A protein extracted from *Metagonimus yokogawai* alleviates inflammation in patients with Ankylosing Spondylitis

Eun Jeong Won  
Asan Medical Center

Yu Jeong Lee  
Graduate School of Chonnam National University

Moon-Ju Kim  
Chonnam National University Medical School and Hospital

Hae-In Lee  
Chonnam National University Medical School and Hospital

Hyun Hee Jang  
Graduate School of Chonnam National University

Seong Hoon Kim  
Graduate School of Chonnam National University

Hee Min Yoo  
Korea Research Institute of Standards and Science (KRISS)

Namki Cho  
Chonnam National University

Min Joo Ahn  
Chungnam National University Hospital

Seung Cheol Shim  
Chungnam National University Hospital

Tae-Jong Kim (ktj1562@jnu.ac.kr)  
Graduate School of Chonnam National University

Short Report

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Abstract

Background

Helminth infections and their components has been recognized to have a positive impact on the immune system. This study aimed to investigate the potential of *Metagonimus yokogawai*-derived proteins (MYp) to provide protection against ankylosing spondylitis (AS) through modulation of immune responses.

Methods

The cytotoxicity of MYp at various doses was first assessed using MTS and flow cytometry. Peripheral blood mononuclear cells (PBMCs) were collected from AS patients, and the production of inflammatory cytokines was analyzed through flow cytometry. In the experiments with SKG mice, MYp or vehicle was administered and inflammation was evaluated through immunohistochemistry and enzyme-linked immunosorbent assay.

Results

The results showed that MYp did not decrease cell viability of PBMCs even after 48 hours. Additionally, the frequencies of IFN-\(\gamma\) and IL-17A producing cells were significantly reduced after MYp treatment in the PBMC cultures. In the SKG mouse model, MYp treatment could reduce serum levels of INF-\(\gamma\), IL-17A, and TNF-\(\alpha\). Overall, MYp treatment significantly suppressed arthritis and enthesitis in the SKG mouse model.

Conclusions

The results suggest the first evidence that MYp can effectively alleviate clinical symptoms and restore cytokine balance in patients with AS.

Background

Ankylosing spondylitis (AS) is a long-lasting inflammatory rheumatic disease that affects the axial skeleton and peripheral joints, as well as other body parts such as the eyes, skin, and gut [1, 2]. With a global prevalence of between 0.1% and 1.4%, AS can cause serious spinal mobility issues and a decline in physical function, affecting the quality of life of those affected [3, 4]. The exact mechanism behind the disease remains unknown, but the role of Th1/17 pro-inflammatory mediators is well established [5, 6]. The hygiene hypothesis, which suggests a correlation between reduced worm infections and the onset of Th1/17-based inflammatory disorders, has been suggested as one explanation for the increasing prevalence [7, 8, 9].
In recent years, there have been attempts to use parasites, such as the nematode *Trichuris suis*, as a form of treatment for autoimmune disorders, with some preliminary results from clinical trials showing promise [10, 11]. However, using helminth products as therapeutic agents has advantages over a live infection. Lee et al. previously clarified the therapeutic effect of *Clonorchis sinensis*-derived protein (CSp) against AS using a murine model [12]. Any trematodes-induced proteins might be active, similar with the basis of the beneficial effect of CSp in the pathogenesis, however, it is still lacking to be known.

This study focuses on *Metagonimus yokogawai*, a prevalent intestinal trematode in Korea that is well adapted to human [13]. We speculate that *M. yokogawai* also can possess the potential to the modulation of AS. Therefore, the aim of this study was to investigate the potential benefits of using *M. yokogawai*-derived proteins (MYp) for the treatment of AS.

**Methods**

**Human samples**

All participants met the modified New York criteria for AS [14]. Blood samples were collected from individuals with active AS and healthy controls (HC). The study was conducted in accordance with the guidelines set forth in the Helsinki Declaration and received ethical approval from the Ethics Committee. All subjects provided written informed consent (CNUH-2011-199).

*M. yokogawai* and their antigen preparation

The *M. yokogawai* metacercariae were collected from the muscles of *Plecoglossus altivelis* caught in the Seomjin-gang river in Jeollanamdo, South Korea, which is known to be an endemic area. The process of preparing the metacercariae was carried out as described previously [15]. The fish flesh was treated with an artificial digestive solution that contained 0.6% pepsin and 0.8% HCl at 37°C for two hours to obtain the metacercariae. The product was washed several times with normal saline and the metacercariae were collected under a stereomicroscope and stored at 4°C until use. The metacercariae were then mixed with 1 mL of homogenation buffer, homogenized, vortexed for five minutes, and centrifuged at 13,000 rpm for 20 minutes at 4°C. The protein extraction was performed using the Pierce BCA Protein Assay Kit according to the manufacturer’s instructions and the resulting extract protein, MYp, was concentrated to a concentration of 1.2–1.5 µg/10 µL.

**Cell viability assay**

To assess the impact of MYp on cell viability, the cells were seeded and exposed to various concentrations of MYp for specified time intervals. The effects of MYp on PBMC cell viability were determined using the Cell Titer 96 AQueous One Solution Reagent (G3580, Promega, USA). The process involved mixing 100 µL RPMI with 20 µL MTS solution, adding it to each well, and then recording the absorbance at 490 nm using a 96-well microplate reader (Molecular Devices, USA). The cell viability was
analyzed using flow cytometry after staining the whole cells with anti-Fixable Viability Dye-eFluor780 (65-0865-14, Invitrogen, USA).

Co-culture of human inflammatory cells with MYp

The Peripheral blood mononuclear cells (PBMCs) were obtained and suspended in a complete growth medium composed of RPMI 1640, 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/mL streptomycin, along with 10% fetal bovine serum. (FBS; Gibco BRL, Grand Island, NY, USA). The cells were then seeded into 96-well plates at a density of 1 x 106 cells per well. MYp was added to the cells in the 96-well culture plates and incubated for 7 hours at 37°C in a CO2 incubator. After being stimulated with PMA (100 ng/mL) and ionomycin (1 µM) for 4 hours, the cells were stained with Pacific Blue-conjugated anti-CD4 (300521, BioLegend, USA) and anti-Fixable Viability Dye-eFluor780 (65-0865-14, Invitrogen, USA). They were then washed, fixed, permeabilized with Cytofix/Cytoperm buffer, and intracellularly stained with FITC-conjugated anti–IFN-γ (552887, BD, USA) and APC-conjugated anti–IL-17A (512334, BioLegend, USA) antibodies before being analyzed using FlowJo Software (BD, USA).

Experimental animal model and clinical score

SKG mice of a BALB/c background were obtained from Clea Japan (Tokyo, Japan) and were kept in a specific pathogen-free environment with ad libitum access to water and standard diet ad libitum. The experiments were approved by the CNU IACUC-H-2019-36 Institutional Animal Care and Use Committee and carried out in accordance with the Laboratory Animals Welfare Act and Guide for the Care and Use of Laboratory Animals. Female mice were used in the study and were divided into three groups: a negative control group (n = 6 mice), a vehicle group (VH, n = 6 mice), and a MYp treatment group (n = 6 mice). The VH and MYp treatment groups received a curdlan (Wako, Osaka, Japan) suspension administered intraperitoneally (i.p.) at a dose of 3 mg/kg, starting at 8 weeks of age. The MYp treatment group received MYp (10 µg/0.2 mL) twice i.p. prior to arthritis induction and once a week thereafter until sacrifice. The control and VH groups received PBS i.p. instead of MYp. Treatment was randomly assigned based on a serial number generation. Clinical signs of the mice were monitored twice a week and scored by two independent observers, with a possible score ranging from 0 to 16, where 0 indicates no symptoms and 16 represents the most severe symptoms, including swelling of ankles and toes, twisting, and severe swelling of ankles and toes.

Histological findings

At the end of the experiment, samples were taken from the ankles of the mice and preserved in 10% formalin for seven days. The samples were then decalcified in Calci-Clear Rapid solution (HS-105, National Diagnostics, USA) by shaking at room temperature for two weeks, and embedded in paraffin. Thin sections of 3.5-5 µm were cut from the paraffin blocks, deparaffinized in xylene (DUKSAN, Korea), hydrated with graded ethanol, and stained with hematoxylin (1.05174.0500, Merck, Germany) and eosin (ab246825, abcam, USA). The staining process followed standard protocols. The pathology of arthritis and enthesitis was scored by two independent readers who were unaware of the group assignments [16, 17].
Serum level of cytokines using ELISA analysis

At the end of the experiment, blood samples were collected from the mice. The concentration of cytokines were measured using ELISA kits for IFN-γ (BMS606-2, Invitrogen, Austria), IL-17A (BMS6001, Invitrogen, Austria), and TNF-α (MTA00B, R&D Systems, USA). The assay was performed as per the manufacturer's instructions and the absorbance was recorded using a SpectraMax® M2 instrument (Molecular Devices Corp., USA) at 450 nm.

Statistical analysis

The symptom score data was analyzed using a two-way ANOVA with time as a dependent variable. The statistical significance of the difference between means was determined using the Kruskal-Wallis test with Dunn's multiple comparisons, the Wilcoxon matched-pairs signed rank test, or the Mann Whitney test. The statistical analysis was performed using Prism 5.0 from GraphPad Software in San Diego, CA. A P value of less than 0.05 was considered significant and represented as follows: * for $P = 0.05 - 0.01$, ** for $P = 0.01 - 0.001$, and *** for $P < 0.001$.

Results

The results showed that there was no significant impact on the viability of cells when they were exposed to up to 30 µg/mL of MYp, suggesting that MYp did not pose any harm to the cells at this concentration (Fig. 1a). The results of the cell viability analysis of PBMCs showed that MYp treatment up to 48 hours did not have any adverse impact on cell survival and functionality (Fig. 1b). This was demonstrated through the MTS assay, and the flow cytometry analysis, which both confirmed the lack of reduction in cell viability (Fig. 1c, 1d).

In order to assess the ability of MYp to modulate the immune response and reduce inflammation in a human setting, experiments were conducted using PBMCs collected from both HC individuals and patients diagnosed with active AS. The demographic characteristics of the enrolled subjects are shown in Additional file 1. The frequency of IFN-γ and IL-17A-producing CD4 + T cells was analyzed in PBMCs obtained from HC using flow cytometry. The frequency of cells producing the inflammatory cytokines IFN-γ and IL-17A in the CD4 T cell subset was significantly reduced after treatment with MYp, as demonstrated by the results of the experiments. This reduction in cytokine production was clearly visible in the flow cytometry analysis, as shown in Fig. 2a and 2b, which indicated that MYp has the potential to suppress the inflammatory response in cells obtained from healthy controls. The frequencies of both IFN-γ and IL-17A producing cells in the CD4 T cells of patients with active AS were significantly reduced after MYp treatment. Figure 2c and 2d show the results of this reduction, which supports the findings from HC in which the frequencies of IFN-γ and IL-17A producing CD4 + T cells were also reduced after MYp treatment. These results indicate that MYp has the potential to modulate and reduce the production of inflammatory cytokines in PBMCs, both in HC and in patients with AS.
To investigate the impact of MYp on the development and progression of AS in a mouse model, the treatment protocol was designed such that the mice were administered MYp or VH one week prior to the curdlan injection. This experimental design was implemented to closely resemble the natural course of the disease and allow for sufficient time for MYp to take effect. The treatment administration was carried out as shown in Fig. 3a. The MYp treatment was found to have a noticeable impact on the development of the disease and significantly delayed the onset of arthritis, as evidenced by the results of arthritis scores determined at the end of the study (Fig. 3b and 3c).

To further confirm the effectiveness of MYp in reducing inflammation, serum samples were collected from the treated mice. The results showed that the levels of INF-γ, IL-17A, and TNF-α were significantly reduced in mice treated with MYp compared to the control group, as depicted in Fig. 4a. To thoroughly evaluate the impact of MYp on local joint and enthesial inflammation, ankle tissues were meticulously analyzed histologically using H&E staining. This method allowed us to assess the extent of inflammation and tissue damage present in the joints and entheses, and to determine the efficacy of MYp in reducing these pathological hallmarks of AS. The representative tissue stains at the end of the experiment were examined in order to assess the effect of MYp on local joint and enthesal inflammation (Fig. 4b). Upon histologic evaluation, it was determined that the mice that were treated with MYp demonstrated a reduction in the severity of both arthritis (Fig. 4c) and enthesitis (Fig. 4d) when compared to the mice that were treated with VH.

**Discussion**

Helminth parasites have evolved their tactics to live and survive within immune-competent hosts for extended periods of time by triggering anti-inflammatory or immune-modulating responses. It has been observed that there is an inverse relationship between autoimmune diseases and helminth infection, indicating a possible protective role of helminth infection in various autoimmune diseases [18]. While much is not known about the potential of intestinal trematodes and specifically *Metagonimus* species, there have been two reports [13, 19]. Kim et al. proposed that *M. yokogawai* infection could improve immune-mediated disorders by changing the gut microbiome, which was linked to an increase in several Lactobacillus species in a mouse model [13]. Lee et al. showed that *Metagonimus miyatai* infection improved DSS-induced colitis in mice and suggested *M. miyatai* as a potential new treatment strategy for autoimmune colitis [19].

To the best of our knowledge, this study is the first to demonstrate the beneficial effect of *M. yokogawai*-related molecules on the symptoms and cytokine imbalances in AS. Previous research showed that CSp treatment could reduce the severity of AS symptoms through the systemic and local suppression of pro-inflammatory mediators [12]. In this study, we aimed to find an alternative therapeutic target for AS. Currently, there is limited research on the therapeutic effects of *M. yokogawai*-related molecules, with only one study [15]. In that study, intralymphatic injection of the protein extract from *M. yokogawai* was found to reduce allergic symptoms and histological findings in an allergic rhinitis mouse model. Additionally,
levels of IFN-γ and IL-17 were decreased in the nasal mucosa and cervical lymph nodes. This result could prompt us to evaluate its potential in Th1/Th17-dominant pathophysiology conditions.

We confirmed that MYp treatment was not toxic, as it did not affect cell viability after 48 hours of treatment. Our results showed a significant decrease in IFN-γ and IL-17A production after treatment with MYp in human PBMCs, suggesting an anti-inflammatory effect. In the SKG mouse model, MYp treatment was able to improve disease symptoms and reduce levels of INF- γ, IL-17A, and TNF-α. The histological findings supported the inhibitory effect of MYp treatment on arthritis and enthesitis. Our findings are similar to those in animals infected with S. mansoni, where a reduction in pro-inflammatory cytokines (INF-γ, TNF-α, and IL-17A) was observed [20]. This common alteration in immune response due to parasite infection may have contributed to the reduced severity of Th1/17-mediated immune disorders, including AS. A previous research has shown that increased recruitment of IL-10 and Treg cells were important factors in the improvement of airway inflammation by M. yokogawai-induced proteins [15, 21, 22, 23]. This study suggests that MYp treatment can reduce the severity of AS symptoms by suppressing pro-inflammatory mediators, offering potential new therapeutic agents for AS.

Conclusions

This study has several limitations. Firstly, the exact mechanism behind the suppression of the inflammatory response is yet to be determined. Secondly, the MYp was used as crude extracts, and further molecular characterization of the MYp will enhance our understanding of the host-parasite interaction that can be applied for a standardized therapeutic approach. Despite these limitations, our results support the idea that MYp has the potential to mitigate the symptoms of AS by suppressing the pro-inflammatory mediators without affecting the host’s ability to fight against the disease. This highlights the possibility of MYp-based therapies as a novel therapeutic target for treating AS.

Abbreviations

AS
Ankylosing spondylitis
MYp
M. yokogawai-derived protein
i.p.
intra-peritoneally
NC
negative control
PBMC
peripheral blood mononuclear cell
PC
positive control
TNF
Tumor necrosis factor.

Declarations

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 Availability of data and materials

The authors declare that all data supporting the findings of this study are available within the article and its Additional files.

Authors’ contributions

EJW and TJK; conceived the study, participated in study design, data analysis, and were responsible for writing and submission of the final manuscript, YJL, MJK, HIL, HHJ, SHK; experiments and analysis, HMY, NC, MJA, SCS; data analysis and interpretations. All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published.

Ethics statement

This study was carried out in accordance with all relevant institutional guidelines. The Ethics Committee of Chonnam National University Hospital approved this study (CNUH-2011-199) and written informed consent was obtained from all subjects. All animal experiments were approved by the Institutional Animal Care and Use Committee (CNU IACUC-H-2019-36).

Consent for publication

Not applicable.

Competing interest

The authors declare that they have no competing interests.
References


**Figures**
Assessment of cell viability with MYp. The viability of PBMCs was evaluated using the MTS assay (a, b). A Kruskal-Wallis test with Dunn's multiple comparisons was performed to determine statistical significance and the results are presented as the mean ± SEM. Additionally, the anti-fixable viability dye was used to stain PBMCs from healthy controls and patients with ankylosing spondylitis, which were then measured by flow cytometry (c, d). The statistical significance was determined through a Mann-Whitney U test. N.S: not significant. MYp: *Metagonimus yokogawai*–derived protein; PBMCs: peripheral blood mononuclear cell.
The MYp treatment reduced the production of inflammatory cytokines from PBMCs of both healthy controls (HCs) and patients with ankylosing spondylitis (AS). After being cultured with MYp for 7 hours, the cells were stimulated with PMA and ionomycin for 4 hours. The representative flow cytometric features are shown (a, c). The percentages of INF-γ and IL-17A positive cells from PBMCs of healthy controls (b) and patients with ankylosing spondylitis (d) were analyzed and are presented as individual
samples. \( *P < 0.05, **P < 0.01 \), by Wilcoxon matched-pairs signed rank test. MYp: *Metagonimus yokogawai*–derived protein; PBMCs: peripheral blood mononuclear cell.

Figure 3

The MYp treatment showed a reduction in clinical symptoms in SKG mice. (a) After being treated with MYp or a vehicle i.p. for 6 weeks, the arthritis scores were determined based on the clinical arthritis
severity in each group (n = 6 mice per group). (b) The results are presented as the mean ± SEM and a significant difference was found with a ***p-value of less than 0.001 through a two-way analysis of variance (ANOVA). (c) A representative mouse from each group is shown at the end of the experiment. MYp: *Metagonimus yokogawai*–derived protein; Control: negative control; VH: positive control with vehicle i.p..
The MYp treatment demonstrated not only a reduced levels of inflammatory cytokines in the peripheral blood, but also a reduction in arthritis and enthesitis in SKG mice. (a) At the end of the experiment, serum samples were collected, and the levels of INF-γ, IL-17A, and TNF-α were measured using enzyme-linked immunosorbent assay kits according to the manufacturer's instructions. Values are the mean ± SEM by Mann Whitney test. (b) The representative example of the ankle tissue stains (H&E) at the end of the experiment is shown in this figure, which demonstrates a noticeable improvement in the tissue condition compared to the untreated mice. (c, d) A visual representation of the scores of arthritis and enthesitis at the end of the experiment further supports the efficacy of the MYp treatment in reducing the symptoms of these conditions. ***P < 0.001, MYp: Metagonimus yokogawai–derived protein; Control: negative control; VH: positive control with vehicle i.p.

Supplementary Files

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