Transcriptional regulation of chicken leukocyte cell-derived chemotaxin 2 (chLECT2) in response to toll-like receptor 3 stimulation

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Title of the manuscript: Expression Analysis of Leukocyte Cell-Derived Chemotaxin 2 (LECT2) by TLR3 Signal in Chicken
ABSTRACT

Objectives Leukocyte cell-derived chemotaxin 2 (LECT2) is associated with several physiological processes including inflammation, tumorigenesis, and natural killer T cell generation. Chicken LECT2 (chLECT2) gene was originally identified as one of the differentially expressed genes (DEGs) in chicken kidney tissue, where in, chicken was fed with different calcium doses. In this study, the molecular characteristics and gene expression of chLECT2 were analyzed under the stimulation of Toll-like receptor 3 (TLR3) ligand to understand the involvement of chLECT2 expression in chicken metabolic disorders.

Methods: Amino acid sequence of LECT2 proteins from various species including fowl, fish, and mammal were retrieved from the Ensembl database and subjected to Insilco analyses. In addition, the time- and dose-dependent expression of chLECT2 were examined in DF-1 cells which were stimulated with polyinosinic:polycytidylic acid (poly (I:C)), a TLR3 ligand. Further, to explore the transcription factors required for the transcription of chLECT2, DF-1 cells were treated with poly (I:C) in the presence or absence of the nuclear factor κB (NFκB) NFκB and activated protein 1 (AP-1) AP1-inhibitors.

Results: The amino acid sequence prediction of chLECT2 protein revealed that along with duLECT2, it has unique signal peptide different from other vertebrate orthologs, and only chLECT2 and duLECT2 has additional 157 and 161 amino acids on carboxyl terminus, respectively. Phylogenetic analysis suggested that chLECT2 is evolved from a common ancestor along with actinopterygii hence, closely related than the mammals. Our qPCR results showed that, the expression of chLECT2 was up-regulated significantly in DF-1 cells under the stimulation of poly (I:C) (p<0.05). However, in the presence of NFκB or AP-1 inhibitors, the expression of chLECT2 is suppressed suggesting that both NFκB and AP-1 transcription factors are required for the induction of chLECT2 expression.
Conclusion: The present results suggest that *chLECT2* gene might be a target gene of TLR3 signaling. For the future, the expression pattern or molecular mechanism of chLECT2 under stimulation of other innate immune receptors shall be studied. The protein function of chLECT2 will be more clearly understood if further investigation about the mechanism of LECT2 in TLR pathways is conducted.

Key words: Chicken, LECT2, Gene expression, Innate immune receptor signaling
INTRODUCTION

Leukocyte cell-derived chemotaxin 2 (LECT2) is a multifunctional protein secreted from liver, and a chemotactic factor for neutrophils. Human LECT2 has a molecular mass of ~16 kDa [1], with three intramolecular disulfide bonds [2]. The amino acid sequence has been well conserved throughout evolution. Accumulating evidence indicate that, LECT2 is associated with several physiologic functions including regulation of liver regeneration [3], tumorigenesis [4, 5], suppression of inflammatory arthritis and cytokine production [6, 7], hepatic natural killer T (NKT) cells generation [8]. It is reported that plasma LECT2 concentration of the patients with bacterial sepsis is significantly lower than healthy human and LECT2 expression is correlated with systemic inflammation [9]. In LECT2-knockout mice, the hepatitis gets more severe due to the increased number of natural killer T cells and their cytokine ligands, and therefore, the homeostasis of NKT cells in the liver is negatively regulated by LECT2 [8, 10]. It was also reported that LECT2 has anti-inflammatory and tumor-suppressive actions in oncogenic β-catenin-induced liver tumorigenesis [5]. Human LECT2 is associated with inflammation and insulin resistance and the suppression of inflammatory and immune responses by LECT2 can inhibit tumorigenesis in liver [11, 12].

In chicken, LECT2 was identified as mim-1 protein, P33, which is chemotactic for heterophils [13], and it also activates antimicrobial responses toward Salmonella enteritidis from chicken heterophils [13]. In Salmonella enteritidis infected chickens, the level of LECT2 protein was decreased in heterophils compared to macrophages without difference at RNA level [14, 15]. In addition, the expression of duck LECT2 was significantly increased in liver and spleen by duck hepatitis virus type I infection [16], or the treatment of polyinosinic-polycytidylic acid (poly (I:C)), implying the LECT2 may play a role in the defense mechanism against the viral infection in duck and be regulated by innate immune receptor signaling pathways, i.e., Toll-like receptors 3 (TLR3)
The recognition of the microbial molecular patterns by TLRs trigger the intracellular signaling cascades where transcription factors, such as nuclear factor κB (NFκB) and activated protein 1 (AP-1) are activated for the transcriptional regulation of target genes [17]. In chicken, flagellin and lipopolysaccharide (LPS), which are bacterial products, activate both NFκB and AP-1 signaling pathways; the engagements of TLRs-TLR ligands lead to the degradation of the inhibitor of NFκB (IκB) to allow the release of NFκB for translocation into the nucleus, and activate mitogen-activated protein kinase (MAPK) to phosphorylate AP-1, leading to the binding to the promoter of the target genes by activated NFκB and AP-1 [18, 19].

In this study, we evaluated transcriptional regulation of chicken LECT2 (chLECT2) in response to a TLR3 ligand engagements and explored signaling pathways responsible for the transcription of chLECT2 with the inhibitors of NFκB and AP-1 in chicken DF-1 cells.

**MATERIALS AND METHODS**

**Bioinformatics analysis**

The LECT2 amino acid sequences of various species (chicken, duck, zebrafish, asian seabass, mouse, rat, pig, cow, human and chimpanzee) were retrieved from the Ensembl database (http://www.ensembl.org/) (Table 1). The amino acid sequences were aligned with the ClustalW implemented in the BioEdit tool. The protein domains were predicted by using the SMART domain search program (http://smart.embl-heidelberg.de/). Phylogenetic analyses were performed with MEGA7 software [20].

**Cell culture**

The chicken DF-1 cell line was purchased from the American Tissue Culture Collection (CRL-12203, Manassas, VA, USA). DF-1 cells were cultured in Dulbecco’s modified Eagle...
medium (DMEM), supplemented with 10% fetal bovine serum, 2 Mm L-glutamine and 100 U/ml each of penicillin and streptomycin (Thermo Scientific, Logan, UT, USA) at 37°C in a humidified atmosphere of 5% CO₂.

Stimulation of DF-1 cells by TLR3 ligand and inhibitors of NFκB and AP-1

Poly (I:C) (TLR3 ligand) was purchased from InVivogen (San Diego, CA, USA) and dissolved in endotoxin and nuclease free water and kept at -20°C until use. DF-1 cells were treated at the concentrations of 0.1, 1, 5, and 10 μg/ml for 24 hr. Also, to determine the time kinetics of mRNA expression, cells were treated for 1, 3, 6, 12, and 24 hr at 10 μg/ml concentration. BAY 11–7085 and Tanshione IIA were purchased from Sigma-Aldrich (Louis, MO, USA). BAY 11-7085 (BAY) inhibits the IκBα phosphorylation, resulting in prevention of NFκB activation, was used as NFκB inhibitor. Tanshinone IIA (Tan-II) may inhibit the binding of AP-1 to DNA in chicken heterophils [19]. DF-1 cells were pretreated with BAY 11–7085 (5 μM) and Tanshinone IIA (25 μM), for 3 hr, of which concentrations and time were determined from the preliminary experiment in this study, and then stimulated with poly (I:C) (5 μg/ml) for 3 hr.

RNA extraction and cDNA preparation

Trizol (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from chicken DF-1 cell. Total RNA was quantified by NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). After quantification, 2 μg of total RNA was reverse-transcribed to synthesize cDNA using the using QuantiTect Reverse Transcription Kit (Toyobo, Osaka, JAPAN) according to the manufacturer’s instructions.
Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Quantitative real-time polymerase chain reaction (qRT-PCR) was conducted with a CFX-96 RT-PCR detection system (BioRad, Hercules, CA, USA) for the analysis of chLECT2 expression. The qRT-PCR conditions were as follows: an initial step at 94°C for 3 min; 39 cycles at 94°C for 10 sec, 58°C for 30 sec, and 72°C for 30 sec; and a final step at 72°C for 10 min. Dissociation was performed at 0.5°C increments from 55°C to 95°C for over 25 min.

The primer sequences of the chicken interleukin 1B (chIL1B), chLECT2 and GAPDH used in this study are shown in Table 2. All samples were measured in triplicates to ensure reproducibility, and Ct values were calculated by the 2^{-ΔΔCt} method [21], to calculate the relative expression level of mRNA on each sample, which was expressed as a ratio relative to GAPDH expression.

Statistical analysis

Results were presented as means ± standard deviation (SD) of triplicate independent experiments. Statistical significance was assessed using a Student’s t-test. A value p<0.05, as compared with the non-treated control, was considered statistically significant.

RESULTS

Phylogenetic analysis of LECT2 sequences

The amino acid sequence of chLECT2 was analyzed and compared with other species. The chLECT2 gene sequence was identified as a DEG from chicken kidney RNA-seq study, where in chickens fed with different doses of calcium [22]. To verify and to compare the chicken-LECT2 amino acid sequences with other vertebrates, the amino acid sequences of LECT2 orthologs from fowl, fishes and mammal including duck, zebrafish, asian seabass,
mouse, rat, pig, cow, human and chimpanzee) were retrieved from Ensemble database and aligned (Figure 1A). The analysis with SignalP v4.0 program (http://www.cbs.dtu.dk/) showed that chLECT2 and duck LECT2 (duLECT2) had the same signal peptide whose amino acid sequences starts at position 1 and ends at position 18. Besides, chLECT2 and duLECT2 had additional amino acids on carboxy terminus (157 for chicken and 161 amino acids for duck) compared to other species. The evolutionary relationships between chLECT2 and LECT2 orthologs were analyzed using phylogenetic tree (Figure 1). Phylogenetic analysis suggested that chLECT2 is evolved from a single gene as a common ancestor with duck. Also, it exhibited LECT2 from fowls are relatively closely related to fishes, while far from mammalian LECT2s.

2. chLECT2 expression from DF-1 cells in response to TLR3 ligand stimulation

To understand the expression patterns of this gene, the transcriptional profile of chicken LECT2 was investigated in chicken DF1 cells under the stimulation with TLR3/MDA5 agonist, poly (I:C). To verify whether the expression of chLECT2 gene is regulated by TLR3 signaling, the expression of chIL1B, which is known to be involved in TLR3 signaling, was also examined along with chLECT2. Stimulation with 10 μg/ml of poly (I:C) significantly upregulated both chLECT2 and chIL1B expressions (Figure 2A and 2C). The levels of the chLECT2 and chIL1B were increased significantly from 1 hr after stimulation with 10 μg/ml of poly (I:C), and reached to maximum at 6 hr after stimulation, which lasted to 24 hr (Figure 2B and 2D).

3. Effects of transcription inhibitors on chLECT mRNA expression

To determine whether transcription factors NFκB and AP-1 are responsible for chLECT2 transcription, DF-1 cells were pre-treated with NFκB inhibitor or AP-1 inhibitor before poly
(I:C) stimulation. The expressions of both *chLECT2* and *chIL1B* were up-regulated after treatment of 5 μg/ml of poly (I:C) without the inhibitors. However, in the presence of transcriptional inhibitors, i.e., BAY or Tan-II, the expressions of *chLECT2* and *chIL1B* were significantly suppressed compared to poly (I:C)-only treated control.

**DISCUSSION**

In mammals, *LECT2* is known as a hepatokine mediating obesity with insulin resistance and associated with the inflammatory response [23, 24]. It was also reported that *LECT2* expression is increased in non-alcoholic fatty liver disease in association with metabolic syndrome such as abdominal obesity and lipid metabolism [25]. However, *chLECT2* may play a different function compared to mammals. In previous report, we found the expression of *LECT2* increased from the kidney of broiler chickens fed with excessive calcium which is required for the growth of chickens including body weight gain and eggshell formation [22]. The high calcium doses in feed cause reduced body weight gain and lead to stress-induced diseases, even though it caused the increased expression of *chLECT2*. In this study, to investigate the role of *chLECT2* in chicken immune response and metabolic disturbance, the expression patterns of *chLECT2* under the stimulation of poly (I:C) revealed that the gene is involved in TLR3 signaling. Therefore, it is suggested that *chLECT2* may be related to the innate immune system upon the viral infection. Interestingly, Bornelov et al. recently reported that *chLECT2* expression in adipose tissue of layer hens is higher than broilers and suggested that the gene expression is related in the regulation of reproduction [25]. Compared to this study, this means the function of *LECT2* in chicken may vary tissue-specifically.

TLR3 plays a key role in initiating the immune response in chicken. As pathogens are detected by TLRs, it leads to the activation of transcription factors such as *NF-κB* and *AP*.
nuclear factor κB (NF-κB) and activated protein 1 (AP-1). Afterwards, the transcriptional pathways stimulate the expression of pro-inflammatory cytokines including interleukin-1β (IL1B) and type I interferon (IFN) and these downstream cytokines regulate the activity of immune-competent cells playing a role in innate immune defense systems. In particular, TLR3, as the receptor for viral dsRNA, triggers the antiviral immune pathway and induces the expression IFN which plays a critical role to antiviral activity.

Recently, Kamimura et al. reported that the transcription of the cytokine was induced by the transcriptional factors, NF-κB and AP-1 after stimulation of different TLR ligands in chicken vaginal cells. Also, they showed that the expression of chIL1B was suppressed by the inhibitors of the transcriptional factors, which are Bay for NF-κB, and Tan-II for AP-1 [26]. These results suggest that in chicken, poly (I:C) can stimulates the expression of chLECT2 and chIL1B —through the activation of both transcriptional factors, NF-κB and AP-1. Therefore, as for the typical TLR3 signal pathway, the expression of chLECT2 can be induced by NF-κB and AP-1 under the stimulation of poly (I:C).

Besides mammal, several reports suggest that LECT2 plays important role in the inflammatory response and immune system in fish. The up-regulation of fish LECT2 expression under bacterial infection has been reported in many fishes, including zebrafish with Aeromonas salmonicida [27], croceine croaker with Vibrio alginolyticus [28], asian seabass with Vibrio harveyi [29], lamprey with Escherichia coli [30]. In asian seabass and croceine croaker, the expression of LECT2 was significantly increased in liver and spleen with bacterial infection compared to the healthy fish. As shown in Figure 1, the amino acid sequences of LECT2 orthologs have generally well conserved sequences but chLECT2 and duLECT2 have the same signal peptides and longer carboxy termini compare to other LECT2 orthologs. The phylogenetic analysis also revealed that chLECT2 is much closer to duck and
fishes than mammals. This might cause the similar but somewhat different function of chLECT2 compared to mammal orthologs.

Poly (I:C) is a prototypical class of PAMPs, which are associated with pathogens. These molecules are recognized by Toll-like receptors (TLRs) and activate the innate immune system. Poly (I:C) is a stable synthetic dsRNA analogue which is generated during the viral replication, and is recognized by endosomally localized TLR3 ligand [31]. It is known that TLR3 prefers to recognize the synthetic poly (I:C) rather than virus-derived dsRNA, suggesting that TLR3 recognizes a unique dsRNA structure, different from other dsRNA-binding proteins [32]. The crucial role of TLR3 in poly (I:C) recognition was demonstrated by TLR3-knockout mice showing reduced inflammatory responses upon the treatment of poly (I:C) [33]. Many TLR3 effects rely on cells of the innate immune system that either express TLR3 or respond to inflammatory mediators that are produced upon TLR3 signaling.

In this study, we investigated the expression of chLECT2 from DF-1 cells in response to TLR3 ligand, and identified that NFκB and AP-1 transcription factors are indispensable for TLR3-mediated expression of chLECT2. The genetic information and the expression patterns of chicken LECT2 gene revealed that chLECT2 is evolutionarily much closer to duck or fishes than the mammal orthologs. Because LECT2 gene is known to be involved in inflammatory response, this report studied its biological role in chicken by investigating its expressional change under the treatment of TLR3 ligand and the inhibitors of transcriptional factors involved in TLR3 signaling. Also, the up-regulation of chLECT2 expression after the stimulation of poly (I:C) suggests that this gene might be related to viral infection in chicken, and the significantly lower expressional level of chLECT2 under the presence of transcriptional inhibitors, Bay and Tan-II verifies that the expression of this gene is involved in TLR3 signal pathway.
CONCLUSION

Exploring the expression profiles of chLECT2 and mechanisms by transcription factors of which activities are under TLR3 signals and regulate transcription of TLR3 target genes may provide the fundamental knowledge for LECT2 in chicken. Further study may confirm the contribution of chLECT2 in the innate immune system against viral diseases and chLECT2 gene can be a promising candidate as a biomarker for the infectious diseases in chickens.

CONFLICT OF INTEREST

The authors declare no conflict of interest. All the authors have approved the manuscript and agreed with submission.

ACKNOWLEDGMENTS

The authors thank Dr. Vijayakumar Gosu for critical reading and editing of this manuscript. This work was supported by grants from the Next-Generation BioGreen 21 Program (No. PJ01324201, PJPJ01315101), Rural Development Administration, Republic of Korea.
REFERENCES


28. Li MY, Chen J, Shi YH. Molecular cloning of leucocyte cell-derived chemotaxin-2 gene


FIGURE LEGENDS

Figure 1. Analysis of LECT2 amino acid sequences. (A) Comparison of amino acid sequences of chicken LECT2 in various species (chicken, duck, zebrafish, Asian seabass, mouse, rat, pig, cow, horse, human, chimpanzee). Only chickens and ducks have the same signal peptide (dotted box) starting at position 1 and ending at position 18. In addition, the chicken and duck have unique 157 and 161 amino acids compared to other species (solid box). (B) Phylogenetic tree of LECT2 in various species. Phylogenetic analyses were performed with the amino acid sequence of each species by MEGA7 software method. ChLECT2 was clustered in the same clade with duck and fish group. The bar indicates 5% amino acid divergence.

Figure 2. Analysis of expression levels of chLECT2 and chIL1B in the cultured chicken DF-1 fibroblast cells with the treatment of different concentration and time of poly (I:C). (A) and (C) Chicken DF-1 cells were treated with 0.1, 1, 5 and 10 μg/ml concentrations of poly (I:C) for 24 hr, and the expressions of chLECT2 and chIL1B was analyzed by qRT-PCR. (B) and (D) Chicken DF-1 cells were treated with 10 μg/ml of poly (I:C) for 1, 3, 6, 12 and 24 hr, and then the expression level of chLECT2 and chIL1B was analyzed by qRT-PCR. The each experiment was repeated for three times (n = 3). Significant differences were determined by
Tukey’s test, and bars with * symbol on top are the cases that significant differences were not found (p < 0.05).

Figure 3. Effects of NFκB inhibitor (BAY11-7085; BAY) and AP-1 inhibitor (Tanshinone II A; Tan-II) on the expressions of chLECT2 (A) and chIL1B (B) after stimulation with poly(I:C) in chicken DF-1 cells. The cells were treated with or without 5 μg/ml of poly (I:C) for 6 hr in the presence or absence of 5 μM of Bay or 25 μM of Tan-II. Values are mean ± SE (n = 3). Significant differences were determined by Tukey’s test, and bars with the same letter (a-d) on top are the cases that significant differences were not found (alpha < 0.05).
Table 1. Ensembl and amino acid sequence ID in the LECT2 gene of various species.

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<th>NCBI Reference Sequence ID</th>
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Table 2. qPCR primers used for mRNA expression analysis.

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<th>Primers 5’-3’</th>
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| **IL1B**     | F- GGA TTC TGA GCA CAC CAC AGT  
               R- TCT GGT TGA TGT CGA AGA TGT C | XM_015297469.1 |
| **LECT2**    | F- GAT ACG GCT GCG GCA ATT AC  
               R- GCC CTT GTG CTT TTC TCC TTT | NM_205478.2 |
| **GAPDH**    | F- TGC TGC CCA GAA CAT CAT CC  
               R- ACG GCA GGT CAG GTC AAC AA | NM_204305.1 |
Figure 1.
Figure 2.
Figure 3.