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Short communication

SmCCL19, a CC chemokine of turbot *Scophthalmus maximus*, induces leukocyte trafficking and promotes anti-viral and anti-bacterial defense

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ABSTRACT

Chemokines are classified into several different subfamilies, of which CC chemokines constitute the largest subfamily in teleost. The prominent structural characteristic of CC chemokines is the presence of an Asp-Cys-Cys-Leu (DCCL) motif. To date, cDNA sequences of several CC chemokines have been identified in turbot (*Scophthalmus maximus*), however, the activity and function of these putative chemokines remain unknown. In this study, we examined the biological effect of the turbot CC chemokine SmCCL19, which has been previously reported as KC70 and shown to be regulated in expression by bacterial infection. To facilitate functional analysis, recombinant SmCCL19 (rSmCCL19) and a mutant form of SmCCL19, SmCCL19M, that bears serine substitutions at the two cysteine residues of the DCCL motif were purified from *Escherichia coli*. Chemotactic analysis showed that rSmCCL19 induced migration of head kidney leukocytes in a dose-dependent manner, whereas rSmCCL19M caused no apparent cellular migration. To examine the *in vivo* effect of rSmCCL19, turbot were administered with rSmCCL19 or rSmCCL19M before being inoculated with viral and bacterial pathogens. Subsequent tissue infection analysis showed that the viral and bacterial loads in rSmCCL19-adminsitered fish were significantly reduced, whereas the pathogen loads in rSmCCL19M-adminsitered fish were largely comparable to those in the control fish. Consistent with these observations, significant inductions of immune relevant genes were observed in rSmCCL19-adminsitered fish but not in rSmCCL19M-adminsitered fish. Taken together, these results indicate that SmCCL19 recruits leukocytes and augments host immune defense in a manner that depends on the conserved DCCL motif.

1. Introduction

Chemokines are a family of cytokines that regulate the migration and immune response of leukocytes [1,2]. These proteins have molecular weights ranging between 8 and 10 kDa and in general possess four highly conserved cysteine residues that form two intramolecular disulphide linkages [3]. According to the number and spacing of the first two conserved cysteines, chemokines are classified into CXC(α), CC(β), C (γ), and CX_3C (δ) subfamilies [4–6]. In addition, a fifth subfamily called CX has recently been discovered in zebrafish, which lacks one of the two N-terminal conserved cysteine residues but retains the third and fourth cysteine residues [7]. In teleost, CC chemokines are most abundant, and at least 30 different members have been identified [8–11]. CC chemokines in fish are suggested to be divided into seven groups named CCL19/21/25, CCL20, CCL27/28, CCL17/22, the fish-specific group, the MIP group, and the MCP group [12]. Chemotactic activity associated with CC chemokines has been reported in a few teleost species including rainbow trout, large yellow croaker, zebrafish, Japanese flounder, and tongue sole [13–17].

Turbot (*Scophthalmus maximus*) is a marine flatfish cultured worldwide. In China, it is one of the major farmed species in the north regions. The high economic value of turbot has led to increased studies on its immune relevant genes. In 2010, Chen et al. reported the cloning and identification of a CC chemokine from turbot, which they named KC70 [18]. They found that KC70 belongs to the mammalian CCL19 group and possesses the conserved Asp-Cys-Cys-Leu (DCCL) motif, which is the signature sequence of CC chemokines. In this study, we renamed KC70 SmCCL19 and examined its chemotactic activity and biological function. Our results

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provide the first evidence that indicates an immunoregulatory function of turbot chemokines.

2. Materials and methods

2.1. Fish

Turbot were purchased from a commercial fish farm in Qingdao, Shandong Province, China, and maintained at 22 °C in aerated seawater. Fish were acclimatized in the laboratory for two weeks before experimental manipulation. Before experiment, fish were randomly sampled for the examination of bacterial recovery from blood, liver, kidney, and spleen, and no bacteria could be detected from any of the examined tissues of the sampled fish. Fish were euthanized with an overdose of tricaine methanesulfonate (Sigma, St. Louis, MO, USA) before tissue collection.

2.2. Plasmid construction

To construct pEtSmCCL19, which expresses SmCCL19, the coding sequence of SmCCL19 without signal sequence was amplified by PCR with primers F1 (5′- CCCGGGATGCAGATTCCCATGGACTGC -3′; underlined sequence, SmaI site) and R1 (5′- CCCGGGCACGTGCTTCACTCTGA -3′; underlined sequence, SmaI site); the PCR products were ligated with pEASYT-T1 Simple (TransGen Biotech, Beijing, China), and the recombinant plasmid was digested with SmaI to retrieve the *SmCCL19* fragment, which was inserted into pET259 [19] at the SwaI site. To construct pEtSmCCL19M, which expresses the mutant SmCCL19 (SmCCL19M) bearing serine substitutions at the two cysteine residues in the DCCL motif, PCR was performed with primers F2 (5′- CCCGGGATGCAGATTCCCATGGACAGCA GCTTGAGCTTC -3′, underlined sequence, SmaI site) and R1, and the PCR products were inserted into pET259 as described above.

2.3. Purification of recombinant protein

Escherichia coli BL21(DE3) (Tiangen, Beijing, China) was transformed separately with pEtSmCCL19 and pEtSmCCL19M. The transformants were cultured in Luria–Bertani broth (LB) medium at 37 °C to an OD_{600} of 0.4, and isopropyl-β-D-thiogalactopyranoside was then added to the culture to a final concentration of 1 mM. After growth at 30 °C for an additional 4 h, His-tagged proteins were purified under denaturing conditions using Ni-NTA Agarose (QIAGEN, Valencia, CA, USA) as recommended by the manufacturer. The proteins were reconstituted as described previously [20]. Briefly, the proteins were dialyzed against the reconstitution buffer containing 50 mM Tris–HCl, 200 mM KCl, 10 mM $MgCl_2$, 10 uM $ZnCl_2$, 5 mM β-mercaptoethanol, 1 mM EDTA, 20% glycerol, and decreasing concentrations of urea (4 M, 2 M, 1 M, 0.5 M, 0.25 M, and 0 M). Reconstituted proteins were eluted in elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole; pH 8.0) and dialyzed overnight against 2 L of PBS. GST was purified from BL21(DE3) transformed with pGEX-4T-1 (GE Healthcare, Piscataway, NJ, USA) as described previously [21]. To remove endotoxin, the proteins were treated with 1% Triton X-114 at 4 °C for 5 min. The mixture was incubated at 37 °C for 45 min. The mixture was then centrifuged at 13,000 *g* for 5 min, and the supernatant was collected for next round of treatment. This process was repeated three times. The purified proteins were dialyzed for 24 h against PBS and concentrated using Amicon Ultra Centrifugal Filter Devices (Millipore, Billerica, MA, USA). The proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized after staining with Coomassie brilliant blue R-250. The concentration of the purified protein was determined using the Bradford method with bovine serum albumin as a standard.

2.4. Leukocyte preparation and chemotaxis analysis

To prepare head kidney leukocytes (HKL), head kidney was taken from turbot (~710 g) under aseptic conditions. The tissues were ground and passed through a sterile 75 μm metal mesh with PBS containing 10% fetal bovine serum (FBS). The cell suspension was centrifuged at 300 *g* for 5 min, and the pelleted cells was washed with PBS containing 10% FBS two times to remove cell debris. The cells were resuspended in PBS containing 10% FBS. Leukocytes were extracted from the cell suspension with 50% Percoll as described previously [17]. Chemotaxis was performed as reported previously [17]. Briefly, rSmCCL19 and rSmCCL19M were diluted in RPMI 1640 medium (Thermo Scientific HyClone, Beijing, China) to 1 ng/ml, 10 ng/ml, and 100 ng/ml. As a control, PBS, in which the purified proteins were dissolved, was similarly diluted. Six hundred microliters of each of the dilutions was applied to the lower chamber of a 24-well Costar Transwell (Corning Costar Co., Cambridge, MA, USA). The upper chamber containing a polycarbonate membrane of 3-μm pore size was placed on top of the lower chamber. One hundred microliters of HKL (~10^5 cells) were added to the upper chamber, and the plate was incubated at 22 °C for 40 min. The number of cells migrated into the lower chamber was counted with a microscope. Chemotactic index was presented as fold increase in the number of migrated cells induced by rSmCCL19 compared to that induced by PBS. The assay was performed four times.

2.5. Effect of rSmCCL19 on bacterial and viral infection

The bacterial pathogen *Edwardsiella tarda* TX1 [22] was cultured in LB medium to mid-logarithmic phase. The cells were washed with PBS and resuspended in PBS. Turbot (average 10.7 g) were divided randomly into groups of equal size ($N = 96$). Turbot in each group were injected intraperitoneally with 20 μg/fish of rSmCCL19, rSmCCL19M, GST, or PBS (control). At 4 h post-injection, the fish in each group were injected intraperitoneally with 1×10^6 CFU of *E. tarda* TX1 or 10^5copies of the megalocytivirus RBIV-C1 [23]. For bacterial infection, kidney was taken aseptically from the fish (three each time) at 12 h, 24 h, and 48 h post-infection. The tissues were homogenized in PBS. The homogenates, after serial dilution, were plated in triplicate on LB agar plates. The plates were incubated at 28 °C for 24 h, and the colonies that appeared on the plates were enumerated. The genetic nature of the colonies was verified by PCR analysis using primers specific to TX1. The PCR products were randomly selected for DNA sequencing to confirm PCR specificity. For viral infection, kidney was taken aseptically from the fish (three each time) at 3 d, 5 d, and 7 d post-infection, and viral copy number in the tissues was determined by absolute quantitative real time PCR as reported previously [23]. The experiments were performed three times.

2.6. Quantitative real time reverse transcription-PCR (qRT-PCR)

Turbot were treated with rSmCCL19, rSmCCL19M, or PBS for 4 h or 24 h as above. Head kidney was taken aseptically from the fish (three from each group) and used for total RNA extraction with E.Z.N.A Total RNA Kit (Omega Bio-Tek Inc., USA). One microgram of total RNA was used for cDNA synthesis with the Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). qRT-PCR was carried out in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) using the SYBR ExScript qRT-PCR Kit (Takara, Dalian, China) as described previously [24]. The immune genes analyzed were interferon regulatory factor (IRF) -1, IRF-3, IRF-5, IRF-7, IRF-8, signal transducers and activators of transcription (STAT)-2, interferon-induced Mx protein (Mx), interleukin (IL)-1β, IL-8, IL-