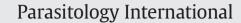
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# The immunosuppression mechanism of hypodermin A on complement

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# ABSTRACT

Hypodermin A (HA), a serine protease secreted by first-instar larvae of *Hypoderma lineatum* (Diptera: Oestridae) is associated with inflammatory and the specific immune responses in cattle hosts. In the present study, the cDNA sequence of HA was synthesized, and found to have fifteen amino acids which differed from the sequence available in GenBank. We then examined the association between recombinant HA and guinea-pig complement component 3 (C3) through a co-immunoprecipitation assay. Cos7 cells stably expressing HA were generated, and were found to be more resistant to lysis by guinea-pig C3 than the controls. HA was also able to degrade the C6 and C5b-9 of guinea-pig C3. The presumed DNA binding site of HA with guinea-pig C3 was detected by an electrophoretic mobility shift assay (EMSA). In contrast, after stable transfection, mHA was unable to reduce the amount of C3 or to inhibit its cytotoxicity, while HA could degrade guinea-pig C3 and inhibit the complement pathway. The findings suggest that recombinant HA could serve as an immunosuppressive agent against organ rejection after xenotransplantation.

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# 1. Introduction

Organ transplantation has become the treatment of choice for many end-stage organ diseases. However, the unavailability of sufficient organs to meet the existing demand for transplantation has resulted in major organ shortage crises. Although heterologous grafts have been widely viewed as alternatives, they unfortunately give rise to another more troublesome problem — transplant rejection. One of the methods used to relieve such a negative response is the application of immunomodulatory agents from parasites, such as *Hypoderma lineatum*. This insect's larvae develop after passing through their hosts' deep connective tissue, a process which involves modulation and evasion of the host's immune system is involved [1]. More specifically, these larvae usually secrete substances which act to weaken host defenses [2,3].

Hypodermin A (HA), secreted by first instar larvae, is able to escape the host immune responses and suppress lymphocyte proliferation [4], and suppress bovine interleukin 2 (IL-2) production via the prostaglandin pathway [5]. HA can also cleave the complement component C3 from bovine serum, block the complement pathway and reduce inflammation [6–8]. The rejection of heterogenic transplantation is attributable to the activation of the host complement system where C3 assumes an important role [9]. HA can inhibit C3-mediated cytotoxicity and cleave the C3 in rat and human. As a result, HA shows enormous potential to slow down the rejection associated with xenotransplantation [10].

To further investigate the role of HA in modulating host immune responses, a cos7 cell model with stable expression of recombinant HA was established in the current study. The recombinant HA exhibited biological properties similar to those of the native form. Possible mechanisms of interaction between HA and C3 were discussed. All the findings will provide increased theoretical support for allotransplantation research.

# 2. Materials and methods

# 2.1. Animals

The first-instar larvae of *H. lineatum* were collected from the oesophagi of slaughtered poephagus grunniens. The larvae were

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preserved in RNA preservation solution after washing in PBS. Six male Guinea-pigs aged seven to ten weeks were selected for the experiment.

## 2.2. RNA isolation and RT-PCR

Total RNA was isolated from the first instar larvae of *H. lineatum* using an RNA isolation kit (TaKaRa Bio, China), and then converted into cDNA using a reverse transcription Kit (TaKaRa Bio, Dalian, China) with random primers, incubated for 15 min at 37 °C, then heated at 85 °C for 5 s. The following primers were then used to amplify the cDNA of HA: forward: 5'-ATGCTGAAGTTTGTTATTTTATT GTGC-3', reverse: 5'-TAGGATCCTTAAATAGATTCTGCATTACTGAC-3' according to the sequence given in GenBank (accession number: X74303). The PCR conditions used were as follows: 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 10 min. The PCR products were analyzed by 1.0% agarose gel electrophoresis, and the fragment was cloned into the TA vector and confirmed by sequencing.

# 2.3. Construction of expression vectors

To obtain the construct pEF1 $\alpha$ -HA-AcGFP encoding HA in the cytoplasm of cells, a signal peptide sequence was added to the 5' end of HA cDNA by overlapping PCR. Meanwhile, a mutant HA vector (pEF1 $\alpha$ -mHA-AcGFP) was established in the potential binding site of the principal chain. The fragments were cloned into the pEF1 $\alpha$ -IRES-AcGFP vector previously digested with BamH I/EcoR I, and the recombination vectors were checked by nucleotide sequencing.

#### 2.4. Cell culture and stable transfection

Cos7 cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Beijing, China) containing 10% fetal bovine serum (FBS) (Invitrogen, Beijing, China), 100 U/mL of penicillin, and 100 µg/mL of streptomycin (HPGC) at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>. Before transient transfection, the cells were seeded into 6-well plates and cultured for 24 to 48 h. Next, the cells were transfected at 60–70% confluence with 2 µg of plasmid DNA pEF1 $\alpha$ -HA-AcGFP (pEF1 $\alpha$ -mHA-AcGFP) encoding HA (mHA) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. To achieve stable expression of HA (mHA), G418 (1 mg/mL) was added to the selection medium after 48 h of incubation. The medium was replaced every two days. After two to three weeks, drug-resistant cell clones were recovered and propagated.

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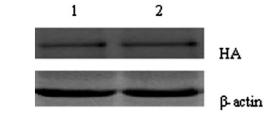


Fig. 2. Western blot analysis of recombinant HA (lane 1) and native HA (lane 2), normalized to  $\beta$ -actin.

#### 2.5. Western blot analysis

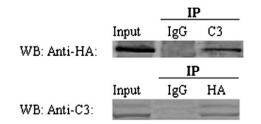
Total proteins were extracted from six first-instar larvae of H. lineatum according to the improved method of Lowry et al. [11]. Proteins (30-50 mg protein/lane) were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes which were then blocked for 30 min with 5% BSA-0.1% Tween 20. The membranes were incubated with the following primary antibodies, HA: rabbit polyclonal, 1:1000 (Abgent Suzhou, China); C3: goat monoclonal, 1:1000 (Thermo Scientific, Rockford, IL); *B-actin*: mouse monoclonal, 1:1000 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), with  $\beta$ -actin used as the loading control. After washing in buffer, the membranes were incubated with corresponding horseradish peroxidase-conjugated secondary antibodies (HA: anti-rabbit, 1:1000; C3: anti-goat, 1:100; B-actin anti-mouse, 1:100, all from Gene Company Ltd., Hong Kong) at room temperature for two hours. Western blot analysis was then performed using odyssey (manufacturer: Gene company limited) for signal detection. Signal intensities were quantified by densitometry using Image J software, and protein expression levels were normalized to  $\beta$ -actin.

#### 2.6. Enzyme-linked immunosorbent assay (ELISA)

Cell culture supernatants were collected from cell stably expressing HA and from control cells. The levels of C3, C6 and C5b-9 were quantified using specific ELISA kits for mice according to the manufacturers' instructions (C3 was from Abnova, Taiwan, China; C6 and C5b-9 were from Shanghai Westang Bio-tech. Co. Ltd., Shanghai, China). The absorbance was read at 450 nm in an ELISA plate reader.

### 2.7. Immunoprecipitation and co-immunoprecipitation assays

The culture medium was centrifuged at 2500 rpm at 4 °C for 3 min. The supernatant was then removed, and 1 µg of rabbit anti-HA polyclonal antibody or control rabbit IgG was added to it at 1:1000, followed by four hours of exposure to dithiothreitol (DTT) and IP buffer at 4 °C. Furthermore, the supernatant was incubated with protein A/G plus sepharose at 4 °C for another two hours with gentle rotation, and subsequently centrifuged at 10,000 rpm at 4 °C for three minutes. The beads were collected and washed three times with IP buffer. The binding proteins were eluted in 20 µL of SDS loading buffer before being boiled for ten minutes and separated by SDS-PAGE for western blot analysis.



**Fig. 3.** The interaction of HA and C3 was detected by Co-IP. (WB: Anti-HA, Input: the protein of first-instar larvae of *H. lineatum*. IgG: the control. C3: the primary antibody of C3 was added to immunoprecipitate the protein of HA. WB: Anti-C3, Input: the protein of guinea-pig serum. IgG: the control. HA: the primary antibody of HA was added to immunoprecipitate the protein of C3.).

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