Developmental and Comparative Immunology (2008) **32**, 1160–1169

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Serum amyloid A: A typical acute-phase reactant in rainbow trout?

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Received 11 October 2007; received in revised form 6 March 2008; accepted 7 March 2008
Available online 7 April 2008

KEYWORDS
Oncorhynchus mykiss;
CpG ODN;
Flavobacterium psychrophilum;
LPS;
Innate immunity;
Head kidney;
Epithelia;
Recombinant trout SAA;
Interrenal cell

Summary
Acute serum amyloid A (A-SAA) has been considered a major acute-phase reactant and an effector of innate immunity in all vertebrates. The work presented here shows that the expression of A-SAA is strongly induced in a wide variety of immune-relevant tissues in rainbow trout, either naturally infected with *Flavobacterium psychrophilum* or challenged with lipopolysaccharide (LPS) or CpG oligonucleotides (CpG ODN). Nevertheless, A-SAA was undetectable by Western blot either in the plasma or in high-density lipoprotein (HDL) of infected or challenged fish, using either an anti-mouse SAA1 IgG or an anti-trout A-SAA peptide serum, which recognise both the intact recombinant trout A-SAA and fragments derived from it. However, the anti-peptide serum was the immunoreactive in all primary defence barriers and in mononuclear cells of head kidney, spleen and liver. These findings reveal that, unlike mammalian SAA, trout A-SAA does not increase significantly in the plasma of diseased fish, suggesting it is more likely to be involved in local defence.

Introduction

Serum amyloid A (SAA) is a family of proteins that comprises acute-phase and constitutive members, both of which are synthesised in the liver under pro-inflammatory conditions. While constitutive SAA (C-SAA) has been identified only in human and mouse, acute-phase SAA (A-SAA) is found in all the vertebrates investigated [1]. Although the liver is the primary site of A-SAA and C-SAA synthesis, extrahepatic production of both SAAs has been also reported in animal models and cell culture experiments of several mammalian

Abbreviations: acute serum amyloid A, A-SAA; pathogen-associated molecular pattern, PAMP; acute-phase reactant, APR; CpG oligodeoxynucleotide, CpG ODN; glutathione–S-transferase, GST; high-density lipoprotein, HDL; head kidney leukocytes, HKL.

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doi:10.1016/j.dci.2008.03.004

species and chicken [2–4]. A-SAA is a 12 kDa major acute-phase reactant (APR), showing up to 1000-fold increase in human plasma during inflammation [5] and its hepatic synthesis is induced by inflammatory cytokines, mainly interleukins 1 and 6, and tumor necrosis factor [6]. The physiological functions of A-SAA, which constitutes a component of the HDL_3 complex in humans, remain unclear; however, the protein appears to play an important role in lipid handling by macrophages [7–9] and has been shown to displace apolipoprotein A-I (ApoA-I) from circulating HDL particles during acute phase and to suffer a coordinated but inverse transcriptional regulation with respect to *apoA-I* expression in mouse hepatocytes [10]. A-SAA is also involved in diverse defensive functions, such as induction of cytokine synthesis, leukocyte recruitment, activation of epithelial immunity, neutrophil priming, opsonisation of Gram-negative bacteria, activation of antimicrobial functions in polymorphonuclear cells and antiviral activity [11–18].

In teleosts, a few studies report the induction of *a-saa* gene transcription in liver and hepatocytes of salmonids [19–21] and more recently, in lipopolysaccharide (LPS)-stimulated rainbow trout macrophages [22] and in the liver and skin of parasite-infected carp [23]. Surprisingly, several studies that have used proteomic approaches to study the acute-phase response in fish, have failed to unequivocally demonstrate the presence of A-SAA protein in the plasma of these fish, while other major acute-phase proteins such as the pentraxins, C-reactive-like protein and serum amyloid P (SAP), have been easily detected in most of them [19,21,24,25].

Due to their immature immune system, juvenile trout are highly susceptible to bacterial and viral diseases (e.g. cold-water disease caused by the Gram-negative bacterium *Flavobacterium psychrophilum*); therefore, early challenges with low doses of different pathogen-associated molecular patterns (PAMPs) such as LPS or CpG ODNs, which participate as ligands of Toll-like receptors, engaged in mounting the innate immune response, have been suggested as an alternative strategy to vaccination, in protecting fish during this period [26].

Accordingly, the aims of this study were to establish the *a-saa* gene expression pattern in a variety of extrahepatic tissues, especially those involved in innate immunity, under normal, pathogen- and PAMP-challenged conditions, and also to assess the presence of A-SAA protein in the plasma and tissues of these fish.

Materials and methods

Fish

Non-vaccinated rainbow trout (*Oncorhynchus mykiss*) were obtained from different fish farms (Cultivos Marinos Chiloe and Granja Marina Tornagaleones, Province of Valdivia, Chile). Healthy controls, infected and challenged fish (10–20 g) were maintained in separate 4000 l flow-through tanks with aerated well water at $15 \pm 1\ ^{\circ}C$ under an 11-h D: 13-h L photo-period. When needed, fish were sacrificed by benzocaine overdose ($250\ mg\ l^{-1}$) following the ethical guidelines for the use of animals in research from the University Committee and dissected to obtain tissue samples for immunohistochemistry and RNA extraction. Trout affected by cold-water disease were obtained from salmon farms and *F. psychrophilum* infection was confirmed by PCR and serology in the Ictiopathology Laboratory of the Universidad Austral de Chile.

Fish challenge with PAMPs

Different groups of eight trout were challenged by intraperitoneal injection (0.1 ml) of a single dose of *Escherichia coli* O26:B6 LPS ($2.5\ mg\ kg^{-1}$, Sigma-Aldrich, Milwaukee, USA), CpG ODN 1668 (*TLR*grade™ Alexis Biochemicals) $0.25\ mg\ kg^{-1}$ prepared in endotoxin-free ($<0.005\ EU\ ml^{-1}$) water (HyClone®) or sterile LPS-free phosphate-buffered saline (PBS), then sacrificed 1, 2, 4 or 7 days later, to obtain tissues for expression studies. The CpG ODN 1668 used has a phosphorothioate backbone for increased stability and contains only one unmethylated CpG motif. Blood samples were extracted from the caudal vein of anaesthetised LPS-challenged fish ($50\ mg\ l^{-1}$ benzocaine) with a heparinised syringe and plasma samples were obtained after centrifugation at 1500*g* for 3 min (twice) to assure the complete removal of blood cells, and after addition of protease inhibitors (1 mM PMSF and 2 mM benzamidine), kept in aliquots at $-20\ ^{\circ}C$. Plasma HDL fractions were isolated from whole plasma of LPS-challenged fish and mice by affinity chromatography on Affi-Gel® Blue (Bio-Rad), as described by Villarroel et al. [27].

For *in vitro* expression studies, head kidney leukocytes (HKL) were isolated from control trout, essentially as described by Anderson et al. [28]. Briefly, organs were resuspended in 0.97% (w/v) Hank's salt solution supplemented with $10\ U\ ml^{-1}$ heparin and 2% (v/v) foetal calf serum (FCS) and passed through a 100-mesh nylon cell strainer (Falcon). The resulting suspension was placed on a 31/46% discontinuous Percoll gradient and centrifuged at 400*g* for 40 min. Finally, leukocytes collected from the interphase were carefully obtained, suspended and washed twice in L-15 medium and viability tested by exclusion of trypan blue dye. Over 95% viable preparations were suspended in L-15 medium supplemented with 10% FCS, $100\ mg\ ml^{-1}$ of streptomycin and $60\ mg\ ml^{-1}$ penicillin and then seeded on 6-well cell culture Nunc® plates (2.5×10^6 cells per well). After 18 h of culture, non-adherent cells were removed and the remaining cells incubated in L-15 medium supplemented with 10% FCS, containing either $25\ \mu g\ ml^{-1}$ *E. coli* LPS or without LPS for 1 h at $16\ ^{\circ}C$. Finally total RNA was obtained for expression assays as described in Section 2.5.

Antiserum preparation

Based on the considerable sequence conservation of some regions of the predicted A-SAA protein between mammals and salmonids, and on the epitopes of human SAA1 that were shown to correspond to good immunogens [29], an 18-residue peptide (ANWKNSDKYFHARGNYDA) was synthesised and conjugated to keyhole limpet hemocyanin (KLH) to raise an antiserum against trout A-SAA in rabbit. Antiserum was prepared as previously described [30]. Briefly, a ratio of 1 mole of peptide per each 50 amino acid residues of KLH was used to prepare the conjugate.