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A plant cell-expressed recombinant anti-TNF fusion protein is biologically active in the gut and alleviates immune-mediated hepatitis and colitis

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ABSTRACT

The orally administered BY-2 plant cell-expressed recombinant anti-TNF fusion protein (PRX-106) (n = 6) consists of the soluble form of the human TNF receptor (TNFR) fused to the Fc component of a human antibody IgG1 domain.
Aim: To evaluate the immune modulatory effect of the oral administration of plant cells expressing PRX-106.
Methods: Mice treated with Concanavalin A (ConA) to induce immune hepatitis was orally treated with cells expressing PRX-106 containing 0.5 or 5 μg PRX 106. In the colitis model, TNBS-colitis was induced in mice followed by the oral administration of plant cells expressing PRX-106. The immune modulatory effect was determined through follow-up to assess the clinical effect, histology, and serum cytokine levels and by FACS analysis for lymphocyte subsets.
Results: The oral administration of BY-2 cells expressing PRX-106 alleviated immune-mediated liver injury. Serum AST and ALT levels decreased and were comparable to those of mice that had received high-dose steroids. The beneficial effect was also observed as a marked decrease in hepatic necrosis. In the colitis model, the oral administration of BY-2 plant cells expressing PRX-106 alleviated weight loss associated with immune-mediated colitis and improved bowel histology. A reduction in I-IkB-alpha phosphorylation in treated mice was also observed. These effects were associated with a significant alteration in the distribution of CD4 + CD25 + FOXP3+ cells.
Conclusions: Plant cells expressing recombinant anti-TNF fusion protein show biological activity when orally administered, exerting an immune modulatory effect through the alleviation of immune-mediated hepatitis and immune-mediated colitis.

1. Introduction

The development of biological agents that target tumor necrosis factor alpha (TNFα) has markedly changed the therapeutic approach to inflammatory diseases (Wiedmann et al., 2009). The parenteral administration of recombinant anti-TNF proteins reduces disease activity and, in some patients, induces remission. However, not all patients favorably respond to anti-TNF antibodies (Bendtzen et al., 2009). Some patients treated with the anti-TNF constructs either do not respond (primary response failure) or show an initial response but have subsequent relapses (secondary response failure), despite increased dosage and/or more frequent administration of the drugs (Bendtzen et al., 2009). Inter- and intra-individual bioavailability and pharmacokinetics differences might contribute to these failures. The immunogenicity of the drugs, leading to the development of anti-drug antibodies (Requena et al., 2010), contributes to treatment failure (Bendtzen et al., 2009).

Etanercept is a recombinant, dimeric, soluble tumor necrosis factor receptor fusion protein that blocks only soluble TNF but not membrane-bound TNF. The parenteral administration of

Abbreviations: ConA, concanavalin A; Tregs, regulatory T cells; TNFα, tumor necrosis factor alpha; TNFR, TNF receptor; ADAs, anti-drug antibodies; RA, rheumatoid arthritis.

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Etanercept has been used to treat rheumatoid arthritis, juvenile rheumatoid arthritis, psoriatic arthritis, psoriasis, and ankylosing spondylitis (Wiedmann et al., 2009; Hoy and Scott, 2007). Monotherapy with subcutaneous etanercept is effective and generally well tolerated in patients with ankylosing spondylitis or psoriatic arthritis, improving health-related quality of life and delaying structural disease progression (Hoy and Scott, 2007).

TNF antagonists, when parenterally administered, are generally well tolerated but carry a risk of side effects. Areas of concern include opportunistic and non-opportunistic infections (bacterial and viral), vaccinations, neurological complications, hepatotoxicity, hematological side effects, malignancies, infusion reactions and autoimmunity. Contraindications, such as heart failure and acute infectious diseases, are also of concern (Stallmach et al., 2010). The use of these agents in the elderly and in young, fertile, or pregnant and breast-feeding women are additional areas of concern (Miehsler et al., 2010). The immunosuppressive capacity of these agents necessitates rigorous long-term safety follow-up, and the potential risks concerning the use of these antagonists should always be considered (Stallmach et al., 2010; Rongioletti et al., 2010).

The oral delivery of therapeutic proteins has been a long-term goal with relatively limited success, answering the unmet need of patients for an oral treatment as a safe, non-invasive method for the delivery of protein-based drugs. Oral delivery might also facilitate daily intake and continuous drug delivery. The requirements for the oral delivery of protein therapeutics include survival in the gastric environment, release from within the cells into the intestine, crossing the intestinal wall and remaining active. In addition, the exertion of an effect at the level of the gut immune system might also contribute to the effects of these proteins.

Orally administered BY-2 plant cell-expressed recombinant anti-TNF fusion protein (PRX-106) consists of the soluble form of the human TNF receptor (TNFR) fused to the Fc component of a human antibody IgG1 domain PRX-106, which has an amino acid sequence identical to Etanercept™.

The aim of the present study was to determine the immune modulatory effect of oral administration of plant cells expressing PRX-106. The data showed that the recombinant anti-TNF fusion protein exerts an immunomodulatory effect, alleviating immune-mediated hepatitis and immune-mediated colitis.

2. Methods

PRX106 is a synthetic DNA sequence rendering the desired amino acid sequence of Etanercept (Drag Bank accession number DB00005). It includes a 27 amino acid leader signal peptide of the Nicitiana Tabaccum calreticulin gene (GenBank accession number ACH72686) and an ER retention signal SEKDEL, synthesized by GENEART AG (Regensburg, Germany). This sequence was optimized according to the codon usage of Nicotiana tabacum genes. Expression of the recombinant proteins was achieved using ICON-TMV vector (Gils et al., 2005). High-yield production of authentic human growth hormone was performed using a plant virus-based expression system (Gils et al., 2005). Expression of PRX106 in line 471 is controlled by induction with Isopropyl β-D-1-thiogalactopyranoside (IPTG).

2.1. In vitro *studies*

2.1.1. *Ability to survive the gastric environment*

To assess the protective nature of the plant cell wall, BY2 cells expressing the TNFRII-Fc fusion protein were lyophilized and incubated in "simulated gastric fluid", followed by shaking at a pH ranging from 1.6 to 5 for 15 and 30 min at 37 °C. At each time point, the reaction was terminated after the addition of Na_2CO_3. The results were compared with commercial Etanercept (20 μg) under the same conditions. To measure the TNFRII-Fc protein content in the cells, the pellet was resuspended in extraction buffer (20 mM phosphate buffer, pH 7.2, 20 mM EDTA, 20 mM L-ascorbic acid, and 1% Triton X-100). The cell lysate was analyzed for protein content through western blotting, using an anti-TNFRII antibody (mouse anti-TNFRII-80 M2; Santa Cruz Biotechnology)

2.2. In vivo *studies*

2.2.1. *Animals*

Male C57BL/6 mice (11–12 weeks old) were obtained from Harlan Laboratories (Jerusalem, Israel) and maintained in the Animal Core at the Hadassah-Hebrew University Medical School. The mice were administered standard laboratory chow, provided water *ad libitum* and maintained under a 12-h light/dark cycle. The animal experiments were performed according to the guidelines of the Hebrew University-Hadassah Institutional Committee for the Care and Use of Laboratory Animals, and approval was also received from this committee.

2.3. *Induction of ConA hepatitis*

ConA (MP Biomedicals, USA) was dissolved in 50 mM Tris, pH 7, containing 150 mM NaCl and 4 mM $CaCl_2$, and injected into the tail vein at a dose of 500 μg/mouse (15 mg/kg).

2.3.1. *Experimental groups*

Six groups of C57B1/6 mice (n = 6) were orally treated once a day for five consecutive days with BY-2 cells or control treatments six hours prior to Concanavalin A (ConA) induction. The treated groups received BY-2 cells expressing PRX-106, equivalent to 0.5 (1x) or 5 μg (10x). Two additional groups of mice were treated with BY-2(−) plant cells in place of the plant cells expressing PRX-106. Mice in the control groups were treated with a high dose of dexamethasone or saline. The mice were sacrificed 15 h after the ConA injection.

2.4. *Assessment of the effect of oral PRX-106 treatment on liver damage*

2.4.1. *Liver enzymes*

Serum was obtained from individual mice. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were determined using an automatic analyzer.

2.4.2. *Cytokine measurement*

Serum IFN-γ and IL-10 levels were measured in each animal using a commercially available "sandwich" ELISA kit (Quantikine, R&D Systems, MN, USA).

2.4.3. *Histological examination of the liver*

Paraffin-embedded liver sections were prepared from each mouse. The livers were cut into 4–5-μm slices and stained with hematoxylin-eosin (H&E). Slides were scored to assess the extent of liver damage using a previously described method (Margalit et al., 2005).

2.5. *Induction of colitis*

2.5.1. *Induction of hapten-mediated (TNBS) colitis*

To induce hapten-mediated colitis, mice were sensitized with 160 μl of the haptenizing agent TNBS (Sigma-Aldrich, Rehovot, Israel) at a concentration of 2.5% in 50% ethanol through skin painting on day −7. On day 0, 120 μl of 2.5% TNBS in 50% ethanol was