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A CCL21 chemokine of tongue sole (*Cynoglossus semilaevis*) promotes host resistance against bacterial infection

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ABSTRACT

Chemokines are a large family of chemotactic cytokines. Based on the arrangement of the first two cysteine residues, chemokines are divided into four groups, one of which is the CC chemokine group. In this study, we characterized a CC chemokine, CsCCL21, from half-smooth tongue sole (*Cynoglossus semilaevis*), and analyzed its activity. CsCCL21 contains two conserved N-terminal cysteine residues in a NCCL motif and is phylogenetically related to the CCL19/21/25 subgroup of CC chemokines. CsCCL21 was constitutively expressed in nine tissues and significantly upregulated by bacterial and viral infection. The recombinant CsCCL21 (rCsCCL21) induced migration of peripheral blood leukocytes. When the two conserved cysteine residues in the NCCL motif were mutated, the chemotactic activity of rCsCCL21 was abolished. rCsCCL21 enhanced the resistance of tongue sole against bacterial infection, but the mutant protein with NCCL mutation lacked this antibacterial effect. Taken together, these results suggest that CsCCL21 is a functional CC chemokine with the ability to recruit leukocytes and is involved in antibacterial immunity in a manner that requires the conserved NCCL motif.

1. Introduction

Chemokines are a large family of chemotactic cytokines that are crucial regulators of the immune response, not only promoting cell trafficking of various types of leukocytes, but also regulating immune responses and differentiation of the recruited cells [1–4]. They are small secreted proteins with molecular weight ranging from 8 to 14 kDa [5]. Chemokines have a typical Greek key shape structure stabilized by disulfide bonds [6]. They are defined by the presence of conserved cysteine residues and are divided into four groups namely, CXC (α), CC (β), C (γ) and CX3C (ε), depending on the arrangement of the first two cysteine residues [7,8]. CC chemokines, which have adjacent cysteine residues in a conserved position, currently make up the largest group of chemokines, and 28 CC chemokines have been identified in mammalian species [9].

In teleost, chemokines have been identified in many species [10–16]. The first identified fish CC chemokine was rainbow trout (*Oncorhynchus mykiss*) CK1, which was reported in 1998 [3]. Since then, many CC chemokines have been identified in a number of species, including channel catfish (*Ictalurus punctatus*) and blue catfish (*Ictalurus furcatus*) [17], gilthead seabream (*Sparus aurata*) [2], snakehead murrel (*Channa striatus*) [18], Atlantic cod (*Gadus morhua*) [19], large yellow croaker (*Pseudosciaena crocea*) [20], zebrafish (*Danio rerio*) [21], turbot (*Scophthalmus maximus*) [22], Japanese flounder (*Paralichthys olivaceus*) [23,24], half-smooth tongue sole (*Cynoglossus semilaevis*) [25,26], and rock bream (*Oplegnathus fasciatus*) [27]. Based on phylogenetic analysis, the fish CC chemokines are divided into seven subgroups [28], including the CCL17/22 subgroup, the CCL20 subgroup, the CCL27/28 subgroup, the CCL19/21/25 subgroup, the MIP (macrophage inflammatory protein) subgroup, the MCP (monocyte chemotactic protein) subgroup and the fish CC subgroup. Currently, most studies of fish chemokines have focused on sequence and expression analysis. Except a few studies, the biological function of teleost CC chemokines remains unknown.

Half-smooth tongue sole (*C. semilaevis*) is an important marine flatfish cultured in China. In this study, we report the biological

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activity and *in vivo* function of a CC chemokine, CsCCL21, from tongue sole. We found that CsCCL21 is upregulated at transcription level by bacterial and viral pathogens, and the purified recombinant CsCCL21 is involved in immune response against bacterial infection.

2. Materials and methods

2.1. Experimental animals

Clinically healthy tongue sole (average weight 14.2 g) were purchased from a commercial fish farm in Shandong Province, China and maintained at 20 °C in aerated seawater. Fish were acclimatized in the laboratory and fed daily with commercial dry pellets as reported previously [29]. Before experiment, fish were confirmed to be free of bacterial infection by examination of potential existence of bacteria in the blood, liver, kidney, and spleen of randomly sampled fish [30]. Before tissue collection, fish were euthanized with tricaine methanesulfonate (Sigma, St. Louis, MO, USA) [31].

2.2. Bacteria

The bacterial pathogens *Edwardsiella tarda* and *Vibrio harveyi* have been reported previously [32,33]. *Escherichia coli* BL21(DE3) was purchased from Tiangen (Beijing, China). Bacterial strains were cultured in Luria–Bertani broth (LB) medium either at 37 °C (for *E. coli*) or at 28 °C (for *E. tarda* and *V. harveyi*).

2.3. Sequence analysis

The cDNA sequence of *CsCCL21* has been reported (GenBank Accession No. XP_008334168.1). The amino acid sequence of CsCCL21 was analyzed using the BLAST program at the National Center for Biotechnology Information (NCBI) and the Expert Protein Analysis System. The molecular mass and theoretical isoelectric point (pI) were predicted using EditSeq in DNASTAR software package (DNASTAR Inc. Madison, WI, USA). Multiple sequence alignment was created with the DNAMAN program. Signal peptide search and subcellular localization prediction were performed with SignalP 3.0 and WoLF PSORT, respectively. Phylogenetic analysis was constructed with ClustalX and the Neighbor-joining algorithm of MEGA 6.0.

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

To examine *CsCCL21* by qRT-PCR under normal physiological conditions, tissues (intestine, blood, kidney, spleen, heart, brain, gill, muscle, and liver) were taken aseptically from five tongue sole and used for total RNA extraction with EZNA Total RNA Kit (Omega Bio-tek, Doraville, GA, USA). cDNA synthesis was performed as reported previously [30]. qRT-PCR was carried out in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) with the SYBR ExScript qRT-PCR Kit (Takara, Dalian, China) [34]. Melting curve analysis of amplification products was performed at the end of each PCR to confirm that only one PCR product was amplified and detected. The expression level of *CsCCL21* was analyzed using comparative threshold cycle method ($2^{-\Delta\Delta CT}$) with beta-actin as the internal control [35]. The experiment was performed three times.

CsCCL21 expression during bacterial and viral infection was examined as reported previously [36,37]. Briefly, *E. tarda* and *V. harveyi* were cultured in LB medium at 28 °C to an OD_{600} of 0.8. The cells were washed with PBS and resuspended in PBS to 2×10^6 CFU (colony forming unit)/ml. Tongue sole (as above) were divided randomly into three groups (20/group) and injected intraperitoneally with 50 μl *E. tarda*, *V. harveyi*, or PBS. Fish (five at each time point) were euthanized at 6 h, 12 h, 24 h, and 48 h post-infection, and kidney, spleen and liver were collected under aseptic condition. For bacterial infections, kidney, spleen and liver were collected under aseptic condition. Total RNA extraction, cDNA synthesis, and qRT-PCR were performed as described above with β-actin (for kidney), L18a (for spleen), and 60S ribosomal protein18s rRNA (for liver) as the internal controls as described previously [35]. The experiment was performed three times.

For viral infection, megalocytivirus RBIV-C1 [38] was suspended in PBS to 5×10^5 copies/ml; tongue sole were divided randomly into two groups (20/group) and injected i.p. with 50 μl megalocytivirus RBIV-C1 or PBS. Fish (five at each time point) were euthanized at 1 d, 3 d, 5 d and 7 d post-infection. Kidney, spleen and liver were collected under aseptic condition. Total RNA extraction, cDNA synthesis, and qRT-PCR were performed with β-actin as the internal control as described previously [35]. The experiment was performed three times.

2.5. Construction of pEtCsCCL21 and pEtCsCCL21M

To construct pEtCsCCL21, which expresses recombinant CsCCL21 (rCsCCL21), the coding sequence of *CsCCL21* without signal sequence was amplified by PCR with primers CsCCL21-F1 (5′-GATATCATGCAAGAGTTCTACGGTAACTGCT-3′; underlined sequence, EcoRV site) and CsCCL21-R1 (5′-GATATCGTTCTTTGGGTTTCTGCTGT-3′; underlined sequence, EcoRV site); the PCR products were ligated with pEASYT-T1 Simple (TransGen Biotech, Beijing, China), and the recombinant plasmid was digested with EcoRV to retrieve the *CsCCL21* fragment, which was inserted into pET259 [39] at the SwaI site, resulting in pEtCsCCL21. pEtCsCCL21M, which expresses the mutant recombinant CsCCL21 (rCsCCL21M) bearing alanine substitutions at ^{27}CC^{28}, was created by two-step PCR as follows: the first PCR was performed with the primer pair CsCCL21M-F1 (5′-GCCGCTCTTGGGCATGTAAAACCAATG-3′) and CsCCL21-R1, and the second PCR was performed with the primer pair CsCCL21M-F2 (5′-GATATCATGCAAGAGTTCTACGGTAACGCCGCTCTTGGGCATGTA-3′; underlined sequence, EcoRV site) and CsCCL21-R1. The products of the PCR were inserted into pET259 as described above.

2.6. Purification of rCsCCL21 and rCsCCL21M

E. coli BL21 (DE3) was transformed with pEtCsCCL21 and pEtCsCCL21M. Protein purification from the transformants was performed as reported previously [40]. Briefly, the transformants were cultured in LB medium at 37 °C to mid-log phase. Isopropyl-β-D-thiogalactopyranoside was added to the culture to a final concentration of 0.5 mM. The growth was continued at 28 °C for an additional 12 h, and recombinant proteins were purified using nickel-nitrilotriacetic acid columns (GE Healthcare, USA) as recommended by the manufacturer. The purified proteins were dialyzed for 24 h against PBS and treated with Triton X-114 to remove endotoxin as reported previously [22]. The proteins were concentrated using PEG20000 (Solarbio, Beijing, China). The proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized after staining with Coomassie brilliant blue R-250.