Full length article

Molecular cloning, biological effect, and tissue distribution of interleukin-8 protein in mandarin fish (Siniperca chuasti) upon Flavobacterium columnare infection

Gai Ling Wang a, b, Ming Cheng Wang b, Xiao Wen Zhang a, Ming Xian Chang a, Hai Xia Xie a, *, Pin Nie a

a State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, Hubei Province, 430072, China
b School of Biological and Food Processing Engineering, Huanghuai University, Zhumadian, Henan Province, 463000, China

A R T I C L E   I N F O

Article history:
Received 18 February 2017
Received in revised form 28 April 2017
Accepted 2 May 2017
Available online 3 May 2017

Keywords:
Interleukin-8
Biological effect
Tissue distribution
Mandarin fish (Siniperca chuasti)

A B S T R A C T

Interleukin-8 (IL-8), a CXC-type chemokine, plays a key role in acute inflammation by recruiting neutrophils in mammals. In the present study, the open reading frame (ORF) of IL-8, encoding 99 amino acids was cloned in mandarin fish, and its function in inflammation was investigated. The IL-8 contains four conserved cysteine residues, with the first two forming the CXC signature motif. The genomic sequence of mandarin fish IL-8 has four exons and three introns, a typical gene organization of the CXC chemokine. Bioactive recombinant IL-8 (rIL-8) exhibited a chemotactic effect on head kidney leukocytes in vitro, and activates the transcription of the inflammatory genes, IL-8 and IL-1β. When mandarin fish was challenged intraperitoneally with the pathogenic bacterium Flavobacterium columnare G4, the steady-state protein level of IL-8 was up-regulated in trunk kidney and head kidney. These results suggest that IL-8 is a functional CXC chemokine in mandarin fish, and plays a key role in the inflammatory responses towards bacterial infection.

© 2017 Elsevier Ltd. All rights reserved.

1. Introduction

Interleukin-8 (IL-8; also known as CXCL-8) is a member of the proinflammatory CXC chemokine family mediating inflammatory and immune responses [1]. CXC chemokines are small proteins (8–12 kDa) containing a conserved CXC residue motif (C-cysteine, X-anything other residue) proximal to their N-termini [2]. IL-8 is synthesized as a precursor of a single peptide, and proteolytically processed to ~8 kDa biologically relevant molecule [3].

IL-8, which is produced mainly by monocytes, can interact with G-protein coupled receptors CXCR1 and CXCR2 to recruit neutrophils as well as trigger cytotoxic effect at sites of infection [1]. Neutrophils are the primary target cells of IL-8, although macrophages, mast cells, endothelials, and keratinocytes also respond to this chemokine in humans [4,5]. IL-8 can also be released from macrophages upon the activation of pattern-recognition receptors [6]. Interestingly, IL-8 also modulates the migration of macrophages through engaging IL-8 receptors expressed in macrophages [7]. IL-8 is one of the first few CXC chemokines discovered in fish, and has been cloned and characterized in multiple teleost fish, including Japanese flounder (Paralichthys olivaceus) [8], rainbow trout (Oncorhynchus mykiss) [9], common carp (Cyprinus carpio L.) [10], channel catfish (Ictalurus punctatus) [11,12], fugu (Takifugu rubripes) [13], haddock (Melanogrammus aeglefinus) [14], Atlantic cod (Gadus morhua) [15], black seabream (Acanthopagrus schlegeli) [16], striped trumpet (Latris lineate) [17], zebrafish (Danio rerio) [18], half-smooth tongue sole (Cynoglossus semilaevis) [19], grass carp (Ctenopharyngodon idellus) [20], large yellow croaker (Larimichthys crocea) [21] and ayu (Plecoglossus altivelis) [22]. Mammalian IL-8 molecule has an ELR motif, while fish IL-8s lack such a motif, with the exception of haddock and Atlantic cod [14,15,23].

Fish IL-8 is a functional homolog of mammalian IL-8 [20]. The recombinant IL-8 protein could stimulate the migration of fish neutrophils and macrophages in vitro and in vivo [3,16,20,22]. IL-8 plays a key role in fish immune responses, and its expression is induced following the injection of LPS, poly I:C, or bacterial...
2. Materials and methods

2.1. Cloning of cDNA and genomic DNA sequences of interleukin-8 (IL-8) from mandarin fish

Degenerate primers (Table 1) were used to obtain a conserved DNA fragment of IL-8 from the mandarin fish. Total RNA was isolated from head kidney of healthy mandarin fish by Trizol reagent (Invitrogen, USA). The first strand cDNA was synthesized using Powerscript II reverse transcriptase with CDS primer (SMART RACE cDNA Amplification kit, Clontech, USA) and inserted into pMD18-T (TaKaRa) for sequencing. To obtain full-length cDNA of IL-8, 5' ends and 3' ends were obtained by rapid amplification of cDNA ends (RACE) approaches using the gene specific primers and adaptor primers. Based on the cDNA full-length sequence, a pair of primers (gIL-8F and gIL-8R) were designed to obtain the full-length genomic sequence. All primers used are listed in Table 1.

The exon/intron structure of the genomic sequence of mandarin fish IL-8 was determined by alignment of the full-length cDNA to the genomic sequence using BLAST2 (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi). The BLAST program from the National Center for Biotechnology Information (NCBI) was used to search similar sequences. Proteins were analyzed at the ExPASy Molecular Biology Server (http://web.expasy.org/translate). Sequence alignment was performed by Clustal W using MegAlign programme.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Primers used to the mandarin fish IL-8 cloning and expression.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name</td>
<td>Primer sequence (5'-3')</td>
</tr>
<tr>
<td>IL-8F</td>
<td>CGCTGCAT(TGGA/AGAC/ACGAGAC)</td>
</tr>
<tr>
<td>IL-8R</td>
<td>AT(TGGA/AGAC/ACGAGACGC/ACAG/CACGCT/CTTCAA</td>
</tr>
<tr>
<td>IL-8F</td>
<td>TCAGTTGCAATCGATACTACATCA</td>
</tr>
<tr>
<td>gIL-8F</td>
<td>ATGAGGAGCAGACGAGATGATCAT</td>
</tr>
<tr>
<td>gIL-8R</td>
<td>TCAGCTTCCTTCTTGGTGAAT</td>
</tr>
<tr>
<td>ExIL-8F</td>
<td>AGGGTGACCCGAGATGAGCT</td>
</tr>
<tr>
<td>ExIL-8R</td>
<td>AGGAAGCTTTCAGGCTTCTTGTTGGA</td>
</tr>
<tr>
<td>qIL-8F</td>
<td>TGGGAGGACAGGACTGATAT</td>
</tr>
<tr>
<td>qIL-8R</td>
<td>CAGGCCTTTTGGTGGTACATG</td>
</tr>
<tr>
<td>ActinF</td>
<td>GAGGAGCAGGAACTCCGCTGGTA</td>
</tr>
<tr>
<td>ActinR</td>
<td>CATACGCGGAGAGAGCG</td>
</tr>
<tr>
<td>UPM</td>
<td>TATACCGACTCACATAGGGC</td>
</tr>
<tr>
<td>API</td>
<td>GTATAATCGACCTCATTAGGCC</td>
</tr>
<tr>
<td>AP2</td>
<td>ACTATAGGCGACCGTGGT</td>
</tr>
</tbody>
</table>

2.2. Production and purification of recombinant IL-8, rIL-8

The mandarin fish IL-8 mature peptide cDNA was cloned using expression primers ExIL-8F and ExIL-8R (Table 1). The PCR product was ligated into pQE-30 (digested with Kpn I and Hind III restriction enzymes) to obtain pQE30-IL-8. This recombinant plasmid was then transformed into Escherichia coli M15 competent cells for protein expression analysis. Overnight E. coli cultures were subcultured at 1:50 into TB medium (12 g bactotryptone, 24 g yeast, 4 ml glycerol per litre) supplemented with 100 μg/ml of ampicillin and 25 μg/ml of kanamycin. The rIL-8 expression was induced at 28 °C for 3 h with isopropyl-b-D-thiogalactopyranoside (IPTG) at a final concentration of 0.5 mM. The cells pellets were stored at −80 °C until purification.

The cell pellet was resuspended in ice-cold lysis buffer, and the protein purification, refolding and dialysis processes were performed at 4 °C as described by Razeghifard [25]. The rIL-8 protein was purified, and then washed with polymyxin B resin (Sigma-Aldrich) following the manufacturer's instruction, before being electrophoresed under either reducing or native conditions. The concentration of rIL-8 was determined with the BCA protein assay kit before being stored at −80 °C in the presence of 15% glycerol.

2.3. In vitro effects of rIL-8 on cell migration, and IL-8 and IL-1β transcription

2.3.1. Isolation of head kidney leukocytes

The head kidney of mandarin fish was dissected and gently pushed through 100 μm nylon mesh in ice-cold leibovitz medium (L-15, Gibco) containing 2% FBS (fetal bovine serum), 10 units/ml heparin (Sigma), and 100 μg/ml penicillin/100 units/ml streptomycin (P/S, Gibco). The resultant cell suspension was layered onto a 34%/51% discontinuous percoll (Amersham Pharmacia Biotech AB) gradient and centrifuged at 400 × g for 30 min [26]. Interface cells were collected and washed twice with L-15 medium and a viable cell count performed using the trypan blue exclusion test (Sigma). Head kidney leukocytes suspension were then adjusted to a concentration of 1 × 10^6 cells/ml in Hanks’ Balanced Salt Solution (HBSS, Sigma) for use in the chemotaxis assay and 5 × 10^5 cells/ml in L-15 medium supplemented with 15% FBS to be treated with rIL-8 to activate the transcription of immune-related genes.

2.3.2. Cell migration assay

The chemotactic capacity of head kidney leukocytes towards the mandarin fish IL-8 was assayed in hanging insert (Millicell,
Millipore) introduced in 24-well culture plates (Costar-Corning Life Sciences). 600 μl HBSS containing four dilutions of rIL-8 (1.5, 15, 150 and 1500 ng/ml) were placed in the wells, with HBSS alone as a negative control. After introducing the hanging insert in each of the wells, 200 μl of cell suspension was loaded to the upper part of the chamber. The upper and lower chambers were separated by a 5 μm pore-sized polycarbonate filter. After 90 min of incubation at 25 °C in a dark humidity chamber, the number of migrated cells was quantified by flow cytometry (FACS Calibur, Becton Dickinson). Cell number was determined at constant flow time (1 min) of the medium in the lower chamber. The cells migrated were analyzed based on forward and side light scatter parameters. All experiments were performed in triplicate and were repeated three times.

To determine the cell types upon which mandarin fish rIL-8 might exert a chemotactic effect, air-dried smears of mandarin fish head kidney cells before migration were stained with Giemsa stain solution (Sigma). The migrated-cells, i.e, cells on the underside of the membrane with pores, were fixed in methanol and also stained. Images of the cells before and after migration were taken under examined under a Zeiss inverted microscope.

2.3.3. Transcription of IL-1β and IL-8 in head kidney leukocytes

Head kidney leukocytes were treated with different concentrations of rIL-8 (0.15, 1.5, 15, 150 and 1500 ng/ml) in a 24-well culture plate and incubated for 24 h at 25 °C. Control cells were treated with protein elution buffer. Each sample was repeated in triplicate wells. Total RNA was extracted using Trizol reagent (Invitrogen, USA) according to manufacturer’s instructions and used as template for reverse transcription-polymerase chain reaction (RT-PCR). RNA samples were treated with RNase-free DNase (Fermentas, Lithuania) before 1 μg RNA was reverse-transcribed with RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Lithuania). All cDNA samples were stored at −20 °C for quantitative real-time PCR assays.

IL-8, IL-1β and β-actin cDNA fragments of mandarin fish were inserted into pMD18T vector. Serial 10-fold dilutions of the recombinant plasmids, ranging from 10 to 1000 copies, were used as the template for a standard curve in each run of real-time PCR. Quantitative real-time PCR was conducted on a Chromo 4™ Real-time Detection System (MJ Research, USA). Amplifications were carried out at a final volume of 20 μl containing 1.0 μl cDNA template, 10 μl 2 x SYBR Green Real-time PCR Master Mix (Toyobo, Japan), 1.0 μl of each primer, and 7.0 μl ddH2O, following the manufacturer’s instructions. PCR amplification was performed in triplicate for each tissue template, using the following conditions: 3 min at 94 °C, followed by 40 cycles of 20 s at 94 °C, 20 s at 58 °C, and 30 s at 72 °C. Data were obtained with the Opticon Monitor software 2.03 version (MJ research). The mRNA profile of mandarin fish IL-8 and IL-1β stimulated by purified rIL-8 was analyzed as reported by Livak and Schmittgen [27]. The relative expression level (fold induction) of genes was presented as 2ΔΔCT method.

2.4. Production of TRX-IL-8 and preparation of antisera

The pET-IL-8 was transformed into E. coli Rosetta-gami DE3 to express a recombinant protein fused with thioredoxin (TRX) and 6 x His tag, with a molecular weight ~ 25 kDa. Positive transformants were inoculated into fresh LB medium containing four antibiotics (34 μg/ml chloramphenicol, 12.5 μg/ml tetracycline, 15 μg/ml kanamycin, 100 μg/ml ampicillin) and incubated at 37 °C. When the OD600 reached ~0.5, the cells were induced by the addition of IPTG to a final concentration of 0.5 mM and incubated for another 4 h at 37 °C. The bacteria were then harvested and suspended in binding buffer. After ultrasonic treatment and centrifugation for 20 min at 14,000 × g, the precipitation was dissolved in binding buffer containing 8 M urea and purified using Ni-NTA His-Bind Resin (Novagen) under denatured conditions according to the manufacturer’s introduction. The recombinant protein purified was checked on 12% SDS-PAGE gel.

The purified recombinant protein was used to immunize rabbits to raise a polyclonal antibody against TRX-IL-8. Briefly, rabbits were injected subcutaneously with ~500 μg purified protein emulsified in complete Freund’s adjuvant (Sigma). After the first injection, two times of booster injections were given with the same antigen amount in an incomplete Freund Adjuvant (Sigma) at two-week interval. Ten days after the last booster, the rabbits were bled and the antisera were stored at ~80 °C until use. Immunoblotting was performed to indicate the specificity of this antibody.

2.5. Infection of mandarin fish with Flavobacterium columnare G4

Mandarin fish from Niushan Lake in Wuhan, China were kept in laboratory tanks at 25 °C ± 1 °C for at least two weeks before experimental manipulation. Flavobacterium columnare G4 were grown shaking at 25 °C for 48 h in Shieh broth, and after 3 washes with PBS, the bacteria were adjusted to 6 x 10^7 CFU/ml. Healthy naive mandarin fish (~500 g) were infected with F. columnare G4. Doses of 6 x 10^8 CFU of F. columnare G4 were injected intra-peritoneally near the dorsal fin, 3 fish per group. As a control, PBS was injected. The fish were anaesthetized with tricaine methanesulfonate (MS222) at 100 mg/l before tissue sampling at 24 h post-infection.

2.6. Steady-state protein level of IL-8 in various tissues of mandarin fish

At 24 h post-infection, tissues including head kidney, gill, intestine, trunk kidney, spleen and liver were dissected, washed with 0.7% NaCl solution, and then homogenized in 500 μl chilled RIPA buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.2, 1% NP-40, 0.1% SDS, 1% Triton X-100, 1% Deoxycholic acid (DOC), 1 mM EDTA, 1 μg/ml leupeptin, 25 μg/ml aprotinin and 1 mg/ml PMSF). The homogenates were centrifuged at 14,000 × g for 30 min. Protein supernatant was collected and the final concentration determined using a BCA protein assay kit (Boster, China). 50 μg protein from each tissue was loaded and separated by electrophoresis on a 16.5% Tricine-SDS-PAGE gel. The protein was transferred onto a 0.22 μm PVDF membrane (Millipore, USA) with a Semi-Dry Transfer Cell (Bio-Rad, USA). The blotting membrane was then incubated in 2.5% glutaraldehyde for 1 h and washed 3 times with TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20) before blocking in 5% skimmed milk. The membrane was incubated overnight at 4 °C with antibody produced in rabbit against TRX-IL-8 at a dilution of 1:500 before incubation with HRP-conjugated goat anti-rabbit IgG (Sigma, USA) at a dilution of 1:2500 for 2 h. Antigen-antibody complex was revealed using SuperSignal West Pico Trial kit (Thermo, USA) and imaged with a ChemiDoc MP imaging system (Bio-Rad).

2.7. Statistical analysis

All statistical analyses were performed with one-way analysis of variance (ANOVA) using SPSS software (version 13.0). All data were expressed as mean plus or minus standard errors of the means (SEM), with P-values less than 0.05 considered significant.
3. Results

3.1. The cDNA and genomic DNA sequences of IL-8 in mandarin fish

The full-length cDNA sequence of IL-8 from mandarin fish was obtained by RACE-PCR based on the internal fragment obtained using degenerate primers. The genomic DNA sequence of IL-8 (GenBank accession No.JN180845) was obtained by PCR amplification of genomic DNA using gIL-8F and gIL-8R primers. It consists of 1259 nucleotides containing a 300 bp open reading frame (ORF) flanked by an 82 bp 5'-untranslated region (UTR) and a 415 bp 3'-UTR (Fig. 1). There are two RNA unstable motifs (ATTTA) and a putative polyadenylation signal sequence (AATAAA) in the 3'-UTR. The ORF was predicted to encode a propeptide of 99 amino acids, preceded by a typical signal peptide of 23 residues. The protein contains four conserved cysteine residues located at positions 35, 37, 61 and 78, with an arginine (R) residue located between the first two cysteine residues.

A schematic gene structure of IL-8 from five teleosts (mandarin fish, zebrafish, fugu, channel catfish and rainbow trout), chicken and human were shown in Fig. 2A. The IL-8 gene structure is highly conserved among all species. Moreover, the lengths of exon 2 (133 bp) and exon 3 (87 bp) are identical, and the lengths of exon 1 (64 or 67 bp) are very similar while the length of exon 4 varies greatly. It must be noted that in channel catfish, the lengths of exons are not as the same as those of other species of fish. In contrast, the introns varied considerably in their lengths among the fish species.

Multiple alignments of the deduced IL-8 amino acid sequence from mandarin fish with other known IL-8 molecules revealed conserved protein domains. All sequences contained four cysteine residues, two of which were separated by a single amino acid, consistent with what is present in typical CXC chemokines. In addition, mandarin fish IL-8 is remarkably similar to many other fish IL-8 proteins in that they lack the Glu-Leu-Arg (ELR) motif (Fig. 2B).

3.2. Effect of mandarin fish rIL-8 on migration of head kidney leukocytes

To determine the biological function of mandarin fish IL-8, the putative mature IL-8 was expressed as a N-terminal 6 × His tagged fusion protein in E. coli M15, purified with Ni-NTA metal affinity chromatography under denaturing conditions, and subsequently refolded. On the SDS-PAGE gel, the rIL-8 protein appears as a dense band with a size of ~10.5 kDa (Fig. 3A), consistent with the predicted molecular weight (mature mandarin fish IL-8 [76 aa] plus vector sequence [16 aa, including His tag sequence]). The protein refolded was then analyzed on native SDS-PAGE gel, where additional two bands with higher molecular weight were observed. These two bands may correspond respectively to a dimer or tetramer of IL-8 based on their molecular weight, as revealed by Coomassie blue staining (Fig. 3A).

The chemotactic activity of the refolded rIL-8 protein was tested using a Millicell hanging inserts. Cells migrating through the membrane pores in response to rIL-8 protein were counted by flow cytometry and the results were shown in Fig. 3B. Compared with the HBSS control, rIL-8 protein showed chemotactic activity toward head kidney leukocytes in a dose-dependent manner when less
than 150 ng/ml rIL-8 was applied. The chemotactic activity of rIL-8 peaked at the dose of 150 ng/ml. Surprisingly, the chemotactic activity of rIL-8 decreased at 1500 ng/ml (Fig. 3B).

To ascertain the cell types of fish rIL-8 recruited, the head kidney leucocytes before chemotaxis assay and migrated cells adhering to the polycarbonate filter after chemotaxis assay were examined microscopically. It was found that mandarin fish rIL-8 mainly induced the migration of lymphocytes (Arrow head), it also

---

**Fig. 2. Comparison of the gene structure of IL-8 homologues, and alignment of IL-8 amino acid sequences in mandarin fish and other fish species.**

A. Diagrammatic comparison of IL-8 gene from mandarin fish, rainbow trout, fugu, channel catfish, zebrafish, chicken and human. Exons are represented by black boxes and lines represent introns. Numbers above the boxes and below the lines denote the exact length of each exon/intron in base pairs. NCBI accession numbers for the genomic sequences of IL-8 are as follows: mandarin fish (*Siniperca chuasti*), JN180845; rainbow trout (*Oncorhynchus mykiss*), AY160987; fugu (*Takifugu rubripes*), NW_004071178.1; channel catfish (*Ictalurus punctatus*), AY145142; zebrafish (*Danio rerio*), NC_007112; chicken (*Gallus gallus*), NC_007112; human (*Homo sapiens*), M28130.

B. Multiple alignment of mandarin fish IL-8 amino acid sequence with those from other species. The four cysteine residues are shown by gray shadow. The ELR motif and other residues at the corresponding positions are boxed. The asterisk (*) represents identical residues, (:) conservative substitution and (.) similar residues.
3.3. Effect of rIL-8 on induction of IL-8 and IL-1β from head kidney leukocytes

To measure the putative response elicited by rIL-8, head kidney leukocytes were exposed to different doses of rIL-8 and the expression of two inflammatory cytokines (IL-8 and IL-1β) were analyzed. Quantitative RT-PCR analysis showed that transcription of IL-8 peaked (9.4-fold change) when rIL-8 was applied at 150 ng/ml, and decreased thereafter. In contrast, the transcription of IL-1β increased with supplementation of rIL-8, and peaked (6-fold change) when rIL-8 was applied at 1500 ng/ml (Fig. 4).

3.4. Steady-state protein level of IL-8 in infected and un-infected mandarin fish

To examine the steady-state protein level of IL-8 under physiological or pathological conditions, we produced the IL-8 antibody. The TRX-IL-8 was purified through Ni-NTA resin under denaturing conditions and was used to produce antibody in rabbits. The identity of purified TRX-IL-8 was confirmed with Coomassie staining and the specificity of IL-8 antibody was corroborated by immunoblotting (Fig. 5A).

To study whether IL-8 protein level could be modulated, F. columnare G4, a pathogenic bacterium of mandarin fish, was used to infect mandarin fish and stimulate inflammation. The protein level of IL-8 was determined by immunoblotting in several tissues, including head kidney, gill, intestine, trunk kidney, spleen, and liver. The results showed that IL-8 protein was induced in trunk kidney, head kidney and liver, while decreased in intestine, spleen, and gill, as compared with the un-infected control (Fig. 5B).

4. Discussion

Members of the CXC chemokine family are usually divided into two categories, the ELR⁺ and ELR⁻ chemokines. ELR motif plays an important role in attracting neutrophils [23], and CXC chemokines lacking an ELR motif, in contrast, attract lymphocytes or monocytes but not neutrophils. In fish, ELR⁺ IL-8 has been found only in the haddock (Melanogrammus aeglefinus) and Atlantic cod (Gadus morhua) [14,15]. Among IL-8 reported in other fish, ELR motif is replaced by an EMH motif and mainly lymphocytes and macrophages are recruited.

In fish, the major lymphoid tissues are the kidney, thymus, spleen and mucosa-associated lymphoid tissues including the gill, intestine, and skin, which provide a barrier to the entry of pathogens and contain leucocyte populations responsible for local immune responses [31–36]. When mandarin fish was stimulated with F. columnare G4, IL-8 protein level was up-regulated in trunk kidney, head kidney, and liver, while down-regulated in gill, intestine, spleen, and gill. Fish trunk kidney is composed of not only renal tubules but also lymphoid-like tissue [37]. In fish kidney, an extensive network exists for the trapping of blood-borne particles [31]. Upon bacterial stimulation, IL-8 expression in kidney could be induced, recruiting some macrophages (Arrow) (Fig. 3C).
immune cells and activating inflammasome. The protein level of IL-8 decreased in gill and intestine upon bacterial stimulations may reside in the delivery of _F. columnare_ through interperitoneal but not through immersion, bypassing the mucosa-associated lymphoid tissues. The highest level of IL-8 protein was detected in spleen under either uninfected or infected conditions, the reason awaits further study.

In mandarin fish, three IL-8 bands were detected from various tissues (Fig. 5B), this is consistent with rIL-8 refolded as was demonstrated on native 16.5% Tricine-SDS-PAGE gel (Fig. 3A, Lane 6). The size of these three bands corresponds to that of a monomers, dimers, and trimers of IL-8, respectively (Fig. 3A). In microchemotaxis assay, the chemo-attractive peaked at 150 ng/ml mandarin fish rIL-8 supplementation, while decreased at 1500 μg/ml. A similar trend was observed in grass carp [20] and in rainbow trout [3]. Under physiological conditions, IL-8 exists as monomers, dimers, or a mixture of monomers and dimers, and could reversibly exist as both monomers and dimers, however, it prefers to exist as a dimer in solution [40]. Both monomers and dimers of IL-8 are able to bind to CXCR1, and the IL-8 monomers engage CXCR1 with a higher affinity than the IL-8 dimers [1,40,41]. Thus, it is possible that the formation of IL-8 dimers at high concentrations decreases the binding affinity of IL-8 to its receptors, and thus leading to a decreased chemotactic.

When rIL-8 was used to induce the migration of cells from mandarin fish head kidney, the majority of migrated cells are lymphocytes and macrophages, consistent with what is observed in turbot [24]. The cell types attracted by mandarin fish IL-8 are a little different from those in human or mouse (mainly neutrophils or macrophages). One possible explanation is that B lymphocytes in fish play an important role in trapping pathogens [42]. We speculated that there would be CXC receptor(s) on fish lymphocytes.

In conclusion, we have described an IL-8 gene from mandarin fish and reported its biological properties, as well as its protein expression patterns in various tissues. We successfully produced a bioactive recombinant IL-8 that is capable of inducing the migration of mandarin fish head kidney leukocytes. Notably, this recombinant IL-8 induces the transcription of IL-1β and IL-8 from mandarin fish head kidney leukocytes. We thus provide the evidence that mandarin fish IL-8 is a potent chemokine involved in active immune responses towards pathogen infections.

Acknowledgements

This study was financially supported by the National Natural Science Foundation of China (grant Nos.31230075 and 31402334), and Foundation and Advanced Technology Research Project of Henan Province (grant No.142300410111).
References


