A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Molecular cloning and characterization of Leukocyte cell-derived chemotaxin Genes in rock bream (*Oplegnathus fasciatus)* and disk abalone (*Haliotis discus discus*)

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요약문

Leukocyte cell-derived chemotaxin (LECT)는 인간의 T cell line에서 분리가 되었으며 호중구의 주화성 기능을 갖고 있다고 처음 보고되었다. 인간의 LECT2의 경우 133개의 아미노산 서열로 이루어져 있으며 16 kDa의 단백질과 3개의 이황화 결합구조를 갖고 있다. 인간의 LECT2는 성인과 태아의 간에서 모두 명확하게 발현되었으며 세포 성장과 분화 및 손상과 치료과정과 같은 면역반응 및 다양한 기능을 갖고 있는 단백질이다. chondromodulin-II는 LECT2와 동일하며, 이는 소의 단백질로써 연골세포와 골아세포를 자극시켜 활성화를 일으킨다. LECT의 중요한 기능으로는 케모카인의 유도와 이에 따른 반응에 주로 관여를 한다고 알려져 있으며 이 밖에도 다양한 면역관련 경로에 관여를 하여 세포의 보호와 종양 성장을 억제한다.

이 연구에서는 돌돔의 GS-FLX database와 전복의 normalized cDNA library로부터 LECT2와 LECT1 유전자를 분리하고 RbLECT2와 AbLECT1의 전체 유전자 서열을 확인하였으며 NCBI BLAST를 통하여 알려져 있는 다른 LECT 유전자 서열들과 비교하여 분석하였다. 그리고 이미 밝혀져 있는 다른 관계된 유전자들과의 유사 관계를 밝히기 위하여 ClustalW pairwise, multiple analysis와 phylogenetic analysis를 통하여 RbLECT2와 AbLECT1 유전자의 특성을 분석하였다. 또한 각 조직별로 유전자의 발현을 알아보기 위하여 돌돔에서는 근육, 아가미, 간, 비장, 신장, 피부, 혈액, 심장, 뇌, 소화관에서 LECT2 유전자의 발현 정도를 측정하였으며 전복의 경우에는 간췌장, 혈구, 아가미, 근육, 소화관, 외투막에서 LECT1유전자의 발현 정도를 측정하였다.

*In vivo*에서 돌돔의 LECT2 유전자 발현 분석을 위해 박테리아와 LPS를 돌돔

에 주입하고 그 조직 특이적 mRNA 발현을 확인하기 위하여 주입 후 3, 6, 12, 24, 48시간 마다 간 조직으로부터 mRNA를 분리하여 RT-PCR을 통해 발현 수준을 측정하였다. 그리고 전복의 LECT1 유전자 발현 분석을 위해 박테리아 복합체를 전복의 근육 내로 주입하고, 그 조직 특이적 mRNA 조절을 확인하기 위해서 주입 후 3, 6, 12, 24, 48시간 마다 혈액세포로부터 mRNA를 분리하여 real time RT-PCR을 통해 발현 수준을 측정하였다. 비교를 위하여 PBS 처리가 된 그룹을 control로 사용하였다.

돌돔의 LECT2의 유전자 서열은 456 bp (151 amino acids)의 open reading frame을 포함하는 768 bp로 확인되었다. 돌돔 LECT2 유전자를 분석한 결과 Peptidase_M23 domain과 N-myristoylation sites를 포함하고 있었다. 돌돔의 LECT2 단백질은 대서양 연어 LECT2, 무지개 송어 LECT2, 북부민물꼬치고기 LECT2와 70% 이상 아미노산 서열이 유사성을 나타내었다. 계통학적 분석에 의해 돌돔의 LECT2 단백질은 다른 어류의 LECT2 단백질과 계통상 거리가 가까운 것으로 확인되었다.

돌돔의 각 조직 내에서 LECT2의 발현을 real time RT-PCR을 통하여 분석한 결과, 간에서 가장 높은 발현량을 보였고 그 다음으로 아가미와 피부 순으로 발현량이 많았으며 근육과 신장에서는 LECT2 발현량이 모든 조직 중에서 가장 낮은 발현량을 나타내어 조직 특이적으로 LECT2가 발현되었다. LECT2의 박테리아와 LPS를 통한 면역반응과 mRNA의 발현 양상을 관찰하기 위하여 각각의 병원체를 돌돔 내로 주입한 후에 각 시간별로 LECT2의 발현 수준을 측정한 결과, 박테리아를 주입 한 경우에는 48시간까지 점진적으로 발현 수준이 증가하는 것을 확인하였다. 돌돔의 LECT2 mRNA는 병원체에 대해서 발현 수준이 control에 비하여 그 수준이 상승하였고 다른 어류에서도 비슷한 양상을 보였다. Peptidase_M23

domain과 병원체 주입에 의한 LECT2 mRNA 발현 수준 상승과 관련하여 다른 어류나 포유동물의 LECT2 단백질들과 높은 유사성을 나타내었고 이는 공통적인 조상에서 나누어졌음을 시사한다.

전복의 AbLECT1의 전체 유전자는 576 bp open reading frame(192 amino acids)을 포함하여 전체 705 bp가 확인되었고 예상되는 분자량과 isoelectric point는 각각 21kDa과 8.9를 나타내었다. AbLECT1의 N-말단에는 신호서열을 포함하고 있었으며 16-17 아미노산 잔기 사이에서 절단되는 것으로 확인되었다. AbLECT1는 BRICHOS domain과 Cys active site motifs를 포함하였고 이 motif는 포유동물에서도 나타난 바 있다. AbLECT2에서 BRICHOS domain 서열이 확인되었고 이는 대부분의 LECT1 단백질들의 공통적인 motif이다. 전복의 LECT1은 *Lactobacillus jensenii* 의 발현 유전자와 비슷하였으나 같은 motif를 갖지 않았으며 지브라피쉬의 LECT1이 BLASTp에서 가장 유사하였다. 무척추동물에서는 아직 LECT1이 아직 보고가 되어 있지 않기 때문에 어류와 포유류의 LECT1을 ClustalW multiple analysis를 통하여 비교분석 하였으며 AbLECT1은 인간의 LECT1과 15.5%의 유사성을 띄었으며 AbLECT1의 BRICHOS domain의 분석을 위하여 소, 인간, 닭, 지브라피쉬와 비교를 하였다. RT-PCR 발현 분석을 통해 박테리아를 전복 내로 주입한 후 각 조직별로 LECT1의 발현량을 분석하였으며 외투막에서 가장 높은 발현량을 나타내었다. LECT1의 면역적 발현 수준을 보기 위하여 박테리아을 주입후 48시간째 혈구세포의 LECT1의 발현 수준이 증가함을 확인하였다.

결론적으로 본 연구에서는 돌돔과 전복으로부터 chemotactic 기능을 갖고 있는 LECT 유전자를 분리하여 그 염기서열의 특성을 분석하고 조직 발현 분석을 수행하였으며 이 연구들은 척추동물과 무척추동물의 LECT를 통한 면역 조절 시스템에 대한 면역학적 연구에 새로운 포석이 될 것이다.

Abstract

Leukocyte cell-derived chemotaxin-2 (LECT2) was first isolated from the culture fluid of the human (Homo sapiens) T cell line SKW-3 and was shown to have neutrophil chemotactic activity. Human LECT2, a 16-kDa basic protein, consisting of 133 amino acid residues and three intramolecular disulphide bonds, is specifically expressed in the adult and fetal livers. Although it was originally demonstrated to have a chemotactic function in vitro, recent data sustain a further multifunctional role of LECT2 that extends from cell growth, differentiation, damage/repair process and carcinogenesis to autoimmune diseases. The in vivo function of LECT2 protein still remains obscure. In order to study the phylogeny of LECT2, a full-length cDNA clone of LECT2 gene, 768 bp in size, was isolated in Rock bream (*Oplegnathus fasciatus*). Its deduced amino acid sequence of 151 residues, presents 73, 56, 45 and 42 % overall identity to Atlantic salmon, Zebra fish, Mouse and Human LECT2 proteins, respectively. In contrast to mammalian LECT2 protein, trout LECT2 protein reveals potential N-myristoylation site and Peptidase_M23 superfamily motif (^{152}AGAATCC-GACCCC^{448}). Phylogenetic analysis shows that Rock bream LECT2 is clustered with the known homologous proteins. RbLECT2 mRNA was significantly ($p < 0.05$) up-regulated after infection with a bacteria and LPS in rock bream liver.

A BRICHOS domain-containing leukocyte cell-derived chemotaxin 1-like cDNA was cloned from the disk abalone (*Haliotis discus discus*) and designated as AbLECT-1. A full-length (705 bp) of AbLECT-1 cDNA was composed of a 576 bp open reading frame that translates into a putative peptide of 192 amino acids. Deduced amino acid sequence of AbLECT-1 had 15.5- and 27.8% identity and similarity to human LECT-1, respectively. Quantitative real-time PCR analysis results showed that the mRNA of AbLECT-1 was constitutively expressed in abalone hemocytes, gills, mantle, muscle, digestive tract and hepatopancreas in a tissue-specific manner. Moreover, the AbLECT-1 transcription level was induced in hemocytes after challenge with *Vibrio alginolyticus, Vibrio parahemolyticus*, and *Listeria monocytogenes* suggesting that it may be involved in immune response reactions in abalone.

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Part I

Molecular cloning and characterization of Leukocyte cell-derived chemotaxin 2 in rock bream (*Oplegnathus fasciatus*)

1. Introduction

Leukocyte cell-derived chemotaxin-2 (LECT2) was first isolated from the culture fluid of the human (Homo sapiens) T cell line SKW-3 and was shown to have neutrophil chemotactic activity. Human LECT2, a 16-kDa basic protein, consisting of 133 amino acid residues and three intramolecular disulphide bonds, is specifically expressed in the adult and fetal livers (Yamagoes S, Yamakawa Y, et al. 1996). The following isolation of the cDNA encoding LECT2 showed that its mRNA is specifically expressed in human fetal liver and human hepatoma cell lines. (Yamagoe S, Akasaka T, et al. 1997). LECT2 is identical to chondromodulin-II, a bovine protein that stimulates the proliferation of chondrocytes and osteoblasts (Shukunami, C., J. Kondo, et al. 1999). Expressed LECT2 protein of various human tissues including the soft tissue surrounding the lymph nodes, cerebrum, bronchial wall, upper layer of the esophagus sweat gland, stomach, vascular endothelium, pancreas, and prostate gland (Nagai, H., T. Hamada, et al. 1998). Although LECT2 was originally isolated as a chemotactic protein in vitro, it may play a role in other cellular functions.

In a previous the study reported that the expression of mouse LECT2 was transiently decreased during con A-induced hepatitis, an experimental model for human autoimmune hepatitis that is induced by the expression of cytokines and cytotoxic molecules associated with effects from other immune cells, such as CD4+ T lymphocytes and macrophages (Tiegs, G., J. Hentschel, et al. 1992). Thus, Current evidence suggests that LECT2 may be a

multifunctional protein, involved in cell growth, differentiation, damage/repair processes and in the autoimmune response. (Hiraki, Y., H. Inoue, et al. 1996; Saito, T., A. Okumura, et al. 2004). However, the function of LECT2 *in vivo* remains unclear.

Proteins homologous to LECT2 have been isolated in many vertebrates in addition to humans, such as bovine (*Bos taurus*) (Yamagoe, S., S. Mizuno, et al. 1998), mouse (*Mus musculus*) (Yamagoe, S., T. Watanabe, et al. 1998) , carp (*Cyprinus carpio*) (Fujiki, K., D. H. Shin, et al. 2000) , rainbow trout (*Oncorhynchus mykiss*) (Kokkinos, P. A., A. Kazantzi, et al. 2005), and zebrafish (*Danio rerio*) (Lin, B., S. Chen, et al. 2007).

The rock bream is very popular for Sashimi and thus is very expensive in both Korea and Japan. In Korea, many fish farmers want to culture rock bream. Nevertheless, diseases were violently occurred in these years, causing grave damage to fish yields and quality. In order to solve the problems, genes of rock bream contribute to immune strategies were put in to study.

In this study, we report the molecular cloning of a full-length cDNA encoding RbLECT2, the LECT2 from the liver of the fish Rock bream (*Oplegnathus fasciatus*), and the phylogenetic relationships of RbLECT2 with the known homologues from other species. The expression profiles of the RbLECT2 gene in healthy and Bacteria infected fish were determined by RT-PCR. Fish LECT2 transcripts were significantly increased after bacterial infection, indicating that LECT2 might participate in immune regulation of fish.

2. Materials and methods

2.1. Rock bream cDNA library construction and isolation of the RbLECT2 cDNA

We have constructed a rock bream cDNA sequence normal by the genome sequence FLX™ genome sequencing technique. Total RNA was isolated using Tri Reagent™ (Sigma, USA) from several tissue pools including gills, blood, liver, spleen, pituitary gland, head kidney and kidney of three healthy rock bream fishes. Then, the mRNA was purified using an mRNA isolation kit (FastTrack® 2.0, Invitrogen, USA). The basic strand cDNA synthesis and normalization were the Creator™ SMART™ cDNA library construction kit (Clontech, USA) and Trimmer-Direct cDNA normalization kit (Evorgen, Russia). After that the GS-FLX™ sequencing of rock bream cDNA was performed according to the manufacturer's instructions (Roche Applied Science, USA). Two primers were designed based on the rock bream LECT2 sequence (RbLECT2F, RbLECT2R) for internal sequencing (Table. 1). Full-length sequence of rock bream Leukocyte cell-derived chemotaxin-2 (RbLECT2) was determined by consecutive internal sequencing reaction using a terminator reaction kit, Big Dye, and ABI 3700 sequence (Macrogen, Korea). From the rock bream cDNA sequence library, a single putative EST gene was identified during homology screening using the Basic Local Alignment Search Tool (BLAST) program available on NCBI (http://blast.ncbi.nlm.nih.gov/Blast/) and was designated as RbLECT2.

2.2. Sequence characterization and phylogenetic analysis

The RbLECT2 full-length sequence was analyzed by BLAST. Similarities were compared with other known LECT2 sequences available in the NCBI and ENSEMBL databases. To obtain the open reading frame (ORF) amino acid sequence of RbLECT2, DNAassist (Version 2.2) was used. Characteristic domains or motifs were identified using the PORSITE profile database and SMART proteomic database. Pairwise and multiple

sequence alignments of RbLECT2 protein were generated, using a ClustalW (version 1.8) program. Identity, similarity and gap percentages were calculated using FASTA program. The phylogenetic relationship of RbLECT2 was determined using the Neighbor-Joining method in the Mega molecular evolutionary genetic analysis software package (Version 2.1) with bootstrapping values taken from 1000 replicates.

2.3. Experimental animals

Healthy Rock breams (body weight of 40–60 g) were obtained from Jeju special self-Governing Province Ocean and Fisheries Research Institute (Jeju Island, Republic of Korea). Rock breams were maintained in flat-bottomed fiberglass tanks (60 L) with aerated and sand-filtered seawater at a temperature of 21 – 23 ℃ and salinity of 34 ± 1 ‰ in our laboratory. All Rock breams were acclimatized for 1 week prior to the experiment before being injected with immune stimulators. Only active rock breams determined by general appearance and the movement were used for tissue extractions.

2.4. Bacterial and LPS challenge of Rock breams

To determine the immune responses of RbLECT2, pathogenic bacterium *Edwardsiella tarda* and lipopolysaccharide (LPS) were used as immunostimulants in time course experiments. For bacterial challenge, *E. tarda* strain was obtained from the Department of Aqualife Medicine, Chonnam National University, Korea and cultured in BHI broth supplemented with 1 % NaCl and incubated at 30℃ for 12h. The bacterial pellet obtained by centrifugation (7000 × g at 4℃ for 5 min) was resuspended in 1 × PBS. For bacterial challenge, the fish were intraperitoneally injected with *E. tarda* (5 × 10^6 CFU/ml) suspended in 1 × phosphate buffered saline (PBS; 100μl/animal). Rock breams were intramuscularly injected with 100 μl per Rock bream of the bacterial having a cell count of 5 × 10^7 cells/ml. The control group was injected with 100 μl of PBS. Rock bream liver

samples were taken from *E. tarda* challenged, and LPS-injected rock bream to determine the transcriptional profiles of RbLECT2. At time point of 3, 6, 12, 24 and 48h post infection/ induction (p.i.) from the animals in order to determine the transcriptional profiles of RbLECT2. PBS-injected control samples were isolated at 3h p.i. muscle, blood, brain, spleen, heart, gill, muscle, liver, kidney, head kidney, skin, tissues obtained from uninduced animals (for tissue-specific expression) were used as blank to compare the effect of PBS injection in rock bream. All samples were obtained and analyzed in triplicate, and the results are expressed as relative-fold change as mean ± standard deviation.

The Rock bream blood was collected in a sterilized syringe from the pericardial cavities of three rock breams. The blood was immediately centrifuged at 3000g for 10 min at 4 ℃. The supernatant was removed and blood cells were collected for RNA extraction. To determine the immune responses of the RbLECT2, two immune stimulation/challenged experiments were devised and conducted using pathogenic bacteria mixture and tissue injury.

2.5. Total RNA isolation and First strand cDNA synthesis

An equal amount (50 μg) of tissue samples was obtained separately from each tissue (three replicate rock breams) to make a pool, before isolation of the RNA. The total RNA was extracted from pooled tissue (150 μg) using Tri Reagent™ (Sigma, USA) according to the manufacturers protocol. The RNA was stored at -80℃ until further use. The RNA concentration and purity were determined by measuring the absorbance at 260 and 280 nm in a UV-spectrometer (Bio Rad, USA). Originally purified RNA was diluted to 1 μg/μl concentration before synthesis of cDNA. 2.5 μg RNA was used to synthesize cDNA from each tissue using a SuperScript III first strand synthesis system for RT-PCR (Invitrogen, USA). Briefly, RNA was incubated with 1 μl of 50 μM oligo$(dT)_{20}$ and 1 μl of 10 mM dNTP for 5 min at 65℃. After incubation, 2 μl of 10 × cDNA synthesis buffer, 2 μl of dithiothreitol (DTT, 0.1 M), 4 μl of 20 mM $MgCl_2$, 1μl of RNaseOUT™ (40 U/μL) and 1 μl

of SuperScript III reverse transcriptase (200 U/μl) were added and incubated for 1h at 50℃. The reaction was terminated by raising the temperature to 80℃ for 5 min. 1μl of RNAse H was add to each cDNA and incubated at 37℃ for 20 min. Finally, The resulting cDNA was diluted 10-fold before (total 200 μl) being stored at -20℃ for further experiments.

2.6. Rock beam LECT2 mRNA expression analysis by real-time PCR

The RbLECT2 mRNA expression was analyzed by quantitative real-time PCR using gene-specific primers (RbLECT2 RT-F; RbLECT2 RT-R). The gene coding for the rock bream beta-actin was selected as a reference gene, beta-actin primers were designed based on the EST of the 1879 bp sequence (Accession No. FJ975145) from rock bream and was amplified using RbBeta-actin-F and RbBeta-actin-R gene specific primers (Table. 1). Tissue-specific mRNA expression was analyzed in rock bream muscle, gill, liver, spleen, head kidney, kidney, skin, blood, heart, brain and intestine. Real-time PCR was carried out in a 20 μl reaction volume containing 4 μl of cDNA from each tissue, 10 μl of 2 × TaKaRa Ex Taq™ SYBR premix, 0.5 μl of each gene-specific primer (20 pmol/μl) and 5 μl dH_2O. The RT-PCR cycle profile was: 1 cycle of 95℃ for 10 s, followed by 35 cycles of 95℃ for 5 s, 58℃ for 10 s and 72℃ for 20 s, and finally, 1cycle of 95℃ for 10 s, 60℃ for 30 s and 95℃ for 15 s. The same RT-PCR cycle profile was used for the internal reference gene. Real-time PCR was carried out using Thermal Cycler Dice™ Real-Time System (TaKaRa, Japan) and the baseline was set automatically using the Thermal Cycler Dice™ Real Time System software (Version 3.0). The relative expression was determined by means of the $2^{-\Delta\Delta ct}$ method. All data represent means ± standard deviation.

3. Results

3.1. Identification and characterization of Rock bream LECT2 cell cDNA

The nucleotide and deduced amino acid sequences of the RbLECT2 from rock bream are shown in Fig. 1. The cloned full-length RbLECT2 consisted of 768 bp. The open reading frame was composed of 456 bp that translate into a putative peptide of 151 amino acid residues. Sequence analysis indicated a 7 bp long 5' untranslated region (5'UTR) and 305 bp 3' UTR, which contains Peptidase_M23 domain (^{49}RIHKGL-MEDKSDP^{147}) following a typical polyadenylation signal (^{736}AATAAA^{742}). There was a signal peptide (^{1}MRRVVVLLAVLCVCDG^{16}) with the RbLECT2 contain ~~four~~ N-myristoylation sites (^{16}GVKFGQLCSGNPDNT^{30}, ^{43}GARRGS^{48}). The RbLECT2 has a putative molecular mass of 17 kDa with 9.4 isoelectric point (p. i). Additionally, a characteristic Peptidase_M23 domain was predicted in the 152–448 amino acid range by the DAS prediction program.

The RbLECT2 amino acid identity and similarity percentages were calculated using FASTA program. We analyzed the RbLECT2 sequence identity from human, mouse, rat, cattle, opossum, chicken, frog, atlantic salmon, rainbow trout, northern pike, sweetfish, zebrafish, monkey and chimpanzee. As it is shown in Table 2, the RbLECT2 demonstrated the highest identity (73.1%) and similarity (81.4%) to Atlantic salmon LECT2. In addition, RbLECT2 was aligned with human, mouse, rat, zebrafish, cattle, opossum, chicken, frog, monkey and chimpanzee sequences in ClustalW multiple analyses (Fig. 2).

3.2. Phylogenetic analysis of rock bream LECT2 gene

Phylogenetic tree analysis showed that RbLECT2 grouped tightly with the fish LECT2, and seemed to be closer to that of sweetfish (CAR94535) LECT2 (Fig. 3). Currently accepted relationships were reflected in the phylogenetic tree of LECT2 sequences (Fig. 3). The RbLECT2 (★) was isolated as single branch. The mammalian LECT2 sequences were

grouped as a separate cluster while teleostei LECT2 was originated as a single branch without connecting to mammalian LECT2 origin.

3.3. Analysis of tissue expression of rock bream LECT2

To determine the tissue-specific RbLECT2 mRNA profile, quantitative real-time PCR was carried out using gene-specific primers designed from the RbLECT2 coding sequence. The relative mRNA expression of each tissue was calculated using rock bream ribosomal protein as a housekeeping gene and result was further compared with muscle expression level to determine the relative tissue-specific expression profile. RbLECT2 mRNA was constitutively expressed in all eleven selected tissues including muscle, gill, liver, spleen, head kidney, kidney, skin, blood, heart, brain and intestine. Further analysis results showed that RbLECT2 mRNA expression was higher in the liver and skin than the gill, where liver showed the highest level of expression with 110-fold compared to that observed for muscle (Fig 4). In contrast, brain, spleen, head kidney and kidney expression was lower than that observed for gill, blood, heart and intestine. Furthermore, in all tissues, the expression level was significantly ($p < 0.05$) different to the expression observed in blood cell. Finally, the RbLECT2 showed tissue specific variation in rock bream tissues.

3.4. Transcriptional responses of rock bream LECT2 after bacteria and LPS stimulation

RbLECT2 mRNA expression levels in the liver following an *in vivo* immune challenge were determined. The relative transcriptional levels of the RbLECT2 were calculated using rock bream beta-actin expression as a reference gene, and the result were further compared to respective PBS-injected control expression levels to determine the fold induction. There was no significant difference in un-induced and PBS-injected controls at 3 h and 48 h p.i. Therefore, the PBS-injected sample collected at 3 h was used to determine the transcriptional

regulation by different stimulants in this study. The RbLECT2 transcript level was up-regulated in liver tissue after all challenges during the period of the experiment, 3- 48 h p.i. (Fig. 4). The LPS induction in RbLECT2 was significantly values ($p < 0.05$) up-regulated after 3 – 24 h and the highest relative expression (2.7 - fold) was observed at 24 h p.i compared to control. Although, subsequently its decreased relative expression (84 %) at 48 h compared with control. The expression levels of those time points were significantly higher ($p < 0.05$) than that observed for the control. As a late-phase response for LPS, RbLECT2 mRNA level had again increased significantly at 3 h – 24 h p. i. RbLECT2 transcript level varied in a slightly different pattern for the *E. tarda* injection form the LPS induction in liver. Initially, it was up-regulated at all-time point with mild alteration.

However the level of expression was significantly ($p < 0.05$) slightly decreased after 12 h and 24 h p.i. subsequently increased RbLECT2 expression at 24 h and 48 h p.i. again. Moreover, the highest level of expression (12- fold) was observed at 48 h compared to control.

4. Discussion

LECT2 was originally noted for its possible neutrophil chemotactic activity (Yamagoe, S., Y. Yamakawa, et al. 1996). In addition, it was independently reported to be a growth-stimulating factor for chondrocytes and osteoblasts and was named chondromodulin-II (Hiraki, Y., H. Inoue, et al. 1996). Although, there is currently a little information available about fish LECT2, studies on mammalian LECT2 might provide functional clue.

The nucleotide and deduced amino acid sequence of the full-length cDNA clone of rock bream LECT2 with a predicted open reading frame of 192 amino acids, including in the N-myristoylation site, a secretary signal sequence 16 amino acids is reported. The deduced amino acid sequences of RbLECT2 are 73% identical to that of *Atlantic salmon* LECT2. Moreover, LECT2 have an about 71% identity to the Rainbow trout LECT2 amino acid sequences and have N-myristylation site (Kokkinos, P. A., A. Kazantzi, et al. 2005). Multiple and pairwise sequence analysis results from the present study also reveal that rock bream LECT2 shares a higher homology with the teleost LECT2 family than other mammalian. The presence of the vertebrate LECT2 domain, sequence similarity, phylogenetic relationship and other common features suggest that the rock bream LECT2 could be a new member of the vertebrate LECT2 family and more closely related to teleost LECT2 family. Phylogenetic analysis, based on the amino acid sequence alignment, showed that rock bream LECT2 protein is clustered primarily with other teleost LECT2 molecules, and secondary with the counterparts from different species (Fig. 3).

In the present study, we successfully isolated and sequenced the leukocyte cell-derived chemotaxin-2 (LECT2) from rock bream and determined the tissue expression profile. The significantly exacerbated arthritis and altered expression of inflammatory cytokines were also found in LECT2-deficient mice (Okumura, A., T. Saito, et al. 2008). Based on the possible roles of LECT2 in liver injury, its tissue-specific expression and expression upon injury has been studied (Yamagoe, S., S. Mizuno, et al. 1998; Segawa, Y., Y. Itokazu, et al.

2001). Some investigations further indicate that mammalian LECT2 might play a role in immune regulation. In LECT2-deficient mice, the proportion of NKT cells in the liver increased and hepatic injury was exacerbated in severe concanavalin A-induced hepatitis (Saito, T., A. Okumura, et al. 2004). previously reported that the LECT2 mRNA in zebrafish liver was up-regulated to 1000-fold upon infection by *Aeromonas salmonicida* and *Staphylococcus aureus*, suggesting that LECT2 served as a positive important phase protein in fish (Lin, B., S. Chen, et al. 2007). Constitutive expression of resembles LECT2 transcripts in a variety of tissues in including liver, brain, heart, intestine, kidney and spleen from healthy and *V. alginolyticus*-infected fish (Li, M. Y., J. Chen, et al. 2008). Croceine croaker LECT2 transcripts have been detected in the various tissues from healthy fish. Similarly, RbLECT2 transcripts are expressed in diversified tissues such as muscle, gill, liver, spleen, head kidney, kidney, skin, blood, heart, brain and intestine. Liver tissue showed the highest constitutive RbLECT2 expression among all selected tissues. It was observed that the RbLECT2 expression pattern was similar to that of rainbow trout LECT2 in previous study (Kokkinos, P. A., A. Kazantzi, et al. 2005). It is difficult at this time to understand the precise reason for the similar tissue distribution profile of RbLECT2. In other word, we can suggest that in rock bream, LECT2 may have common tissue expression patterns since their tissue-function specificity is similar compared to vertebrates. Higher constitutive expression in gill and skin may be associated with the rock bream was external environment and frequent exposure to contaminants and pathogens, which are coming through the environment water and materials.

Identification of expression responses of this neutrophil chemotactic response will be useful for better understanding of host immune defense mechanisms against various pathogens. Furthermore, LPS is a structural component of the Gram negative bacterial outer membrane that has shown different effects on the host immune system. Moreover, LPS is widely used as a potent immune stimulant for inducing different genes (Xiang, L. X., B. Peng, et al. 2008). Hence, we conducted further expression studies on the responses of RbLECT2 to different immune stimulants including bacteria and LPS induction. Our results

show RbLECT2 mRNA level is induced by bacteria and LPS stimulation in rock bream liver. However, RbLECT2 expression was lower at 48 h p.i. than at 24 h in LPS immune stimulation experiments. The reason for this is not clear at the moment. In general, RbLECT2 mRNA was induced in rock bream liver by a bacteria and LPS showing a specific expression profile to each immune stimulation or challenge done in this study. In contrast to liver tissue displayed a similar set of response patterns for the challenges. It is noteworthy that in comparison to liver expression we instigated to a high level. *E. tarda* induced a strong early -phase instigation, whereas LPS cause a mild instigation of the RbLECT2 transcription.

The most important outcome of this immune stimulation is the inducibility of RbLECT2 transcripts. It is presumed that RbLECT2 were involved chemotactic and immune defense responses against pathogens and immune stimulants. The constitutive and omnipresent expression of RbLECT2 in liver as well as in other tissues with transcriptional regulation against challenges would suggest that it has an important function of regulating the host defense against pathogens.

In conclusion, a new leukocyte cell-derived chemotaxin 2 (RbLECT2) from rock bream (*Oplegnathus fasciatus)* was identified and characterized at both primary and tertiary levels. With molecular characterization, phylogenetic analysis showed its similarity to other LECT2 members. Constitutive RbLECT2 mRNA expression was observed in a tissue-specific manner, suggesting its immune system importance. Furthermore**,** RbLECT2 expression was up-regulated upon being challenged with different immune stimulants, indicating its neutrophil chemotactic role against immune related protein. LECT2 may regulate not only neutrophil functions buy also other cellular functions. It had been reported that LECT2 immune responded prevent fish diseases, buy the mechanism responsible for this phenomenon was unknown. Further research is needed to clarify the physiological role of LECT2

Fig.1. The complete nucleotide and deduced amino acid sequences of the rock bream leukocyte cell- derived chemotaxin-2 cDNA. The start (ATG) and stop (TGA) codons are in boldface. Predicted signal peptide is bold and underlined. The predicted Peptidase_M23 superfamily motif (^{49}RIHKGL-MEDKSDP^{147}) is italics and underlined. The Predicted N-myristoylation site are in box. The polyadenylation signal (AATAAA) is in bold italics and the poly-(A) tail is underlined at the end of the nucleotide sequence.

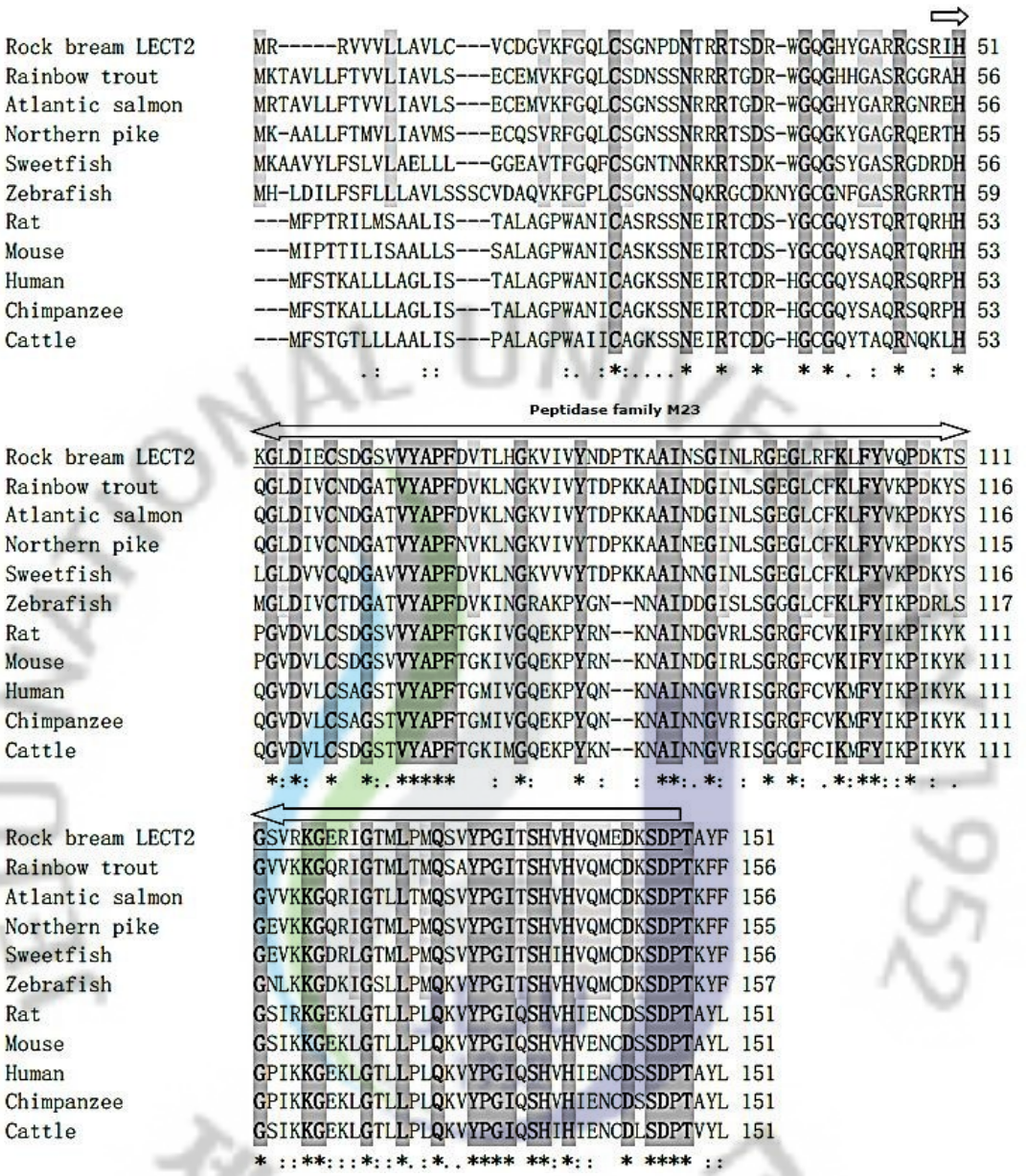

Fig. 2. ClustalW multiple sequence alignment of the deduced aa sequence of RbLECT2 with known homologous leukocyte cell-derived chemotaxin-2 aa sequences. Residues shaded with dark grey and boldface represent the completely conserved aa, indicated by (*) and residues strongly conserved and weakly conserved are indicated by colons (:) or dots (.), respectively. Only the light grey shade represents identical residues among rock bream and other teleost LECT2 sequences. The Peptidase family M23 site motif is underlined with the arrow and name on the top. The GenBank accession numbers are same as the numbers used in Fig. 3.

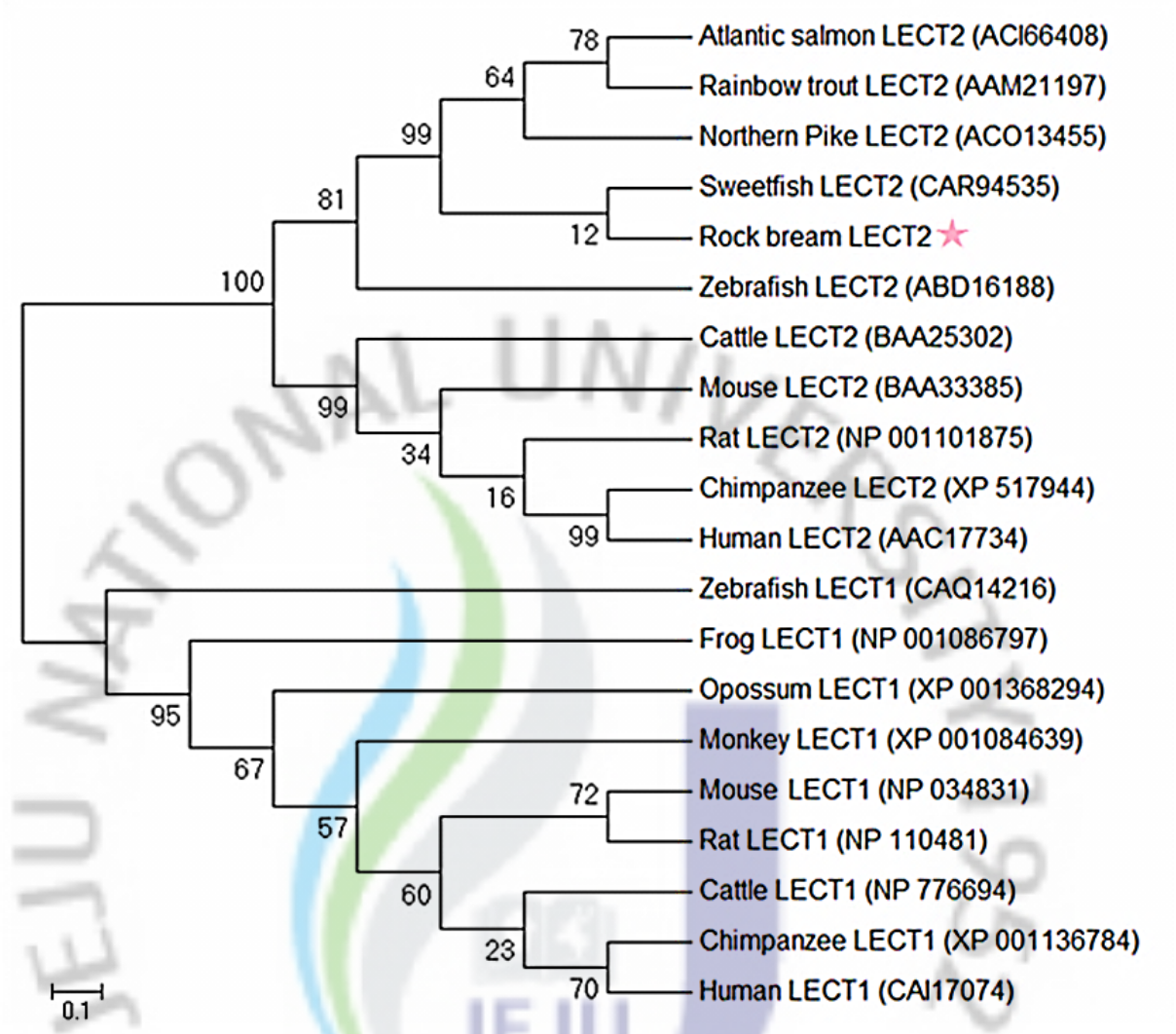

Fig. 3. Phylogenetic analysis of rock bream LECT2 with selected different vertebrate LECT2 and LECT-1 members. Phylogenetic analysis was done by the Neighbor-joining algorithm using the MEGA program (version 3.1) based on ClustalW (version 1.81) alignment using LECT2 and LECT-1 amino acid sequences. The Numbers at tree nodes indicate the bootstrap confidence values from 1000 replicates. The accession numbers are show next to each species.

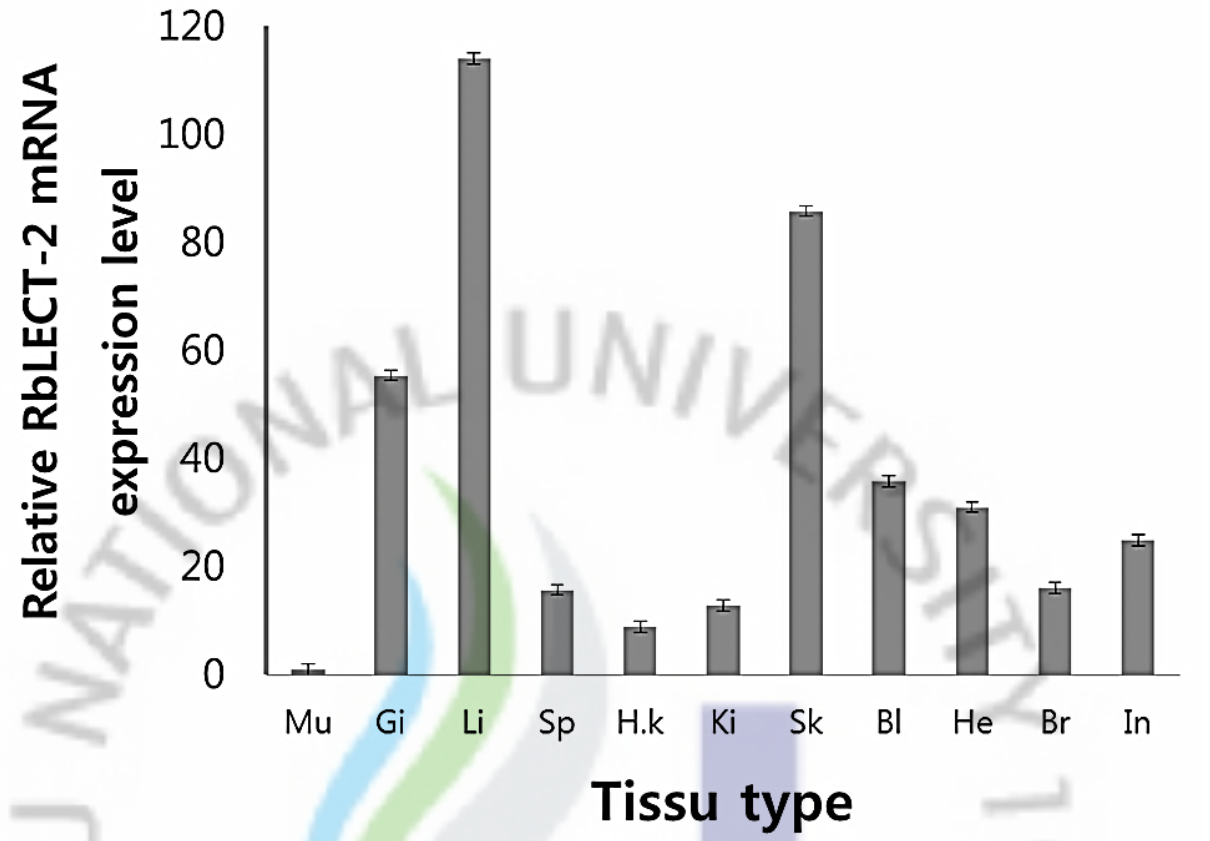

Fig. 4. Tissue specific expression of RbLECT2 mRNA determined by quantitative real-time PCR. The relative RbLECT2 mRNA expression of each tissue was calculated by $2^{-\Delta\Delta ct}$ method using rock bream beta-actin protein as a reference gene. Then, the relative mRNA level was compared with muscle expression to determine the tissue-specific expression. Mu – muscle, Gi – gill, Li – liver, Sp – spleen, H.k – head kidney, Ki – kidney, Sk – skin, Bl – blood, He – heart, Br – brain, In – intestine. The bars represent the standard deviation.

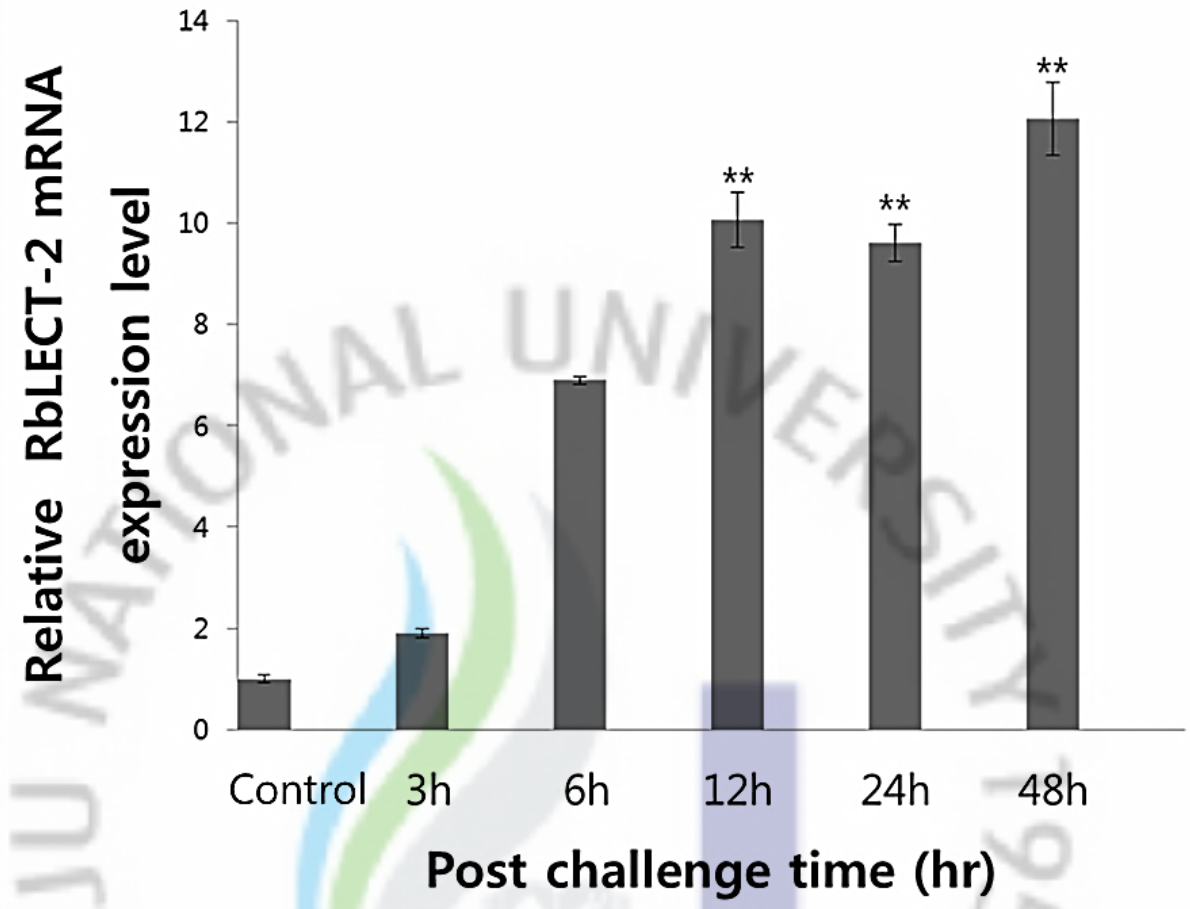

Fig. 5. RbLECT2 mRNA expression by SYBR green real-time PCR in liver tissue after bacteria challenge. The relative mRNA expression was calculated by $2^{-\Delta\Delta ct}$ method using rock bream beta-actin protein as a reference gene and PBS as a control. The bars represent the standard deviation. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range test using SPSS 11.5 program. Differences were considered statistically significant (**) at $p < 0.05$.

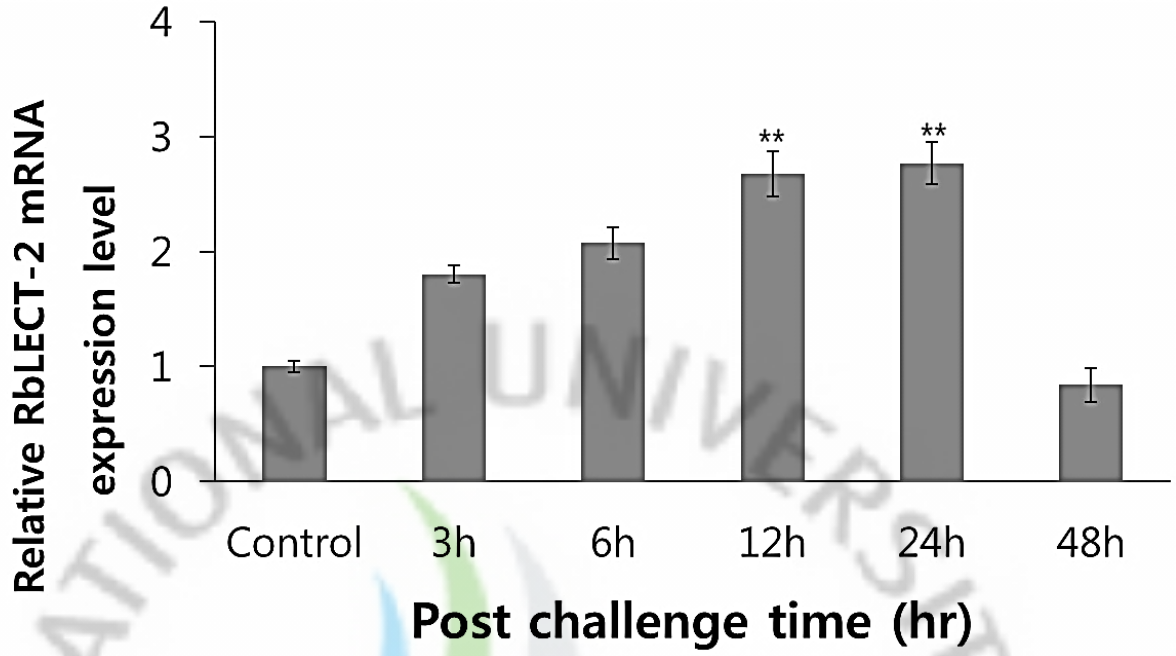

Fig. 6. RbLECT2 mRNA expression by SYBR green real-time PCR in liver tissue after LPS challenge. The relative mRNA expression was calculated by $2^{-\Delta\Delta ct}$ method using rock bream beta-actin protein as a reference gene and relative to PBS control. The bars represent the standard deviation. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range test using SPSS 11.5 program. Differences were considered statistically significant (**) at $p < 0.05$.

Name	Target	Orientation	Sequence
RbLECT2-1F	ORF amplification	Forward	gagagagaattcATGAGACGTGTCGTCGTTTTGCTG
RbLECT2-1R	ORF amplification	Reverse	gagagaaagcttTCAGAAGTACGCCGTGGGGT
RbLECT2 RT-F	RT-PCR amplification	Forward	TGATGTGACACTCCACGGAAAGGT
RbLECT2 RT-R	RT-PCR amplification	Reverse	AACTTGAAACGCAGACCCTCTCCT
RbBeta-actin-F	Internal PCR control	Forward	TCATCACCATCGGCAATGAGAGGT
RbBeta-actin-R	Internal PCR control	Reverse	TGATGCTGTTGTAGGTGGTCTCGT

Table. 1. A description of primers used in this study.

	# Identity	# Similarity	# Gaps
Atlantic salmon	114/156 (73.1%)	127/156 (81.4%)	5/156 (3.2%)
Rainbow trout	111/156 (71.2%)	123/156 (78.8%)	5/156 (3.2%)
Northern pike	109/155 (70.3%)	123/155 (79.4%)	4/155 (2.6%)
Sweet fish	106/156 (67.9%)	121/156 (77.6%)	5/156 (3.2%)
Zebra fish	89/159 (56.0%)	114/159 (71.7%)	10/159(6.3%)
Human	65/153 (42.5%)	94/153 (61.4%)	4/153 (2.6%)
Chimpanzee	65/153 (42.5%)	94/153 (61.4%)	4/153 (2.6%)
Rat	69/155 (44.5%)	94/155 (60.6%)	8/155 (5.2%)
Mouse	71/155 (45.8%)	94/155 (60.6%)	8/155 (5.2%)
Cattle	65/155 (41.9%)	95/155 (61.3%)	8/155 (5.2%)

Table. 2. Percentage of identity, similarity and gaps of the Rock bream LECT2 amino acid sequences of with known other LECT2 amino acid sequences.

Patr II

BRICHOS domain-containing leukocyte cell-derived chemotaxin 1-like cDNA from disk abalone (*Haliotis discus discus)*

1. Introduction

The BRICHOS domain contains approximately 100 amino acids and it was originally identified by comparison of Bri protein, chondromodulin (ChM-1) and surfactant-associated protein C (proSP-C) sequences. This domain has been found in several unrelated proteins such as integral membrane protein (ITM2) 2 family, chondromodulin1 family (leukocyte cell-derived chemotaxin), and SP-C. It was reported that BRICHOS domain proteins are associated with major diseases such as dementia syndrome (similar to Alzheimer‘s disease) respiratory distress syndrome and cancer (Sanchez-Pulido, L., D. Devos, et al. 2002). It has been proposed that BRICHOS-containing proteins may be involved in intramolecular chaperon-like function, intracellular protease processing, and inducing secretary pathways (Sanchez-Pulido, L., D. Devos, et al. 2002) and (Azizan, A., N. Holaday, et al. 2001). Several studies have been demonstrated that neutrophils are important chemokine-generating leukocytes that can produce chemokines and respond to chemotactic cytokines (Kunkel, S. L., N. Lukacs, et al. 1995). In human cells, leukocyte cell-derived chemotaxin-1 or chondromodulin-1 is a 25 kDa glycoprotein generated from a larger transmembrane precursor after post-translational modification and proteolytic cleavage. It stimulates the proliferation, proteoglycan synthesis, and colony formation of cultured growth plate chondrocytes *in vitro* (Inoue, H., J. Kondo, et al. 1997) and showed the inhibition of DNA synthesis, and tumor growth *in vivo*(Hayami, T., C. Shukunami, et al. 1999).

Even though there have been several reports on leukocyte cell-derived chemotaxin-2 in lower-order species including fish *Plecoglossus altivelis*(Chen, J., X. J. Lu, et al. 2010),

rainbow trout (Kokkinos, P. A., A. Kazantzi, et al. 2005), carp *Cyprinus carpio*(Fujiki, K., D. H. Shin, et al. 2000), only zebrafish (CAQ14216) leukocyte cell-derived chemotaxin-1 has been identified from fish. Furthermore, there is no information on cloned chemotaxin genes in mollusks to date. Therefore, molecular understanding of leukocyte cell-derived chemotaxin-1-like gene from abalone could be useful to understand properties of new chemotactic factors in mollusks.

In this study, we report the identification, molecular characterization and expression analysis of a BRICHOS domain-containing leukocyte cell-derived chemotaxin 1-like cDNA from disk abalone *Haliotis discus discus* which was denoted as AbLECT-1.

2. Materials and method

2.1. Identification and sequence analysis of AbLECT-1

We identified the complete coding sequence of AbLECT-1 from the disk abalone normalized cDNA library (EST database). The basic procedures of cDNA library construction, normalization and sequencing were described in a previous report (De Zoysa, M., W. A. Pushpamali, et al. 2008). The Basic Local Alignment Tool (BLAST) program was used to search nucleotide and protein sequences similar to AbLECT-1 (Altschul, S., W. Gish, et al. 1990). Characteristic domain or motifs were identified using PROSITE profile analysis (Bairoch, A., P. Bucher, et al. 1997). Pairwise and multiple sequence alignment were analyzed using the CLUSTALW (version 1.83) program (Pearson, W. 1990). The signal peptide was predicted by the SignalP program (Nielsen, H., J. Engelbrecht, et al. 1997).

2.2. Experimental Animals

Health disk abalone (*H. discus discus*), with an average body length of 6–7 cm, were obtained from Youngsoo abalone farm (Jeju Island, Republic of Korea). Abalones were maintained in fiberglass tanks (65 × 50 × 22 cm) with aerated and sand-filtered seawater at 18–20 °C in our laboratory. The salinity and pH of the seawater were maintained at 32‰ ± 1 and 8.0 ± 0.5, respectively. Water quality parameters were monitored by digital meter (YSI-USA). All abalones were acclimatized for 1 week prior to the experiment. A maximum of 20 animals per tank were maintained during the experiment. Only active abalones, as determined by general appearance and foot movements, were used for tissue extraction.

2.3. Abalone tissues and hemolymph isolation

To study the tissue-specific mRNA expression of AbLECT-1, gills, mantle, muscle, digestive tract, hepatopancreas were isolated from three healthy (un-treated) abalones. The abalone hemolymph (1 mL/abalone) from three healthy animals was withdrawn from the cephalic arterial sinus, accessed anteriorly at the angle between the foot and the head using a syringe fitted with a 22-gauge needle. The hemolymph was immediately centrifuged (3000 × g for 10 min at 4 °C) and obtained the hemocytes after removal of supernatant. All the tissues and hemocytes were immediately snap-frozen in liquid nitrogen and stored at -70°C.

2.4. Bacterial challenge of abalones

To determine the immune responses of AbLECT-1, with three bacteria strains were used to challenge the abalones including *V. alginolyticus* (KCTC2472), *V. parahemolyticus* (KCTC2729), and *L. monocytogenes* (KCTC3710). The purpose of using bacteria mixture was to make greater pathogenic effect to abalone. The choice of two Vibrio species was justified by the pathogenicity of these strains for abalone based on the previous reports. Briefly, *V. alginolyticus and V. parahemolyticus* were cultured on LB marine plates at 25 °C overnight. From each plate a single colony was selected to inoculate 4 mL of marine broth at 25 °C for 16 h. Similarly, *L. monocytogenes* was cultured on an LB agar plate at 30 °C overnight, and then inoculated in LB broth at 30 °C for 16 h. Then, all three bacterial cultures (1.5 mL) were centrifuged at 7000 × g at 4 °C for 5 min. The supernatant fluid was removed and the bacterial pellets were re-suspended in phosphate-buffered saline (PBS). Equal concentration from each bacterium was mixed to make bacterial stock. Abalones were intramuscularly (i.m.) injected with 50 µL (per abalone) of bacterial mixture having cell count of 5×10^7 cells / mL in bacterial stock. The control group was injected with same

volume of PBS. Abalone hemocytes samples were removed at 3, 6, 12, 24, and 48 h post challenge of bacteria-injected abalones. All of the tissue samples and hemocytes were immediately snap-frozen in liquid nitrogen and stored at −80 °C until used for RNA isolation.

2.5. RNA isolation and cDNA synthesis of abalones

The total RNA was extracted from pooled tissues of three abalones using Tri Reagent™ (Sigma, USA) according to the manufacturer‘s protocol. Originally purified RNA was diluted to 1 μg/μL concentration prior to synthesis of cDNA. 2.5 μg RNA was used to synthesize cDNA from each tissue using a SuperScript III first-stand synthesis system for RT-PCR (Invitrogen, USA). Briefly, RNA was incubated with 1μL of 50 μM oligo(dT) $_{20}$ and 1μL of 10 mM dNTP for 5 min at 65℃. After incubation, 2μL of 10X cDNA synthesis buffer, 2μL of dithiothreitol (DTT, 0.1M) 4μL of 25mM $MgCl_2$, 1μL of RNaseOUT^{TM} (40 U/μL) and 1μL of SuperScript III reverse transcriptase (200 U/μL) were added and incubated for 1 h at 50℃. The reaction was terminated b raising the temperature to 85℃ for 5 min. Finally, 1 μL of RNase H was added to each synthesized cDNA and incubated for 20 min at 37 ℃. The resulting cDNA was diluted 10 fold (total 200 μL) before being stored at -20℃

2.6. Transcriptional analysis of abalone LECT-1 by quantitative real-time PCR

The AbLECT-1 transcriptional response against bacterial infection was determined in hepatopancreas, hemocyte, gill, mantle, muscle and digestive tract using time-frame analyzed by quantitative real-time PCR (qRT-PCR). AbLECT-1 gene-specific primers were designed based on the AbLECT-1 coding sequence (forward:-5′- ACTTCTGTGACGGTT GCAAAGTGC-3′ and reverse: 5′-TCTTGGACAGCCTCTTGACAGCTT-3′). The abalone ribosomal protein (Accession: EF103443) was selected as a reference gene, and it was amplified using (forward:-5′-GGGAAGTGTGGCGTGTCAAATACA-3′ and reverse: 5′-TCCCTTCTTGGCGTTCTTCCTCTT-3′) gene-specific primers. Real-time PCR was carried

out in a 20 μL reaction volume containing 4 μL of 1:10 diluted original cDNA, 10 μL of 2X SYBR Green Master Mix, 1.0 μL of each primer (10 pmol/μL), and 4.0 μL of PCR grade water using Thermal Cycler Dice™ Real Time System (TaKaRa, Japan). The real-time PCR cycling protocol was: one cycle of 94℃ for 3 min ; followed by 35 cycles of 95℃ for 20s, 58℃ for 20s, and 72℃ for 30s. The base line was set automatically using the Termal Cycler Dice™ Real Time System Software (version 2.00). The relative expression was determined by means of the $2^{-\Delta\Delta CT}$ method (Livak, K. and T. Schmittgen 2001). All data represent means ± standard deviation, and were subjected to one-way analysis of variance (ANOVA), followed by Duncan's Multiple range test, using SPSS version 11.5. Differences were considered statistically significant at $p < 0.05$

3. Results and discussion

3.1. Identification and characterization of AbLECT-1 cDNA

In this study, complete cDNA sequence of disk abalone BRICHOS domain-containing leukocyte cell-derived chemotaxin 1-like gene (GenBank accession number FJ864723) was determined (Fig. 1). The full-length (705 bp) of AbLECT-1 gene was shown to have a 576 bp open reading frame (ORF) that translates into a putative peptide of 192 amino acid peptide. The ORF was predicted to produce a 21 kDa molecular mass protein with 8.9 isoelectric point (pI). Signal peptide prediction results showed that AbLECT-1 has an N-terminal signal peptide with the cleavage site between 16 and 17 amino acids. A characteristic BRICHOS domain (93 aa) was identified in the amino acid sequence ranging from 53–146. In addition, it was shown that there was a 55 bp long 5′ untranslated region (5 ′UTR) and 74 bp 3′ UTR with a typical polyadenylation signal (^{579}AATAAA^{585}). There were seven cysteines in the mature peptide of AbLECT-1.

Data base search by NCBI BLASTp revealed that the most highest match (Identity = 28%; E-value = 0.55; gaps-25%) of AbLECT-1 to putative adhesion exoprotein of bacteria *Lactobacillus jensenii* (ZP_04646030) sequence which has extraordinary higher amino acid length (2766 aa) than to AbECT-1 (192 aa). However, motif scan analysis results showed that there was no BRICHOS domain in *L. jensenii* putative adhesion exoprotein. Further BLASTp results shown that AbLECT-1 was similar to zebrafish leukocyte cell-derived chemotaxin 1 as well (Identity = 22%; E-value = 4.6; gaps-18%). Therefore, we justified that identified abalone sequence could be a putative homologue of leukocyte cell-derived chemotaxin 1 in mollusk. However, AbLECT-1 showed very low identity to known LECT-1 counterparts displaying only 15.5 and 13.8% identity to human, and mouse LECT-1 sequences, respectively. ClustalW multiple analysis was done using the BRICHOS domain of leukocyte cell-derived chemotaxin-1 of abalone, bovine, human, mouse, chicken and

zebrafish (Fig. 2). Results showed that the BRICHOS domain length (94 aa) of AbLECT-1 was within the range of other known sequences. Even though a higher number of strongly and weekly conserved amino acids were available, only a few identical amino acids are aligned with all selected sequences. Low identity and similarity of AbLECT-1 may be due to limited LECT-1 sequences available from lower-order species. It was reported that the BRICHOS domain includes a pair of conserved cysteine residues that probably form a disulfide bridge (Sanchez-Pulido, L., D. Devos, et al. 2002). Similarly, the BRICHOS domain of AbLECT-1 contains highly conserved two cysteine residues at C^{80} and C^{137}. The presence of the BRICHOS domain-containing genes in *Caenorhabditis elegans* and *Drosophila melanogaster* suggests an ancient origin close to the appearance of animals (Sanchez-Pulido, L., D. Devos, et al. 2002). In this study we identified a BRICHOS domain-containing cDNA sequence which is similar to leukocyte cell-derived chemotaxin 1-like gene suggesting that similar chemotactic genes may be present in other species of the phylum mollusk. However, leukocyte cell-derived chemotaxin 1 seems to be more widely conserved in vertebrates compared to invertebrates (Inoue, H., J. Kondo, et al. 1997; Hayami, T., C. Shukunami, et al. 1999).

3.2. Expression profile and transcriptional responses of AbLECT-1 against bacterial infection

To determine the transcriptional level of AbLECT-1 in various abalone tissues, qRT-PCR was performed and compared with the level of expression in hepatopancreas (Fig. 3). AbLECT-1 mRNA was constitutively expressed in all selected abalone tissues. The highest and lowest mRNA expression was detected in mantle and hepatopancreas, respectively. Gill tissue showed the second highest expression (154-fold) among selected tissues. Expression of AbLECT-1 mRNA was induced at 3 h and then slightly decreased near to basal level by 6 h, then gradually increased up to 48 h post bacterial challenge (Fig. 4). There has been no

evidence for tissue-specific expression analysis of LECT-1 for fish or other invertebrates animals. However, constitutive expression of chemotaxin 2 was detected in brain, heart, kidney, liver and spleen of several fish species including *Plecoglossues altivelis* (Chen, J., X. J. Lu, et al. 2010), rainbow trout (Kokkinos, P. A., A. Kazantzi, et al. 2005), and *Pseudosciaena crocea* (Li, M. Y., J. Chen, et al. 2008). It has been reported that chemotactic factors play important role in activating neutrophils during inflammatory conditions (Kunkel, S. L., N. Lukacs, et al. 1995). More specifically, chemotaxin 2 was induced in several fish species including *Plecoglossues altivelis* by bacteria (Chen, J., X. J. Lu, et al. 2010); *P. crocea* by *V. alginolyticus* infection (Li, M. Y., J. Chen, et al. 2008); zebrafish by *Aeromonas salmonicida*; and, *Staphylococcus aureus* (Lin, B., S. Chen, et al. 2007). Similarly, AbLECT-1 was induced by three bacteria including challenge by *V. alginolyticus*, *V. parahemolyticus*, and *L. monocytogenes* suggesting that it may participate innate immune defense system of disk abalone.

In conclusion, we identified a BRICHOS domain-containing leukocyte cell-derived chemotaxin 1-like gene from disk abalone, which showed constitutive expression in various tissues as well as transcriptional induction in hemocytes after bacterial challenge. The characterization of leukocyte cell-derived chemotaxin 1 counterparts from abalone could expand our understanding of evolutionary relationships and their immune responses especially in invertebrate mollusks.

Fig. 7. The complete nucleotide and deduced amino acid sequences of the disk abalone leukocyte cell-derived chemotaxin-1-like cDNA. The start (ATG) and stop (TAA) codons are in boldface. The predicted signal peptide sequence is bold shaded. The predicted BRICHOS domain is underlined. The polyadenylation signal (AATAAA) is in bold italics and the poly-(A) tail is underlined at the end of the nucleotide sequence.

Fig. 8. ClustalW multiple alignment of the predicted BRICHOS domain of abalone LECT-1 and other known LECT-1 from bovine, human, mouse, chicken and zebrafish. Identical amino acid residues are shaded indicated by asterisks (*). Residues which are strongly conserved and weakly conserved are indicated by colons (:) or dots (.), respectively. The dashes indicate the gaps introduced to maximize the alignment.

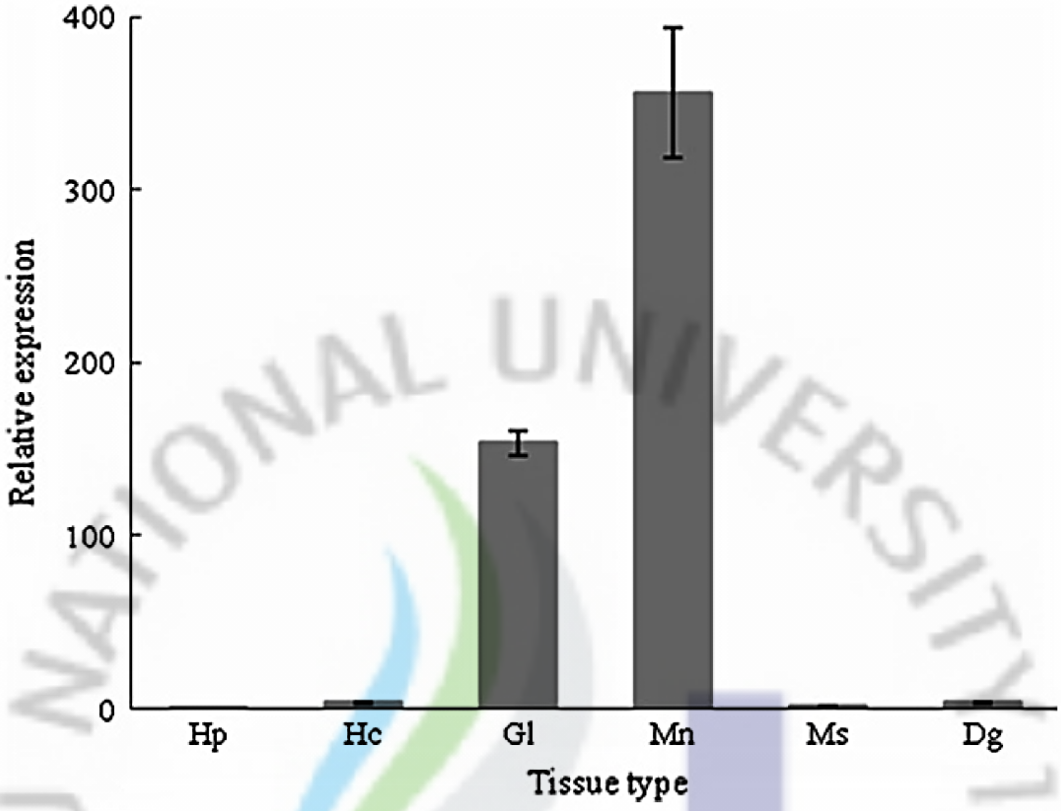

Fig. 9. Tissue specific mRNA expression of AbLECT-1 in disk abalone. Analysis of mRNA was done by qRT-PCR. The relative expression fold was calculated by the $2^{-\Delta\Delta CT}$ method using abalone ribosomal protein as a reference gene. Hp-hepatopancreas, Hc-hemocytes; Gl-gills; Mn-mantle; Ms-muscle; Dg-digestive tract.

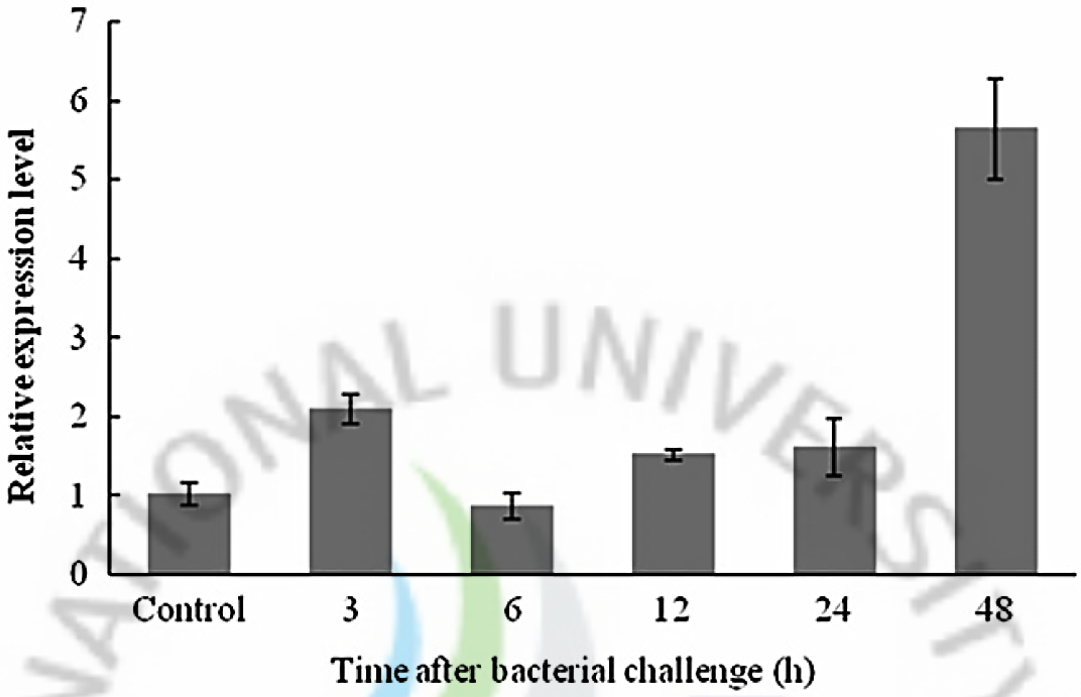

Fig. 10. Transcriptional regulation of AbLECT-1 in hemocytes after bacteria (*L. monocytogenes, V. alginolyticus* and *V. parahemolyticus*) challenge. Analysis of AbLECT-1 mRNA expression was done by qRT-PCR. The expression fold- was calculated by the $2^{-\Delta\Delta CT}$ method using abalone ribosomal protein as a reference gene. The relative expression fold at each time point was compared to that of PBS-injected sample as a control.

Summary

Leukocyte cell-derived chemotaxin-2 (LECT2) was first isolated from the culture fluid of the human (*Homo sapiens*) T cell line and was shown to have neutrophil chemotactic activity. Human LECT2, a 16-kDa basic protein, consisting of 133 amino acid residues and three intramolecular disulphide bonds, is specifically expressed in the adult and fetal livers. LECT2 is identical to chondromodulin-II, a bovine protein that stimulates the proliferation of chondrocytes and osteroblasts. Although it was originally demonstrated to have a chemotactic function in vitro, recent data sustain a further multifunctional role of LECT2 that extends from cell growth, differentiation, damage/repair process and carcinogenesis to autoimmune diseases.

In this study, both LECT2 isolated from rock bream sequence database by genome sequence FLX and LECT1 isolated from the disk abalone normalized cDNA library and cDNAs were sequenced to determine the full length sequences of RbLECT2 and AbLECT1. the resulting full-length RbLECT2 and AbLECT1 sequences were compared with other known sequences available in the National Center for Biotechnology Information (NCBI) database. after having the full length of RbLECT2 and AbLECT1, sequence characterization, ClustalW pairwise and multiple analysis, and phylogenetic analysis were performed in order to establish the relationship between known respective genes.

Over and above for the RbLEC2 expression analysis, isolated muscle, blood, brain, spleen, heart, gill, muscle, liver, kidney, head kidney, skin, tissues obtained from uninduced animals were used as blank to compare the effect of PBS injection in rock bream. Tissue-specific mRNA expression of AbLECT-1, gills, mantle, muscle, digestive tract, hepatopancreas were isolated from healthy abalone.

For the *In vivo* to determine the immune responses of RbLECT2, pathogenic bacterium *Edwardsiella tarda* and lipopolysaccharide (LPS) were used as immunostimulants in time course experiments. Rock bream liver samples were taken from *E. tarda* challenged, and

LPS-injected rock bream to determine the transcriptional profiles of RbLECT2. At time point of 3, 6, 12, 24 and 48h post infection/ induction (p.i.) from the animals in order to determine the transcriptional profiles of RbLECT2. Similarly, to determine the immune responses of AbLECT-1, with three bacteria strains were used to challenge the abalones including bacteria mixture. Abalone hemocytes samples were removed at 3, 6, 12, 24, and 48 h post challenge of bacteria-injected abalones. The control group was injected with same volume of PBS.

The cloned full-length RbLECT2 consisted of 768 bp. The open reading frame was composed of 456 bp that translate into a putative peptide of 151 amino acid residues. Sequence analysis indicated contains Peptidase_M^{23} domain (^{152}AGAATCCAC-AGTGACCCC^{448}) following a typical polyadenylation signal (^{736}AATAAA^{742}). There was a signal peptide (^{8}ATGAGACGT- GTGTGATGG^{54}) with the RbLECT2 contain four N-myristoylation sites(^{45}GGTGTA-AACACA^{91} , ^{127}GGAGCC-GGGAGC^{135}). The RbLECT2 has a putative molecular mass of 17 kDa with 9.4 isoelectric point (p. i). The RbLECT2 demonstrated the high identity (over 70%) to teleostei LECT2. Phylogenetic analysis, based on the amino acid sequence alignment, showed that rock bream LECT2 protein is clustered primarily with other teleost LECT2 molecules, and secondary with the counterparts from different species.

Further analysis results showed that RbLECT2 mRNA expression was higher in the liver and skin than the gill, where liver showed the highest level of expression with 110-fold compared to that observed for muscle. RbLECT2 mRNA expression levels in the liver following an in vivo immune challenge were determined. The RbLECT2 transcript level was up-regulated in liver tissue after all challenges during the period of the experiment, 3- 48 h p.i. The LPS induction, RbLECT2 was up-regulated after 3 – 24 h and the highest relative expression (2.7 - fold) was observed at 24 h p.i compred to control. As a late-phase response for LPS, RbLECT2 mRNA level had again increased significantly at 3 h – 24 h p. i. RbLECT2 transcript level varied in a slightly different pattern for the E. tarda injection form the LPS induction in liver. Initially, it was up-regulated at alltime point with mild alteration.

Previously reported that the LECT2 mRNA in zebrafish liver was up-regulated to 1000-fold upon infection by *Aeromonas salmonicida* and Staphylococcus aureus, suggesting that LECT2 served as a positive important phase protein in fish.

The full-length (705 bp) of AbLECT-1 gene was shown to have a 576 bp open reading frame (ORF) that translates into a putative peptide of 192 amino acid peptide. The ORF was predicted to produce a 21 kDa molecular mass protein with 8.9 isoelectric point (pI). Signal peptide prediction results showed that AbLECT-1 has an N-terminal signal peptide with the cleavage site between 16 and 17 amino acids. A characteristic BRICHOS domain (93 aa) was identified in the amino acid sequence ranging from 53–146. There were seven cysteines in the mature peptide of AbLECT-1. Data base search by NCBI BLASTp revealed that the most highest match (Identity = 28%; E-value = 0.55; gaps-25%) of AbLECT-1 to putative adhesion exoprotein of bacteria Lactobacillus jensenii (ZP_04646030) sequence which has extraordinary higher amino acid length (2766 aa) than to AbECT-1 (192 aa). However, motif scan analysis results showed that there was no BRICHOS domain in L. jensenii putative adhesion exoprotein. Further BLASTp results shown that AbLECT-1 was similar to human leukocyte cell-derived chemotaxin 1 as well (Identity = 15.5%). ClustalW multiple analysis was done using the BRICHOS domain of leukocyte cell-derived chemotaxin-1 of abalone, bovine, human, mouse, chicken and zebrafish.

To determine the transcriptional level of AbLECT-1 in various abalone tissues, qRT-PCR was performed and compared with the level of expression in hepatopancreas. AbLECT-1 mRNA was constitutively expressed in all selected abalone tissues. The highest and lowest mRNA expression was detected in mantle and hepatopancreas, respectively. Expression of AbLECT-1 mRNA was induced at 3 h and then slightly decreased near to basal level by 6 h, then gradually increased up to 48 h post bacterial challenge.

Taken together, isolation, sequence characterization and tissue expression analysis of LECT2 and LECT1 gene in rock bream and abalone could be considered as new era of immunological research with to vertebrate and invertebrate LECT regulatory immune system.

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