor A (VWA) domain of human capillary morphogenesis protein 2 (CMG2) is an exceptionally effective anthrax toxin receptor (ATR) proficient in helping to resolve this issue.

Here we describe in planta production of chimeric recombinant protein (immunoadhesin) comprised of functional ATR domain fused with the human immunoglobulin Fc fragment (pATR-Fc). The fusion design allowed us to obtain pATR-Fc in plant green tissues in a soluble form making it fairly easy to purify by Protein-A chromatography. Standardized pATR-Fc preparations (purity > 90%) were shown to efficiently bind anthrax PA as demonstrated by ELISA and Western blot analysis. Recombinant pATR-Fc was also shown to protect J774A1 macrophage cells against the anthrax toxin. This study confirmed that plant-derived pATR-Fc antibody-like protein is a prospective candidate for anthrax immunotherapy.

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Introduction

The occurrence of dangerous side effects associated with the current anthrax vaccine is halting mass vaccination efforts [1]. The development of preventive and/or post-exposure treatments has become urgent in view of the threat of bio-terrorism [2].

The search for efficient immunotherapy has confirmed that many molecules, peptides, and other compounds are able to block Bacillus anthracis toxins [3–6]. The PA-specific 2 antibodies that would be excellent for anthrax prophylaxis [7,8] might not be effective when used against genetically altered strains [9].

The recently discovered VWA domain of either TEM8 or the CMG2 receptors was shown to bind PA very efficiently [10] and was therefore capable of protecting cells against toxicity in vitro [11,12]. The soluble receptor decoy consisting of CMG2 domain (sCMG2) was able to protect animals against a lethal toxin challenge in vivo [13,14].

The main advantages of plants for use as bioreactors are their low-cost and a greater potential for scalability compared to microbial or animal systems [15,16]. An additional advantage, from the public health point of view, is greater safety, since plants do not contain mammalian pathogens.

Here we report the plant-based production of a potent ATR-based agent, the immunoadhesin, comprised of the VWA domain of CMG2 fused with human antibody Fc part fragment (pATR-Fc). The fusion of heterologous peptides with Fc is known to provide better yields, stability and facilitate purification process in different systems, including plants [17–21]. The retention of this recombinant protein in the endoplasmic reticulum (ER) has allowed for its abundant expression and accumulation in plants without any adverse effects and being mostly in soluble form makes it feasible for simple protein-A-based purification. In vitro characterization of pATR-Fc preparations (purity > 90%) confirmed that it binds to commercial PA and is able to protect J774A1 macrophage cells against the anthrax toxin. At this point, the study suggests pATR-Fc as a functional recombinant plant-based therapeutic agent suitable for post-infection therapy of anthrax.

Materials and methods

Genetic engineering of recombinant plant codon-optimized ATR-Fc fusion

The 171 amino acid part of CMG2 protein (VWA domain, aa 44–214, Accession Number pdb|1TZN|AA) was selected for stable plant-based expression as part of a chimeric Fc fusion recombinant
The resulting product was diluted 1:1000 and amplified using the regular PCR reaction with the following primers: F: 5'-CCC ATG GAT CTT TAT TTT GTT CTT-3' and R: 5'-CGC AAG AAT AGA ATT AAT-3'. The fragment was gel extracted and sub-cloned into pL2 Impact Vector [Plant Research International, Wageningen, The Netherlands] using NcoI–NotI sites. After sequence verification, the fragment was placed under the Rubisco promoter as a fusion with human Fc using a flexible hinge [21] and carried an apoplastic (Apo) signal, with or without ER C-terminal signal (KDEL); both expression cassettes contained 6× His and c-myc detection/affinity-purification tags (Fig. 1). For plant transformation, the expression cassettes were further sub-cloned (Pacl–AscI) into the binary pBIN-PLUS vector (pATR-FcApo and pATR-FcER) and put in Agrobacterium tumefaciens LBA4404 strain suitable for stable tobacco plant transformation targeting to apoplastic region or ER, respectively.

**Plant transformation**

A stable agrobacterium-mediated transformation procedure was performed according to standard protocol [22]. Transgenic tobacco lines (cv Wisconsin 38) were selected using kanamycin at a final concentration of 100 mg/l. Rooted shoots were then transferred to soil and grown to maturity in greenhouse conditions.

**Isolation and purification of recombinant proteins**

Total and soluble plant proteins were isolated as described previously with minor modifications [23]. Frozen tobacco plant leaves were homogenized in PBS buffer containing 0.5% Tween-20 and clarified by centrifugation at 15,000g for 30 min. The supernatant was filtered through a miracloth [Calbiochem, La Jolla, CA] applied as a positive control (+). WT indicate cytokinesis from wild-type tobacco (WT) and the human IgG were used as corresponding negative and positive controls. Molecular weight measured in kilodaltons (kDa).

**Affinity pull down assay**

Commercial preparation (0.5 μg) of anthrax PA (Psa3) protein #171A from List Laboratories [Campbell, CA] was mixed with 10 μg pATR-Fc and brought to 100 μl with Tris buffered saline (TBS) plus 0.05% Tween-20 (TBST), 3% BSA, and 1 mM MgCl2 for overnight incubation at 4 °C. Commercial anti-PA goat antiserum [List Laboratories] (10 μg) was used as positive control. Bound protein complexes were rescued from the solution using MagnaBind Protein-A Beads [Pierce, Rockford, IL] following the manufacturer’s protocol with some minor modifications. Elu-

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**Fig. 1.** Production of recombinant ATR-Fc protein in plants. (A) Schematic diagram of pATR-Fc expression cassette arrangements in the binary vector pBIN-Plus for Agrobacterium-mediated plant transformation, resulting in pATR-FcApo and pATR-FcER constructs. Synthetic cDNA fragment encoding CMG2 protein domain (ATR) fused with human IgG-Fc via the flexible linker peptide (L) placed between the ribulose promoter (P) and terminator (T), with addition of N-terminal apoplastic signal (Apo) with or without ER C-terminal retention peptide (KDEL) and both having C-terminal c-myc and His tags. Both cassettes are placed in the transfer DNA region between the left (LB) and right (RB) borders that contain NPTII gene expressing cassette for kanamycin selection. (B) The presence of pATR-Fc protein in extracts of transgenic tobacco plants of cv Wisconsin 38 (left panel) was confirmed by Western blot analysis (reducing conditions) using anti-c-myc monoclonal antibodies (right panel). Samples are shown for the constructs carrying ER retention signal (pATR-FcER) or apoplastic signal (pATR-FcApo). E. coli expressed foreign peptide with c-myc tag used as a positive control (+). WT indicate cytokinesis from wild-type tobacco (WT) and the human IgG were used as corresponding negative and positive controls. Molecular weight measured in kilodaltons (kDa). (C) The purified preparation of pATR-Fc was tested on SDS-PAGE at reducing conditions and either visualized by Coomassie blue staining (left), or tested with human Fc-specific antibodies on corresponding (diluted 1:10) Western blot (right). The band of expected molecular weight (47.7 kDa) of pATR-Fc is indicated by arrowhead. The extracts from wild-type tobacco (WT) and the human IgG were used as corresponding negative and positive controls. Molecular weight measured in kilodaltons (kDa).
tion by ImmunoPure Gentle Ag/Ab Elution Buffer [Pierce] was done in a small volume of 40 μl and analyzed on Western blot using anti-PA mAbs [Biodiesel, Saco, ME].

**Sandwich ELISA analysis of binding pATR-Fc to PA$_{83}$**

Binding of pATR-Fc with PA was detected using sandwich ELISA as described by [11] with some minor modifications. The 96-well plates [Costar, Corning, NY] were coated with PA$_{83}$ [List Laboratories] diluted in TBS (0.3 μg/50 μl per well) overnight at 4°C. After 1 h at room temperature, plates were blocked with 3% BSA in TBS containing 0.025% Tween-20. Dilutions of pATR-Fc started at 600 ng/ml (50 μl per well) with additional 1 mM MgCl$_2$; plates were incubated for 1 h and washed twice for 5 min in TBST. Primary anti-c-myc [Invitrogen, Carlsbad, CA] antibody was added at 1:1500 dilutions, incubated for 2 h and then washed 3 times for 5 min with TBST. After 1 h incubation with the secondary antibody AP-anti-mouse A3562 conjugate [Sigma, Saint Louis, MO] was added at 1:2000 dilution and plates were developed with pNPP substrate [Sigma] as recommended and OD$_{405nm}$ was determined.

**In vitro neutralization assay**

Neutralizing-antibody activity was determined in monococyte–macrophage cells J774A.1 [American Type Culture Collection, Manassas, VA] essentially as described [26]. Briefly, preparations of PA and lethal factor [50 μl each] at 0.1 μg/ml were combined with dilutions of pATR-Fc, negative serum and positive control PA$_{83}$-immunized serum. The antiserum–toxin mixtures were added to J774A.1 at 4 × 10$^6$ cells per well. Lactate dehydrogenase activity was measured by a Cytotoxicity Detection kit [Roche, Indianapolis, IN]. The percentage of cell lysis was calculated as the mean of (A$_{490}$ of serum/PA toxin/target cell mix) minus (A$_{490}$ of target cell control) divided by (A$_{490}$ of cells treated with 2% Triton X-100) minus (A$_{490}$ of cells incubated with medium).

**Results**

**ATR-Fc constructs for stable plant transformation and targeted expression**

The expression cassette encoding the plant codon-optimized fragment (GenBank #GQ478119) of the CMG2 protein [VWA/I Domain, aa 44-214] was fused at the C-terminus with the human IgG-Fc fragment and assembled in the plant expression vector producing two constructs (pATR-Fc$_{apo}$ and pATR-Fc$_{ER}$) suitable for efficient stable transformation and apoplast/ER targeted expression in dicot plants (Fig. 1A).

**Transgenic plants expressing pATR-Fc fusion protein**

The presence of the ATR-Fc fragment in 30 independent transgenic lines of tobacco Wisconsin 38 was confirmed by PCR (not shown). All plants were grown to maturity and were subjected to Western blot analysis using anti-c-myc specific antibodies (Fig. 1B). Expression of recombinant product was detected mostly in soluble fractions and was more abundant with the pATR-Fc$_{ER}$ construct. The best transgenic lines were used for further protein isolation and then propagated to T1 generation.

**Isolation and purification of pATR-Fc in soluble form**

Recombinant pATR-Fc protein was efficiently extracted from fresh plant tissues using a previously established protocol [21,23,24] yielding up to 3 mg per kg of raw weight. The final prep-

**pATR-Fc binds to PA$_{83}$ in a specific manner**

Two independent experiments were conducted to demonstrate the ability of the plant-derived ATR-Fc fusion protein to bind PA. In the first one, the commercial anthrax PA$_{83}$ protein (0.5 μg) was shown to bind pATR-Fc protein as compared to binding with anti-PA mAbs (positive control) or TBS buffer (negative control). The protein complexes rescued by Protein-A beads were verified by Western blot with anti-PA mAbs (Fig. 2A). It appears that plant-derived ATR-Fc binds to PA83 comparable to binding of PA83 with anti-PA mAbs.

In another experiment, the pATR-Fc preparation was tested for its ability to bind PA in ELISA. The pATR-Fc binding to commercial PA83 was detected and found to depend on the presence of Mg$_{2+}$ ions (Fig. 2B).

**In vitro protection of macrophage cells against anthrax toxin with plant-derived ATR-Fc**

Neutralization of anthrax toxin by pATR-Fc was tested by in vitro macrophage lysis inhibition assay (Fig. 2C). The inhibition of PA–LF anthrax toxicity was tested in J774A.1 cells by different amounts of pATR-Fc (from 0.15 to 2.4 μg/ml). Neutralization of anthrax toxin was shown in the pATR-Fc preparation to be dose dependent with an IC$_{50}$ of 0.6 μg/ml. No neutralization activity was detected in the serum of mice injected with non-transgenic plant proteins. The serum of mice immunized with commercial preparation of PA$_{83}$ at different concentrations efficiently neutralized anthrax toxin. Cell viability was calculated as a percentage of surviving cells to the one lysed completely.

**Discussion**

Anthrax post-infection treatment demands additional prophylaxis resources. The recently discovered receptor molecules for anthrax toxin have a clear advantage over other toxin-specific inhibitors/antibodies because of their higher affinity and universal character. These peptides/receptors could make an important contribution in the fight against engineered decoy-resistant forms of the toxin while preserving their normal cellular–receptor use.

ATR might be active in the protection against mutant forms of anthrax toxins [14]. The soluble form of ATR expressed in vitro was shown to shield against a lethal toxin challenge in cell culture and animals [11,27]. A safe and reliable source of inexpensive functional ATR is considered. A soluble form of ATRs was successfully expressed in bacteria [27,28]. Expression of these molecules in eukaryotic systems might have an advantage, though the relative yields in mammalian cells were significantly lower and require extensive purification [13,29,30]. The idea of utilizing plants as an alternative production of bioreactors for such recombinant proteins fulfills advantages, such as low-cost of production and greater potential for safety and scalability, as compared with microbial or animal systems.

Here we describe successful production in plants of chimeric peptide that is a fusion of human CMG2 and IgG-Fc fragment. The design of immunoadhesin antibody-like protein [17] gave a noticeable advantage of being efficiently expressed in eukaryotic plant cells. It was particularly abundant when targeted/retained in the cellular ER apparatus. Furthermore, the protein accumulated in plant tissues in a soluble form, making it easy for downstream purification as a one-step procedure using the protein-A method.
shown that PA-binding mAbs may require Fc–Fc Receptor interaction for enhanced neutralization of anthrax toxin [18]. To date, several Fc fusion proteins have been shown highly active in virus neutralization [31] or tumor growth inhibition [19,32].

The functionality of plant-derived ATR-Fc was confirmed by ELISA and Western blot analysis, where binding to PA was as efficient and comparable to the high standards of human cell-derived receptor molecules. VWA/I domain of the native CMG2 protein binds PA in a divalent cation-dependent manner [11]. Purified plant-derived ATR-Fc was also shown to protect the monocye–macrophages cells J774A.1 in vitro from being killed by the PA–LT complex, opening the way for in vivo experiments.

In summary, we have established the efficient production of a recombinant plant-derived ATR-Fc fusion protein that is in a soluble and easily purifiable form. After establishing proof that it is functionally active for passive anthrax immunotherapy and appropriately satisfies all safety standards, we are seeking to use it as a prospective candidate for in vivo testing in anthrax post-infection treatment.

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References


