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The bacterial exotoxin AIP56 induces fish macrophage and neutrophil apoptosis using mechanisms of the extrinsic and intrinsic pathways

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ABSTRACT

It has been previously shown that the exotoxin of the important fish pathogen *Photobacterium damselae* ssp. *piscicida* is a key pathogenicity factor and is responsible for the extensive systemic apoptosis of macrophages and neutrophils seen in acute fish photobacteriosis. The focus of the present study was to further characterize the AIP56-induced apoptosis of sea bass professional phagocytes by assessing the involvement of caspases, mitochondria and oxidative stress. The resulting data indicate that the apoptotic response in peritoneal macrophages and neutrophils treated *ex vivo* with AIP56 involves activation of caspase-8, -9 and -3, and mitochondria as shown by loss of mitochondrial membrane potential, release of cytochrome c and over-production of ROS. These results together with previous data from this laboratory suggest that both the extrinsic and intrinsic apoptotic pathways are involved in the AIP56-induced phagocyte apoptosis.

1. Introduction

Apoptosis is an evolutionary conserved, genetically controlled process of active cell death that plays an important role in several physiological processes, but which can also be used by pathogens to subvert the host immune defences and to produce pathology. Several examples of bacterial pathogens which induce pathogenic apoptosis of phagocytes have been described (reviewed in refs [1–3]); these bacteria exert their apoptogenic activity using either cytotoxins directly injected into the host cells through secretion systems, as is the case of Ipa of *Shigella flexneri* [4], SipB of *Salmonella* spp. [5] and YopP/J of *Yersinia* [6,7], or exotoxins that do not require direct contact of the pathogen with the target cells [8].

Photobacterium damselae ssp. *piscicida* (*Phdp*) is a Gram-negative pathogen responsible for acute fish photobacteriosis, a very serious bacterial septicaemia. This infection is one of the most threatening bacterial diseases in mariculture worldwide due to its wide host range, massive mortality, ubiquitous geographical distribution, widespread antibiotic resistance and lack of efficient vaccines [9]. Our previous results showed that, in experimental and natural photobacteriosis, virulent *Phdp* strains induce extensive systemic apoptosis of macrophages and neutrophils in the tested teleosts [10,11]. The factor responsible for the apoptogenic activity of *Phdp* towards fish phagocytes has been isolated, purified and identified; this factor is a novel plasmid-encoded apoptosis-inducing protein of 56 kDa (AIP56) secreted by the virulent strains of *Phdp* [12,13]. AIP56 is a exotoxin that operates as the key virulence factor of *Phdp* [11,12,14]. The AIP56-induced apoptotic destruction of both professional phagocytic cells has been observed both *in vivo* and in an *ex vivo* model [10,12]. By affecting both macrophages and neutrophils, the pathogen is able to evade the crucial host phagocytic defences, allowing the unrestricted, massive extracellular multiplication of Phdp. The disseminated phagocyte apoptosis induced by *Phdp* was found to proceed to apoptotic secondary necrosis, which results in extensive lysis of the phagocytes with release of cytotoxic molecules, including neutrophil elastase [11]; therefore, this pathogenicity mechanism, besides impairing the host phagocytic defence, directly participates in the production of the severe infection-associated pathology of photobacteriosis.

Fish possess apoptotic pathways equivalent to those of mammals [15] and present several advantages as models for the study of apoptosis in health and disease including in infection [16]. The study of the mechanism of the apoptotic process used by AIP56 to destroy the two professional phagocytes of susceptible fish is relevant in the context of the emerging theme of apoptosis-associated microbial pathogenicity [1–3]. The objective of the present study was to characterize the pathways by which AIP56 induces apoptosis in sea bass macrophages and neutrophils. The results

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presented here disclose novel information on the interaction of AIP56 with those phagocytes and therefore greatly contribute to the better understanding of this important pathogenicity mechanism.

2. Materials and methods

2.1. Experimental fish

Sea bass (*Dicentrarchus labrax*) were purchased from a commercial fish farm and maintained in recirculating aerated seawater, at 22 °C. Water quality was maintained with mechanical and biological filtration and ozone-disinfection and the fish were fed *ad libitum* on commercial pellets. All work was carried out in accordance with the EC Directive 86/609/EEC for animal experiments.

2.2. Preparation of recombinant AIP56

Recombinant AIP56 (rAIP56) was purified by affinity chromatography from the soluble fraction of transformed *Escherichia coli* BL21(DE3) cells containing the plasmid pETAIP56H+, as described elsewhere [12]. The concentration of rAIP56 was determined by densitometry [12]. Recombinant protein was stored in 20 mM Tris–HCl, pH 8.0 at −80 °C until use. For one experiment, rAIP56 was heat inactivated at 60 °C for 1 h.

2.3. Cells

The cells used in the *ex vivo* studies were peritoneal leukocytes collected from inflamed peritoneal cavities of fish weighing between 100 and 300 g. Inflammatory peritoneal exudates were induced with the intraperitoneal injection of 100 μl of Incomplete Freund's Adjuvant 15 days before collection. Peritoneal leukocytes were collected as previously described [17] using L-15 medium (Gibco) adjusted to 322 mOsm and supplemented with 10% inactivated (60 °C, 40 min) fetal bovine serum (FBS, Gibco), 1% penicillin/streptomycin (P/S, Gibco) and 20 U ml^{-1} heparin (Braun). Unless otherwise stated cell suspensions were adjusted to 1×10^6 cells ml^{-1}. For routine leukocyte morphological analysis and labelling of phagocytes, cytospin preparations were obtained. Due to the different fixation needed for the enzymatic labelling of the phagocytes this had to be done separately. Neutrophils were labelled following fixation (10% of 37% formaldehyde in absolute ethanol; 45 s) by peroxidase detection using the Antonow's technique [17,18]. Macrophages were fixed with Baker's 10% neutral formol and labelled by α-naphtyhl acetate esterase (ANAE) activity [19,20].

2.4. Cell treatment with rAIP56

Peritoneal leukocytes at the cell density of 1×10^6 cells ml^{-1} were incubated with 2 μg ml^{-1} rAIP56 at 22 °C for a maximum of 4 h. The 2 μg ml^{-1} dose was chosen following previous studies performed in our laboratory were recombinant protein in the range of 0.1–10 μg ml^{-1} was tested for apoptogenic activity in these cells. To obtain a sufficient amount of cellular proteins for western blot analysis 1×10^7 cells ml^{-1} were used.

2.5. Determination of the apoptogenic activity of rAIP56

The apoptogenic activity of AIP56 towards peritoneal leukocytes was assessed in phagocytes incubated *ex vivo* with 2 μg ml^{-1} rAIP56, using a set of indicators, which allows for the safe identification of apoptosis [12,21–24]. These indicators included morphological alterations (cell rounding and shrinkage, nuclear fragmentation and/or chromatin condensation, cell blebbing and formation of apoptotic bodies), and activation of caspase-3. Microscopic quantification of cells was done in a blinded fashion by scoring a total of 200 cells per slide.

2.6. Immunodetection of activated caspase-3

Detection of activated caspase-3 was performed by immunocytochemistry on cytospin preparations of rAIP56-treated cells using a 1:500 dilution of an affinity-purified rabbit antibody as described previously [14].

2.7. Flow cytometric analysis (FC)

FC analysis was performed on a BD FacsSort (Becton Dickinson Biosciences, San Jose, CA) flow cytometer equiped with a 488 nm blue laser. Red fluorescence was collected through a 585/42 nm detection filter and green fluorescence was collected through a 530/30 nm detection filter. Ten thousand cells per sample were analysed at low flow rate. All data were analysed using FlowJo software (San Diego, CA).

2.8. Caspase activity assays

Caspase-3 activity was determined using a commercial fluorimetric caspase-3 assay kit (Sigma–Aldrich), following the manufacturer's instructions. Briefly, soluble extracts were prepared from 1×10^6 peritoneal leukocytes by incubation with 33 μl of lysis buffer (50 mM HEPES pH 7.4, 5 mM CHAPS and 5 mM DTT) for 20 min on ice. Cellular debris was eliminated by centrifugation and 18 μl of the supernatant was assayed for caspase-3 activity as described previously [12].

Activation of caspase-8 and -9 were measured using the substrates Ac-IETD-pNA and Ac-LEHD-pNA from Caspase-Glo™ 8 and 9 assay kits (Promega), respectively, according to the manufacturer's instructions. Briefly, an equal volume of cell suspension and Caspase-Glo® reagents were added to the wells of white-walled 96-well plates and incubated at room temperature (RT) for 10 min. The luminescence of each sample was measured in a plate-reading luminometer (Spectramax Gemini XS, Molecular Devices), with the resulting luminescent signal being directly proportional to the amount of caspase activity present in the sample. Results for caspase activities were expressed as relative fluorescence units (RFU) for caspase-3 activity and in relative luminescence units (RLU) for caspases-8 and -9 activities.

2.9. Caspase inhibition assays

In order to assess the involvement of caspases in rAIP56-induced apoptosis of peritoneal leukocytes, cells were pre-incubated for 1 h with 25 μM of the general caspase inhibitor Z-VAD-FMK (from a 50 mM stock in DMSO), 25 μM Z-IETD-FMK, a caspase-8 inhibitor and with 25 μM Z-LEHD-FMK, a caspase-9 inhibitor (both from 20 mM stocks in DMSO), before stimulation with rAIP56. Control cells consisted of untreated cells and cells incubated with same amount of DMSO as the inhibitor-treated. At specific times, cytospins were done for morphological analysis and cells were collected for caspase-3 activity determination as described above.

2.10. Mitochondrial membrane potential

Mitochondrial function/integrity was evaluated by FC using the fluorescent dye rhodamine 123 (Rh123, Molecular Probes), a lipophilic cationic fluorochrome which is incorporated by