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Evaluation of *Opisthorchis viverrini* calreticulin for potential host modulation

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ABSTRACT

The multifunctional calreticulin (CALR) was identified as a major calcium-binding protein of the endoplasmic reticulum before being recognized as a chaperone in the same place. Only later were activities of calreticulin outside the endoplasmic reticulum described that for example affect cell proliferation and the innate immune system. In the present work we have investigated those extracellular activities of CALR from the cancerogenic human liver fluke *Opisthorchis viverrini* (OvCALR), as they might be important in host/parasite interaction. We first demonstrate that OvCALR is released from the parasite and stimulates a specific humoral immune response. Recombinant OvCALR is then shown to suppress proliferation of primary endothelial cells, their motility and sprouting activities. The potential of OvCALR to interfere with the complement system is established, firstly by demonstrating its direct binding to C1q and, secondly by suppression of hemolysis of sensitized red blood cells. These findings suggest that OvCALR is an important parasite antigen that could modulate diverse host functions and support parasite survival.

1. Introduction

The liver fluke *Opisthorchis viverrini* is an important human parasite in the northeastern regions of mainland Southeast Asia. Inflammatory responses in the bile ducts during heavy infections and development of cholangiocarcinoma in chronic infections have been connected to released and surface-exposed parasite antigens, for example Ov-grn-1, a granulin-like growth factor that was found to stimulate proliferation of host cells (Smout et al., 2009, 2011; Papatpremsiri et al., 2015). Calreticulin is a conserved protein in eukaryotes and extracellular effects of calreticulin that support parasite survival have been identified in a variety of parasites as follows. In nematodes CALR has been analyzed in several important parasitic species with a focus on its immunomodulatory effects. In filaria it was first described in *Onchocerca volvulus* as antigen RAL-1 which stimulated the production of autoantibody against human CALR (Meilof et al., 1993; Rokeach et al., 1994). Molecular characterization of *Dirofilaria immitis* CALR demonstrated its secretion in larvae and adult worms, ubiquitous localization, especially hypodermis and syncytial muscle cells, as well as calcium-binding of its recombinant form (Tsuji et al., 1998). Recently, interaction of *Brugia malayi* CALR with human complement protein C1q was reported (Yadav et al., 2014) following studies to this effect in the hookworm *Necator americanus* and the important *Haemonchus contortus*. Hookworm CALR was identified as an allergen, stimulating histamine release and an IgE response (Pritchard et al., 1999, 2007; Griffiths et al., 2008). Furthermore, it was demonstrated that it can directly inhibit human complement protein C1q (Kasper et al., 2001), an activity previously found in human CALR (Kishore et al., 1997). In a vaccine trial immunization of mice with recombinant *N. americanus* CALR led to 43–49% reduced worm burden (Winter et al., 2005). CALR secreted by *Haemonchus contortus* was found to have an inhibitory effect on the host complement pathway through interaction with C1q (Suchitra and Joshi, 2005; Suchitra et al., 2008; Naresha et al., 2009). Immunomodulatory activity was also noted for CALR from the rodent intestinal nematode *Heligmosomoides polygyrus*, the protein stimulated IL-4 release from CD4(+) T cells of infected mice (Rzepecka et al., 2009). Interference with host defense mechanisms were also reported for secreted CALR of nematodes parasitizing plants (Jaubert et al., 2005; Jaouannet et al., 2013).

Several studies investigated host immune responses to *S. mansoni* CALR, it was reported as a good T- and B-cell antigen (El Gengehi et al., 2000) but only 10% of schistosomiasis-resistant individuals showed a proliferative response to the antigen (El Naglaa et al., 2004). Furthermore, while antibodies against calreticulin were detected in a majority

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of patients sera cross-reactivity was observed with sera from healthy controls (El Aswad Bel et al., 2011).

In cestodes *Taenia solium* CALR (Mendlovic et al., 2004) has been investigated as an oral vaccine with cholera toxin as adjuvant in hamsters and showed protection depending on the type of cysticerci (León-Cabrera et al., 2009). Further studies on the kind of elicited immune responses indicated a mixed Th1/Th2 cellular response (Fonseca-Coronado et al., 2011; Mendlovic et al., 2015).

Considering the significant immunomodulatory properties of calreticulin in the mentioned helminths it should be of interest to investigate the roles of OvCALR in opisthorchiasis. In recent work we have already demonstrated that OvCALR has the properties of a calcium-binding chaperone and that it is present in tissues of the reproductive system, the digestive system, tegumental cells and during embryogenesis in intrauterine eggs (Chaibangyang et al., 2017).

2. Material and methods

2.1. Parasites

Juvenile and mature *O. viverrini* were collected as previously described (Chaibangyang et al., 2017). All animal experiments in this research were approved by the Animal Ethics Committee of Thammasat University (No. 014/2557) in accordance with the Ethics of Animal Experimentation of the National Research Council of Thailand.

2.2. Expression of recombinant proteins in Escherichia coli

Recombinant OvCALR, mouse CALR (rMmCALR), and *Schistosoma japonicum* glutathione S-transferase (rSjGST) were prepared as previously described (Chaibangyang et al., 2017). Following dialysis against phosphate buffered saline (PBS), pH 7.4 bacterial endotoxin was removed twice on Pierce High Capacity Endotoxin Removal Spin Columns (Thermo Scientific, IL, USA) following the manufacturer's instructions.

2.3. Parasite crude worm extract and excretory-secretory product preparation

Mature parasite were homogenized in 10 mM PBS, pH 7.2, 150 mM NaCl, 0.5% [v/v] Triton X-100, 1 mM PMSF, 1 mM EDTA. Insoluble material was pelleted by centrifugation at 12,000 × g, 4 °C for 15 min. The supernatant was used as soluble crude worm (CW) extract.

Parasite excretory-secretory (ES) product was collected from mature *O. viverrini* obtained from experimentally infected hamsters. Alive parasites were washed in 0.85% (w/v) NaCl and then cultured in 10 mM PBS, pH 7.2 at 37 °C, 5% CO_2 for 4 h. The medium was collected and insoluble materials including eggs were pelleted by centrifugation at 5000 × g, 4 °C for 20 min. The ES product was concentrated using a centrifugal concentrator with 3 kDa cut-off (GE Healthcare, Buckinghamshire, UK). Quantity and quality of parasite CW extract and ES product were determined by a Bradford assay (Bio-Rad, CA, USA) and SDS-PAGE, respectively. The proteins were aliquoted and stored at −80 °C.

2.4. OvCALR and ES product antisera

The preparation of mouse anti-OvCALR antiserum used in this study was described in Chaibangyang et al., 2017 (Chaibangyang et al., 2017). Anti-ES product antisera were prepared by intraperitoneally injection of two female 6–8 weeks-old BALB/c mice with 20 µg ES product per mouse for priming and 10 µg ES product per mouse for boosting, three times in a 3-week interval. Titer Max Gold (Sigma, MO, USA) was used as adjuvant. Mouse pre-immune sera were collected one week before immunization and final antisera were collected two weeks after the last immunization.

2.5. Immunohistochemistry

Liver tissue of *O. viverrini*-infected hamster was fixed in 10% neutral buffered formalin (NBF), embedded in paraffin, sectioned and processed for antigen detection as previously described (Chaibangyang et al., 2017). Briefly, antigen retrieval was performed by heating the liver sections in Tris-EDTA buffer (10 mM Tris-Base, 1 mM EDTA, 0.05% Tween 20, pH 9.0). After blocking in 1% glycine and 4% BSA in PBS, pH 7.2 each for 30 min at room temperature the parasite sections were incubated with either mouse anti-rOvCALR antiserum or pre-immune serum (1:2000) in 1% BSA in PBS, pH 7.2 at 4 °C overnight. Endogenous peroxidase activity was blocked by incubation in 3% H_2O_2 (Merck, Hohenbrunn, Germany). The sections were then incubated in biotinylated polyclonal rabbit anti-mouse immunoglobulin (1:200) (Dako, Denmark) at 37 °C for 1 h, washed and chromogenic detection was performed using an avidin-biotin complex (ABC) peroxidase staining kit (Thermo Scientific, IL, USA) with 3-amino-9-ethyl carbazole (AEC) as substrate (Vector, CA, USA). The reaction was stopped by washing in PBST (10 mM PBS, pH 7.2, 0.1% Tween 20).

2.6. SDS-PAGE and western analysis

Duplicate samples of 10 µg each of parasite CW extract, ES product, and 100 ng rOvCALR were size-separated by 12.5% Tris-Glycine SDS-PAGE and transferred onto an Immobilon-NC membrane (Merck Millipore, Darmstadt, Germany) by semi-dry blotting. Immunodetection with mouse anti-rOvCALR antiserum and mouse pre-immune serum was performed as previously described (Chaibangyang et al., 2017).

2.7. ELISA infected hamster sera

Retro-orbital blood samples were collected from experimentally infected hamsters (n = 10) prior to infection and 2, 4, 8, and 12 weeks postinfection. The blood samples were centrifuged at 5000 × g for 10 min at room temperature and sera were aliquoted and kept at −20 °C. A 96-well plate was coated with 100 ng recombinant OvCALR in carbonate buffer, pH 9.6 and incubated overnight at 4 °C. The wells were blocked with 0.5% skim milk (Oxoid, Hants, UK) in coating buffer at room temperature for 30 min. Preinfection sera and 12-week postinfection sera at dilution 1:200 in 0.5% skim milk in PBS, pH 7.2 were added into separate wells and all samples were assayed in duplicate. The plate was incubated at 37 °C for 1 h and washed several times afterwards. Goat anti-hamster IgG (H + L) HRP antibody (Invitrogen, MD, USA) at dilution 1:3000 was added and incubated at 37 °C for 1 h. The wells were washed, 200 µl of o-phenylenediamine dihydrochloride substrate (OPD, Sigma, MO, USA) was added, and colorimetric detection proceeded in the dark at room temperature for 30 min. The reaction was stopped by adding 50 µl of 2 M H_2SO_4 and the absorbance was measured at 492 nm on a plate reader. Pooled hamster preinfection sera and pooled *O. viverrini* postinfection sera for each collection week were assayed as described above.

2.8. Cell culture

Pre-screened human umbilical vein endothelial cells (HUVECs) were purchased from Lonza Australia and grown in endothelial growth medium (EGM-2, Lonza). Cell passaging was done using ReagentPack subculture reagents (Lonza). *In vitro* angiogenesis assays were done using HUVECs passage numbers 3 to 6 with cell viability greater than 80%.

2.9. MTT assay

HUVECs were seeded at 1×10^4 cells/well into a 96-well cell culture plate (Corning). The plate was incubated at 37 °C, 5% CO_2 for 4 h to allow attachment of the cells. Solutions of rOvCARL, rMmCALR (positive control) and rSjGST (negative control) were prepared at concentrations of 0.1, 1, 10, 20 µM in 100 µl of endothelial growth medium (EGM-2, Lonza) and