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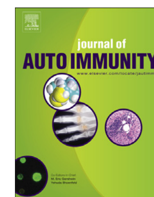

Phosphorylcholine-tuftsin compound prevents development of dextransulfate-sodium-salt induced murine colitis: Implications for the treatment of human inflammatory bowel disease

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ABSTRACT

Improved clinical findings of inflammatory bowel disease (IBD) upon treatment with helminthes and their ova were proven in animal models of IBD and in human clinical studies. The immunomodulatory properties of several helminthes were attributed to the phosphorylcholine (PC) molecule. We assessed the therapeutic potential of tuftsin-PC conjugate (TPC) to attenuate murine colitis. Colitis was induced by Dextransulfate-Sodium-Salt (DSS) in drinking water. TPC was given by daily oral ingestion (50 μg/0.1 ml/mouse or PBS) starting at day −2. Disease activity index (DAI) score was followed daily and histology of the colon was performed by H&E staining. Analysis of the cytokines profile in distal colon lysates was performed by immunoblot. Treatment of DSS induced colitis with TPC prevented the severity of colitis, including a reduction in the DAI score, less shortening of the colon and less inflammatory activity in histology. The immunoblot showed that the colitis preventive activity of TPC was associated with downregulation of colon pro-inflammatory IL-1β, TNFα and IL-17 cytokines expression, and enhancement of anti-inflammatory IL-10 cytokine expression. In the current study, we demonstrated that TPC treatment can prevent significantly experimental colitis induction in naïve mice. We propose the TPC as a novel potential small synthetic molecule to treat colitis.

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1. Introduction

Inflammatory bowel disease (IBD) is comprised of two major phenotypes, Crohn's disease (CD) and ulcerative colitis (UC). The progress in deciphering the molecular mechanisms underlying the pathogenesis of IBD and other autoimmune diseases, has led to the development of new-targeted biological treatments [1]. Nevertheless, existing therapies for IBD such as thiopurines, methotrexate, calcineurin inhibitors and anti-TNF often causes severe side effects [1].

In Western countries, a significant increase in the prevalence of autoimmune and auto-inflammatory syndromes is strongly correlated with improved sanitation and hygiene. Unsurprisingly, a strong correlation was reported between high prevalence of parasitic worms (helminthes) in certain geographic areas and immune protection from atopic, autoimmune, and auto inflammatory diseases [2–5]. Through co-evolution of the helminthes with their hosts, the helminthes were able to modulate inflammatory response in the host in order to promote parasite survival. This may have also generated a predisposition in the host towards the development of autoimmunity in the absence of infection [6].

These observations led to the successful application of helminthes and their ova, which resulted in ameliorated clinical and laboratory findings in a large number of autoimmune diseases, including multiple sclerosis [7,8], torheumatoid arthritis [9,10], type

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I diabetes mellitus (T1DM) [11–13], and IBD [5,14–18]. Since the "inflammatory bowel disease hygiene hypothesis" [19] has risen, many groups have investigated the effect of helminthes on colitis, and different helminthes were shown to suppress colon inflammation by modulating several immune pathways [18,20–24]. Clinical trials using helminthes to treat IBD were conducted by Weinstock et al. [15,16]: ulcerative colitis (UC) and Crohn's disease (CD) patients were treated with ingestion of live *Trichuris suis* ova (TSO), resulting in a significant clinical improvement in nearly 80% of the patients with CD and a more modest effect in patients with UC [15,16,25]. To date, in all clinical trials conducted, adverse events associated with helminthic treatment were uncommon, suggesting relative safety [15,16,26].

Many of the helminthes species employed in experimental animal models were shown to limit the inflammatory activity in a variety of immune-modulated diseases [13,18,27,28]. This implies that the beneficial effect of the helminthes is not specific to any single helminth species. Filarial nematodes secrete immunomodulatory molecules mainly comprising of phosphorylcholine (PC)-moieties glycoporteins, into the host environment [28–33]. ES-62 is the most studied one among them. Its immunosuppressive activity was attributed to the PC moieties, covalently attached to N-type glycans [28,31,32]. Other nematodes like *Ascaris suum* also express the PC-immunomodulatory moiety [34]. PC in nature is presented by apoptotic cells, Gram-positive bacterium including *Streptococcus pneumonia*, *Clostridium*, *Lactococcus*, *Lactobacillus*, and the Gram-negative bacterium *Haemophilus influenzae* attached directly to sugar residues, generally considered to be N-acetylgalactosamine [34–38].

The idea of finding a synthetic product which can mimic the helminthes-immune-modulatory effect have led us to evaluate the protective potential of PC based compound on DSS-induced colitis in mice. Currently, in the field of autoimmunity there is need for a treatment with small molecules which have a minimal side effects [37]. The PC is a non-immunogenic natural small molecule, therefore we conjugated it to tuftsin, coined as TPC, using the tuftsin as a self-natural adjuvant [36,39]. Tuftsin is a physiological tetrapeptide (Thr-Lys-Pro-Arg) fraction of the IgG-heavy-chain molecule produced by enzymatic cleavage of the Fc-domain of the heavy chain of IgG in the spleen [40]. Our data are an experimental proof-of-concept that TPC therapeutic efficacy is associated with prevention of murine colitis development manifested by a reduction in weight loss, intestinal bleeding, colonic length, and histological damage, resulting in both increased and improved survival. This has important implication in suggesting TPC as a synthetic small molecule which mimics helminthes' derivate for treating colitis.

2. Materials and methods

2.1. TPC

Tuftsin extended at its C-terminal i.e THR-LYS-PRO-ARG-*Gly-Tyr*, was synthesized manually following solid phase peptide technology (GLS peptide synthesis, Shanghai, China). The peptide was coupled to diazotized 4-aminophenyphosphoryl chloride to form an azo bond between the PC (Sigma–Aldrich L-4391 St Louis, MO, USA) and the tyrosine moiety of the spacer employing procedure as previously described [41]. The conjugate was characterized by mass spectra and amino acid analysis as well as by HPLC.

2.2. Mice

C57BL/6 male mice weighting 25–30 g were purchased from Harlan, Israel. The mice were maintained in a conventional animal housing facility at Sheba Medical Center Israel. The mice were kept in individually ventilated cages, under specific pathogen-free (SPF) environment. All experiments were approved and executed according to the protocols of the Ethical Committee of the Israeli Ministry of Health no.696/11.

2.3. Induction of colitis and protocol of treatment

Colitis was chemically induced by 2% dextransulfate sodium salt (DSS) reagent grade MW-36,000–50,000 (MP Biomedicals Eschwege, Germany) in tap water and given to the mice for 5 days. Three groups of mice were employed in each experiment: 1) DSS induced colitis mice treated with TPC (50 μg/0.1 ml PBS) by oral ingestion for 11 days using a feeding needle, beginning at day -2 before disease induction. 2) DSS induced colitis mice treated with a control PBS as a vehicle for 11 days, starting at day -2. 3) Healthy, non-treated mice. Each group comprised 10 mice.

2.4. Assessment of DSS colitis

Throughout the experiment, mice health status was monitored daily by the determination of weight loss, rectal bleeding, stool consistency, and survival. Changes of body weight were indicated as percentage loss of the baseline body weight. Occult intestinal bleeding was followed by using the Hemoccult test (SENSA, Beckman counter, USA) while rectal bleeding signs or gross bleeding were observed. The disease activity index (DAI) was calculated by grading on a scale of 0–4 the following parameters: change in weight (0, $\leq$1%; 1, 1–5%; 2, 5–10%; 3, 10–20%; and 4, >20%), intestinal bleeding (0, negative; 2, hemoccult; 4, gross bleeding), and stool consistency (0, normal; 2, loose stools; 4, diarrhea). Ten days following disease induction the mice were sacrificed and the colon was harvested and evaluated for colon length and microscopic colonic damage.

2.5. Histopathology analysis

The distal portions of the colon were excised and fixed in 4% formalin. The tissue was dehydrated in alcohol (1 h each in 70, 80, 90, and 100%) and xylene (three steps, 1 h for each step) and embedded in paraffin. The paraffin block was sliced at a 7 μm thickness, stained with Hematoxylin-Eosin (H&E) and observed microscopically. Histological damage and inflammation was assessed in a blinded fashion by an expert pathologist. The following manifestations were included in the evaluations: the extent of inflammation, depth of inflammation and layers involved, distribution of lesions, and nature of mucosal changes.

2.6. Protein isolation and Western blotting

Colon tissues were suspended in ice-cold lysis buffer containing 50 mM Tris (pH7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 1 mM PMSF, 1 mM sodium vanadate, 0.1% protease inhibitor mixture (Sigma–Aldrich L-4391 St Louis, MO, USA) for 30 min on ice and centrifuged at 13,000 rpm for 20 min. Protein concentration was defined by BCA Protein Assay Kit (Pierce, Thermo scientific, Rockford, IL, USA). Tissue lysates were boiled for 5 min in non-reduced sample buffer and applied to electrophoresed on 10% SDS-PAGE, and transferred to nitrocellulose membrane. Following blocking with 10% skim milk, the membranes were exposed to the following antibodies: goat-anti-IL-10 monoclonal antibodies, goat anti-IL-17A, goat anti-TNFα, goat-anti-IL-1β and anti-GAPDH, all purchased from Santa Cruz. Blots were developed using horseradish peroxidase-conjugated secondary antibodies and the proteins were visualized by chemiluminescence (Santa Cruz Biotechnology, Santa Cruz, CA, USA), using Kodak BioMax film. the ECL detection system (Pierce).

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