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Immunosuppressive mechanism of *Hypoderma lineatum* secreted serine esterase, a potential modulatory method used to inhibit transplant rejection

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ABSTRACT

Background: Although immunosuppressive therapies have made organ transplantation a common medical procedure worldwide, chronic toxicity has a major issue for long-term treatment. One method to improve therapies and methods is the application of immunomodulatory agents from parasites such as *Hypoderma lineatum*. Hypodermin A (HA) is a serine esterase secreted by the larvae of *Hypoderma lineatum*, several studies demonstrated its immunosuppressive mechanism *in vitro*, and recently we discovered that HA inhibits the expression of interferon (IFN)-γ and interleukin (IL)-2 and activates IL-10 expression. Therefore, we hypothesized that it might be a potential agent used to block allograft rejections. However, most studies of the immunosuppressive mechanisms associated with HA were undertaken at the cellular level. In order to augment these studies, we evaluated the immunosuppressive effects of HA *in vivo* using an HA transgenic mouse model.
Result: Our results revealed similar findings to those reported by *in vitro* studies, specifically that HA induced prostaglandin E_2 expression, downregulated IFN-γ and IL-2 expression, and promoted IL-10 secretion *via* E-type prostanoid receptor 4. Additionally, we observed that HA overexpression inhibited lipopolysaccharide-induced TLR4 activation. These findings provide insight into a new potential agent capable of blocking graft rejection.
Conclusion: Our founding suggested that HA-related treatment could be a promising option to improve the viability of grafts in human.

1. Introduction

Organ transplantation is an effective way to treat many end-stage organ diseases; however, immunological rejection of the graft remains the primary reason for transplant failure. Therefore, to improve graft-viability rates, improved therapies and methods are required to inhibit transplant rejection. One method involves application of immunomodulatory agents from parasites, such as *Hypoderma lineatum*. *H. lineatum* infestation of cattle is difficult to circumscribe in the northern hemisphere due in part to the insect larvae modulating and evading the host immune system following invasion of host connective tissue.

Hypodermin A (HA), which is secreted by *H. lineatum* larvae, contains 771 bp and encodes 257 amino acid residues. HA has serine protease activities and plays an important role in immunosuppression during *H. lineatum* infestation [1]. Our previous study demonstrated that HA downregulates interferon (IFN)-γ and interleukin (IL)-2 expression and promotes IL-10 secretion *in vitro* [2]. The effect of HA on cytokine expression led us to hypothesize that HA might be a potential agent capable of preventing allogeneic rejection.

To investigate the role of HA in host immune response *in vivo*, we established a HA transgenic mouse using pronucleus microinjection and evaluated the effect of HA on cytokine expression related to graft-rejection response. Our results indicated that compared to the control, high levels of prostaglandin E_2 (PEG_2) and IL-10 and low levels of IFN-γ and IL-2 were detected in transgenic mice. Previous studies showed that toll-like receptor 4 (TLR4) activation regulates graft rejection. Here, we found that HA overexpression significantly

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inhibited lipopolysaccharide (LPS)-inducted TLR4 activation both *in vitro* and *in vivo*. Our findings suggested that HA could be a potentially effective immunosuppressant agent for reducing organ-transplant rejection.

2. Materials and methods

2.1. Animals, cell cultures, and lentivirus construction

Friend virus B-type (FVB) mice were purchased from the Animal Center of Xuzhou Medical University, Xuzhou, China. HA-overexpressing FVB transgenic mice were generated by Cyagen Biosciences (Santa Clara, CA, USA), and subsequent HA expression was verified *in vivo* [3]. Normal 6- to 8-week-old female mice were used in the experiments and were maintained under clean conditions. RAW264.7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, Waltham, MA, USA), 0.25 μg/mL fungizone, 100 U/mL penicillin, 10 μg/mL streptomycin sulfate, and 5 μg/mL gentamicin and incubated at 37°C in 5% CO_2.

2.2. Protein analyses via western bloting

Back skin samples taken from mice were homogenized using a PK-02200PMGXL (Proscientific, USA), protein was isolated using lysis buffer (65 mM Tris–HCl, 4% sodium dodecyl sulfate [SDS], 3% DL-dithiothreitol, and 40% glycerol). 30 μg of protein was separated on a SDS-polyacrylamide electrophoresis gel and transferred onto nitrocellulose membranes. Membranes were blocked in 5% skim milk, and probed with a rabbit polyclonal antibody against HA, or a rabbit monoclonal antibody specific to β-actin overnight. Membranes were then washed three times in TBST buffer (20 mM Tris–HCl, 150 mM NaCl, 0.05% Tween-20), and incubated with horseradish peroxidase-conjugated anti-rabbit (Beyotime Biotechnology, China) (diluted 1:1000) at room temperature, and visualized with Odyssey® CLx Infrared Imaging System (LI-COR, Lincoln, NE, USA).

2.3. RNA isolation and real-time reverse transcription polymerase chain reaction (RT-PCR)

A lentiviral vector containing a green-fluorescent protein tag and the full-length coding sequence for the HA gene (Lv-HA) was generated by GenePharma (Shanghai, China). Our previous study demonstrated its ability to be transduced into RAW264.7 cells and effectively express the HA protein [2]. TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total cellular RNA from RAW264.7 cells, followed by conversion to cDNA using reverse transcriptase (Roche Applied Science, Mannheim, Germany). cDNA samples were standardized based on the content of GAPDH cDNA as a housekeeping gene. The sequences of both the forward and reverse primers used in this study were designed with Primer Premier 5.0 (Premier Biosoft, Palo Alto, CA, USA) and are listed in Table 1.

Table 1
Primers used in this study.

Primer	Sequence 5′ → 3′
IL-1β-F	GCAACTGTTCCTGAACTCAACT
IL-1β-R	ATCTTTTGGGGTCCGTCAACT
TNF-α-F	GTGGAACTGGCAGAAGAGGC
TNF-α-R	AGACAGAAGAGCGTGGTGGC
GAPDH-F	GGCAAATTCAACGGCACAGT
GAPDH-R	TAGGGCCTCTCTTGCTCAGT

2.4. Enzyme-linked immunosorbent assay (ELISA)

Blood was obtained from the eyeballs of mice, and the concentrations of IL-2, IFN-γ, IL-10, and PGE_2 in serum were quantified using specific ELISAs (R&D Systems, Minneapolis, MN, USA) according to manufacturer instructions. RAW264.7 cells were cultured in DMEM and after a series of treatments, supernatants were collected and levels of IL-β1 and tumor necrosis factor (TNF)-α measured by ELISA (R&D systems). Absorbance was determined with an EL312 Bio-Kinetics microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA).

2.5. Statistical analysis

All experiments were conducted in triplicate to ensure that the results were reproducible. Data are presented as the mean ± standard deviation. A $P < 0.05$ was considered significant, whereas a $P < 0.01$ was considered highly significant.

3. Results

3.1. HA downregulates the production of IL-2 and IFN-γ and promotes IL-10 production in vivo

Our previous study showed that HA overexpression in RAW264.7 cells downregulates IL-2 and IFN-γ expression and upregulates IL-10 expression. To extend our study *in vivo*, we generated FVB transgenic mice capable of overexpressing HA, and western blot was performed to detect the expression of HA. The result showed that HA was expressed successfully in the transgenic mice (Fig. 1a). Then we measured the concentrations of IL-2, IFN-γ, and IL-10 in serum of mice. Relative to control mice, HA transgenic mice showed lower levels of IL-2 and IFN-γ (Fig. 1b and c); however, IL-10 concentration was higher in transgenic mice (Fig. 1d).

3.2. HA regulates the production of IL-2, IFN-γ, and IL-10 through PGE_2 induction in mice

The regulatory effect of HA on IL-2, IFN-γ, and IL-10 was demonstrated *in vitro* [2]. To verify the effect of HA overexpression *in vivo*, we measured PGE_2 expression levels. As shown in Fig. 2a, PGE_2 concentration in transgenic mice was significantly elevated relative to that observed in wild-type mice. It was demonstrated that HA markedly reduces IL-2 production in phytohemagglutinin-stimulated bovine peripheral blood mononuclear cell cultures *via* a prostaglandin-dependent pathway [4], and we previously reported a similar result in RAW264.7 cells. Here, we determined whether the regulatory role of HA on IL-2, IFN-γ, and IL-10 was related to PGE_2 induction *in vivo*. Following injection of mice with the cyclooxygenase-2 inhibitor NS-398, we observed significant suppression of PGE_2 expression (Fig. 2a) and abrogation of the regulatory effect of HA on IL-2, IFN-γ, and IL-10 (Fig. 2b–d). These results suggested that HA regulated the production of IL-2, IFN-γ, and IL-10 through PGE_2 induction in mice.

3.3. Selective blockage of EP4 receptors prevents HA-induced regulation of cytokine expression

The effects of PGE_2 are mediated by four distinct G protein-coupled EP receptors (EP1–4). To identify the EP receptor subtype(s) responsible for HA-induced regulation of IL-2, IFN-γ, and IL-10 expression, transgenic mice were injected with 10 μg/kg SC-51089 (an EP1 antagonist), 50 mg/kg PF-04418948 (an EP2 antagonist), 20 mg/kg L798106 (an EP3 antagonist), or 50 mg/kg ER-819762 (an EP4 antagonist), and 1-d later, the expression levels of IL-2, IFN-γ, and IL-10 were measured by ELISA. We observed no effects on cytokine expression following treatment with the EP1, EP2, or EP3 antagonists;