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Short communication

A synthetic peptide derived from the D1 domain of flagellin induced the expression of proinflammatory cytokines in fish macrophages

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ABSTRACT

Flagellin is the main protein component of flagellum in Gram negative and positive bacteria, and it is also the ligand that activates the Toll-like receptor 5 (TLR5) in fish and mammals. In higher vertebrates, flagellin induces the activation of the membrane-bound TLR5 (TLR5M), which promotes the expression of proinflammatory cytokines and chemokines, and other immunological functions. We have previously reported that recombinant flagellin from *Vibrio anguillarum* and its ND1 domain are able to upregulate the expression of genes encoding major the proinflammatory mediators in gilthead seabream and rainbow trout macrophages. Considering the key role of D1 domain of flagellin for binding to TLR5M and its immunostimulatory activity, we designed and chemically synthesized a peptide derived of this region. The effects of the synthetic peptide were evaluated *in vitro* using head kidney macrophages from gilthead seabream (*Sparus aurata* L., Perciformes, Sparidae) and rainbow trout (*Oncorhynchus mykiss* W., Salmoniformes, Salmonidae). In both species the expression of genes encoding the proinflammatory cytokines interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α), and the chemokine IL-8, was induced upon stimulation of macrophages with the D1 domain synthetic peptide. IL-1β and IL-8 were the most upregulated genes and to a lesser extent TNF-α. Interestingly, however, the induction activity of the synthetic peptide was higher in gilthead seabream than in rainbow trout macrophages. The results were confirmed at the protein levels for IL-8. Collectively, these results suggest that synthetic peptide derived from flagelling could be a promising approach for the immunostimulation and vaccination of farmed fish.

1. Introduction

Flagellin is a pathogen associated molecular pattern (PAMP), which is recognized by the pattern recognition receptor (PRRs) called toll-like receptor 5 (TLR5) [1]. The recognition of PAMPs by TLRs allows the innate immune system to distinguish between the target molecules present in different classes of pathogens and co-ordinate the immune response, triggering the differential induction of cytokines, co-stimulatory molecules, and the repertoire of TLRs

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expressed in different cells depending on the type of PAMP and the types of cells stimulated [2]. Flagellin is the main protein component of the flagellum in Gram positive and negative bacteria and it is one of the most powerful PAMPs, can activate a broad range of cell types within the innate and adaptative immune system to promote cytokine production [3,4].

Sequence comparisons have revealed that the flagellin proteins of different bacterial species are conserved in the N- and C-terminal of the D1 domain, ND1 and CD1, respectively, and these regions are responsible for the immunostimulatory activity of flagellin [5–7]. This domain is formed by three α-helices (ND1a, ND1b, and CD1) that fold to form a super-secondary structure [8,9]. Previous reports in FliC flagellin from *Salmonella enterica* serovar *Typhimurium* have shown the relevance of 13 amino-acid residues of the D1 domain essentials for the activation of TLR5 in mammals [10]. More precisely, three amino acids located in ND1 domain of FliC flagellin from *S. Typhimurium* (L89, Q90, L94), seems to be essential for the activation of TLR5 in mammals [11]. On basis of this information, the present study proposes the use of synthetic peptides as a tool to mimic the effects of flagellin during the immune regulation in fish. Thus, one peptide was designed considering the essential conserved residues in the sequence of D1 domain of flagellin B from the fish pathogenic bacterium *Vibrio anguillarum*. The activity of this peptide was evaluated *in vitro* using macrophages from two phylogenetically distant teleost species, the gilthead seabream (*Sparus aurata* L., Sparidae) and the rainbow trout (*Oncorhynchus mykiss* W, Salmonidae). The results showed an upregulation on the levels of expression of genes encoding major proinflammatory cytokines, namely IL-1β and TNF-α, and a pivotal chemokine, i.e. IL-8, involved in the innate immune response. This contribution is the first step for considering the use of synthetic peptide as a tool for providing immunostimulatory candidates for use in aquaculture.

2. Materials and methods

2.1. Peptide design

Peptide 518 was designed from the sequence of flagellin from *V. anguillarum* (Access Nr. UniProtKB/Swiss-Prot: Q56572.3), using ClustalW alignement. Amino acids described in *Salmonella typhimurium* flagellin (Access Nr. UniProtKB/Swiss-Prot: P06179.4) as responsible of mammal TLR5 activation were identified in the regions L89 – E115 and I293 – R313 of *V. anguillarum* flagellin. Both sequences were chemically synthesized and linked through two proline residues. The three-dimensional structure of the peptide was generated by overlapping in the model structure of *V. anguillarum* flagellin obtained from *S. typhimurium* flagellin (1UCU).

2.2. Peptide synthesis, purification and characterization

The selected peptide was chemically synthesized by solid-phase peptide synthesis on a 433A Peptide Synthesizer (Applied Biosystems) using resin matrix 0.4 [12] and Fmoc-protected amino acids. The peptide was cleaved and final deprotection was performed with trifluoroacetic acid: (TFA/H_2O/triisopropylsilane/ethanedithiol) (92.5:2.5:2.5:2.5) (v/v) for 90 min at room temperature. The peptide was precipitated with diethyl ether, extracted with water and purified by RP-HPLC to a final value higher than 95%, lyophilized and analysed by MALDI-TOF to confirm its molecular weight. Additionally, circular dichroism (CD) spectroscopy was carried out in a JASCO J-815 CD Spectrometer coupled to a Peltier JASCO CDF-426S/15 for temperature control (Jasco Corp., Tokyo, Japan) in the far ultra-violet (UV) range (190–250 nm) using quartz cuvettes of 0.1 cm path length and 1 nm bandwidth at 0.1 nm resolution. The spectrum was recorded as an average of four scan repetitions in continuous scanning mode with 50 nm/min scanning speed and a response time of 1 s (Figure supplementary 1A and B).

2.3. Fish maintenance

Gilthead seabream specimens with a mean mass of 150 g were kept at the Oceanographic Centre of Murcia (Spain) in a 14 m^3 aquarium maintained with running seawater (6 ppm dissolved oxygen and a flow rate of 20% aquarium vol/h) under natural temperature and photoperiod. The gilthead seabream were fed twice daily with a commercial pellet diet (Trouvit, Burgos, Spain). Rainbow trout approximately 8–10 cm in length obtained from Lillogen (Leon, Spain), were kept at the Miguel Hernández University (Spain) (UMH) in an aquarium maintained between 12 and 14 °C and were fed daily with a commercial diet (Trouw, Leon, Spain). The fish were fasted for 24 h before sampling. All experiments complied with the guidelines established for the use of laboratory animals by the European Union Council (86/609/EU) and the Bioethical Committee of the University of Murcia (Approval #537/2011).

2.4. Cell culture and treatments

Head kidney leukocytes of rainbow trout were isolated following the method previously described by Secombes [13]. Briefly, fish were sacrificed by overexposure to MS-222 and the anterior kidney removed aseptically and passed through a 100 μm nylon mesh using Leibovitz medium (L-15, Gibco) supplemented with penicillin (100 IU ml^{-1}), streptomycin (100 μg ml^{-1}), heparin (10 units ml^{-1}) and 2% foetal calf serum (FCS). The resulting cell suspension was placed onto Percoll gradients with a density of 51%/34% and then centrifuged at 500 × g for 30 min at 4 °C. The interface cells were collected and washed twice and centrifuged at 500 × g for 5 min in L-15 containing 0.1% FCS. Viable cell concentration was determined by Trypan blue exclusion, and the cells were resuspended in L-15 with 5% FCS. The cells were dispensed into 24-well plates at a concentration of 5 × 10^5 cells ml^{-1} for extraction of total RNA and also into 12-well plates at a concentration of 1.5 × 10^5 cells ml^{-1} for extraction of total proteins. Seabream head kidney leukocytes obtained as described by Sepulcre et al. (2002) [14] and maintained in sRPMI [PRMI 1640 culture medium (Gibco, Madrid, Spain)] adjusted to gilthead seabream serum osmolarity (353.33 mOs with 0.35% NaCl) and supplemented with 5% FCS (Gibco), 100 I.U. ml^{-1} penicillin, and 100 mg ml^{-1} streptomycin (Biochrom, Cambridge, UK). Macrophage monolayers were obtained after overnight culturing. Adherent cells were exposed for 3 h to different concentrations of peptide 518. Bs-Fla, Flagellin of *Bacillus subtilis* (Invivogen) and VarFla, recombinant flagellin of *V. anguillarum* [15] were used as positive controls. After 3 or 6 h of incubation, leucocytes were washed and total protein or total RNA were extracted from the cells. Total protein was quantified by the bicinchoninic acid method (Pierce BCA® kit) [16] and used for detection of IL-8 by ELISA.

To exclude non-specific effects by chemical contaminants from peptide synthesis and confirm the importance of the 13 amino acid residues conserved. A control peptide (518C) was synthetized with all of the 13 amino acid mutated to alanine (Fig. S1B). This peptide was used to stimulate isolated cells and evaluate the ability to induce the overexpression of proinflammatory genes.

2.5. RNA-extraction and cDNA synthesis

Total RNA was extracted from macrophages monolayer with TRIzol Reagent (Invitrogen), following the manufacturer's