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Molecular and functional characterization of goldfish (*Carassius auratus L.*) Serum Amyloid A

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ABSTRACT

Quantitative expression analysis of goldfish SAA revealed the highest mRNA levels in the kidney, spleen and intestine with lower mRNA levels in muscle and liver. Goldfish SAA was differentially expressed in goldfish immune cells with highest mRNA levels observed in neutrophils. To functionally assess goldfish SAA, recombinant protein (rgSAA) was generated by prokaryotic expression and functionally characterized. Monocytes and macrophages treated with rgSAA exhibited differential gene expression of pro-inflammatory and anti-inflammatory cytokines. rgSAA induced gene expression of both pro-inflammatory (TNFα1, TNFα2) and anti-inflammatory cytokines (IL-10, TGFβ) in monocytes. rgSAA induced IL-1β1 and SAA gene expression in macrophages. rgSAA was chemotactic to macrophages and neutrophils, but not monocytes. rgSAA did not affect respiratory burst induced by heat-killed *Aeromonas salmonicida*. rgSAA treatment of macrophages down-regulated their production of nitric oxide. rgSAA exhibited antibacterial properties against *Escherichia coli* in a concentration dependent manner.

1. Introduction

Serum Amyloid A (SAA) is a major acute phase protein, whose serum levels can increase 1000-fold in humans during the acute phase response (APR) in response to various insults [1]. It is primarily synthesized in the liver and its production regulated by cytokines such as interleukin-6 (IL-6), IL-1, and tumor necrosis factor-alpha (TNFα) [2]. The SAA family is composed of acute phase and constitutive proteins. Constitutive SAA (C-SAA) is present in humans and mice only, whereas the acute phase SAA (A-SAA) has been documented in all vertebrates [1]. In humans, A-SAA is encoded by 2 genes, and in mice 3 genes, respectively. In invertebrates such as echinoderms and lower vertebrates such as teleosts, there is a single identified SAA gene, suggesting that SAA multi-gene family only evolved in mammals [3,4]. The presence of SAA dates to echinoderms, and based on the high amino acid conservation throughout 500 million years of evolution, and lack of SAA-deficient animals, SAA appears to be vital to survival [5,6]. Despite the importance of SAA for well being of organisms, its functions remain to be fully elucidated, particularly in lower vertebrates.

In humans, SAA appears to have cytokine-like properties [7]. The addition of SAA induces production of cytokines [8,9], chemokines [10] and matrix metalloproteinases (MMPs) [11] in human monocytes. SAA has been demonstrated to act as a chemoattractant for phagocytes, T lymphocytes, and granulocytes (neutrophils) [12–14]. SAA has been shown to bind to a diverse group of receptors, including TLR2 [15], TLR4 [16] formyl peptide receptor-like 1 (FPRL1) [17] and CD36 [18]. Given this, it is not surprising that the physiological effects of SAA are pleiotropic. For example, SAA acts as a priming agent of neutrophils [19], induces production of cytokines such as TNFα and IL-1β in immune cells [8,9,20] and induces antimicrobial effector functions [17]. SAA has also been reported to have anti-inflammatory effects, such as inhibition of oxidative burst in neutrophils [21,22], and stimulation of production of anti-inflammatory cytokines such as IL-10 [8,9].

In fish SAA was first identified in salmonids [23]. Thereafter, it has been identified in several bony fish species including carp (*Cyprinus caprio*) [24], rainbow trout (*Oncorhynchus mykiss*) [3], zebrafish (*Danrio rerio*) [25] atlantic cod (*Gadus morhua* L.) [26] (*Epinephelus coioides*) [27] Asian Seabass (*Lates calcarifer*) [28], and rock bream (*Oplegnathus fasciatus*) [29]. Up-regulation of SAA has been observed in various fish species in response to numerous chemical stimuli and pathogens, supporting evidence that SAA may play an important role in immunomodulation, like it does in

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mammals. However, there is limited information on the functional roles of SAA in fish. In this study, we report on the molecular and functional characterization of goldfish SAA. Goldfish SAA constitutive mRNA levels were measured in normal goldfish tissues, and in different immune cell populations. The gene expression of pro- and anti-inflammatory cytokines was examined in monocytes and macrophages treated with recombinant goldfish SAA (rgSAA). Additionally, we examined whether the rgSAA modulated antimicrobial responses in cultured goldfish monocytes and macrophages.

2. Materials and methods

2.1. Fish

Goldfish (*Carassius auratus L.*) (3–6 cm in length) were purchased from Aquatic Imports (Calgary, Alberta, Canada) and maintained in the Aquatics Facility in the Biological Sciences Building at the University of Alberta. Fish were kept in a continuous flow-through water system at 20 °C, on a stimulated natural photoperiod (14 h light, 10 h dark), and fed trout pellets daily until satiated. Fish were acclimated for at least three weeks prior to use in experiments. Prior to handling or manipulation, fish were sedated with TMS (tricaine methane sulfonate; 40 mg/L solution). The care of experimental animals followed guidelines of the Canadian Council of Animal Care (CCAC-Canada) and University of Alberta Animal Ethics protocol AUP00000069.

2.2. Trypanosoma carassii and infection of goldfish

T. carassii strain TrCa (syn. *Trypanosoma danilewskyi*) was used in this study. Parasites were cultured *in vitro* in TDL-15 medium supplemented with 10% heat-inactivated goldfish serum at 20 °C as previously described [30,31]. Fish were infected with *T. carassii* as previously described [32] with an additional infection dose of 1×10^7 of *in vitro* grown parasites. Collection and enumeration of parasitemia was accomplished as previously described [30,33]. Number of parasites per mL was log transformed and presented as mean ± SEM parasites per mL of blood of 6 fish per experimental group. Preparation of cDNA from goldfish tissues and quantitative PCR thermocycling conditions were previously described [32].

2.3. Isolation of goldfish leukocytes

2.3.1. Isolation of goldfish primary kidney macrophage (PKM) and splenocytes

The procedures for the isolation and cultivation of primary kidney macrophages (PKM) [34,35] and splenocytes [36] have been previously described. Briefly, the medium (NMGFL-15) used for culturing contained 100 U/mL penicillin/100 μg/mL streptomycin (Invitrogen), 100 μg/mL gentamycin (Gibco), 10% newborn calf serum (NCS: Hyclone, Loan, UT), and 5% carp serum. Splenocyte cultures were re-suspended in complete medium prior to use. PKM cultures were established by seeding freshly isolated kidney leukocytes into 75 cm^2 tissue culture flask containing 15 mL complete medium and 5 mL of cell-conditioned medium (CCM) from previous flasks. The PKM cultures consisted of a heterogeneous population of cells, which include early progenitor cells, monocytes, and mature macrophages. Day 3–4 PKM cultures consisted primarily of monocytes, whereas older cultures (6–8 days) consisted primarily of mature macrophages. In this paper, day 3–4 PKM cultures are referred to as monocytes and day 6–8 PKM cultures are referred to as macrophages. These designations are based on previously established parameters, day 6–8 PKM cultures were shown to contain mature macrophage and day 3–4 PKM cultures contained monocytes based on internal complexity and size using the FacsAria flow cytometer (Becton Dickson) [34,35].

2.3.2. Goldfish kidney neutrophils

The procedure for isolation of kidney neutrophils has been previously described [37]. Briefly, kidneys were aseptically removed from fish and passed through steel screens, homogenates collected, and layered on a 51% Percoll solution, and centrifuged at 400× *g* for 25 min. The pellet containing red blood cells and kidney-derived granulocytes were re-suspended in 1× red blood cell lysis buffer (144 mM NH_4Cl, 17 mM Tris, pH 7.2) to lyse the red blood cells. The cells were washed twice in NMGFL-15 (230× *g* for 10 min) to collect neutrophils.

2.3.3. Goldfish peripheral blood leucocytes (PBLs)

The procedure for the isolation of PBLs has been previously described [36]. Blood was collected from the caudal vein of individual fish using heparinized needles and suspended in NMGFL-15 containing 100 U/mL penicillin/100 μg/mL streptomycin. PBLs were pelleted by centrifugation at 400× *g* for 10 min, and the red blood cells were lysed using 1× red blood cell lysis buffer (144 mM NH_4Cl, 17 mM Tris, pH 7.2) for 30–40 min on ice. The sample was washed twice in NMGFL-15 (230× *g* for 10 min) to collect PBLs.

2.4. DNA sequencing and in silico analyses of goldfish SAA

Primers were designed based on the nucleotide sequences against the SAA gene of common carp (Accession No. AB016524.1), and zebrafish (Accession No. NM_001005599.1). RACE PCR (Clonetech, USA) was performed to obtain a full open reading frame and untranslated 5′ and 3′ sequences. All amplicons were gel purified using the QIA Gel Extraction kit (Qiagen) and cloned into pJET1.2/blunt cloning vector (Fermantas). Colony PCR was performed to identify positive clones using vector specific sequencing primers, plasmids isolated using QIAprep spin Miniprepkit (Qiagen) and sequenced using the DYEnamic ET terminator cycle sequencing kit and a PE Applied Biosystems 377 automated sequencer. Sequences were analyzed using 4peaks software (http://mekentosj.com/4peaks/) and BLAST programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Fig. 1 and Suppl. Fig. S1). The phylogenetic analysis was done using CLUSTAL-W program and unrooted phylogenetic tree constructed using neighbor-joining methods of the MEGA 5 software (Suppl. Fig. S2). The complete list of primers used for homology-based PCR, RACE PCR and Q-PCR (Suppl. Table S1).

2.5. Prokaryotic expression of recombinant goldfish SAA (rgSAA)

PCR fragment encoding the mature, signal-cleaved goldfish SAA was amplified using primers that allowed cloning into pET SUMO expression vector (Invitrogen). PCR products were gel purified (QIAquick Gel Extraction Kit, Qiagen), ligated into pET SUMO expression vector (Invitrogen), and transformed into competent *Escherichia coli* (NEB10, Biolabs), plated onto LB-kanamycin plates (50 μg/mL) and incubated at 37 °C overnight. Positive clones were identified by colony PCR, cultured in LB-kanamycin, and plasmid DNA purified from recombinant clones using QIAprep spin Miniprepkit (Qiagen). To verify that the insert was in the correction orientation and frame, the purified plasmids were sequenced using vector specific primers. The pET SUMO vector has an N-terminal 6XHis tag for purification and detection of the recombinant molecules.