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Research Article
Expression of Chicken Leukocyte Cell-Derived Chemotaxin 2 in the Embryonic Bursa of Fabricius

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Abstract
Background and Objective: A previous transcriptomics analysis identified the expression of a pleiotropic cytokine, termed leukocyte cell-derived chemotaxin 2 (LECT2) in the bursa of Fabricius of the chick embryo. However, a role for the LECT2 gene product in the bursal microenvironment is unknown at present. The goal for this research project was to validate the expression of the LECT2 gene at the mRNA and protein level in the chick embryo bursa. **Materials and Methods:** The LECT2 gene transcript levels were determined by quantitative PCR with RNA derived from embryonic B-cells isolated at two different periods of bursal development. Whole protein extracts from the embryonic bursa were evaluated by mass spectrometry. **Results:** Expression of the LECT2 gene is significantly higher in B-cells from the early stage of bursal development. Mass spectrometry analysis revealed 9 different peptides from the LECT2 protein in the same embryonic period. **Conclusion:** The LECT2 gene is expressed at both the mRNA and protein level in the early period of bursa development. We postulate that LECT2 may contribute to B-cell migration into microenvironments established by non-lymphoid cells.

Key words: Bursa of Fabricius, B-cell, leukocyte cell-derived chemotaxin 2, chick embryo, protein level

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

In the chicken, the bursa of Fabricius controls the maturation of B-cells[1]. Committed B-cell progenitors, termed prebursal stem cells express surface IgM at the time the bursa is colonized between embryonic day (ED) 8 and ED14[2]. The prebursal stem cells migrate across the embryonic mesenchyme and enter the stratified epithelial lining at the border between the mesenchyme and bursal lumen[3,4]. Prebursal stem cells initiate cell division giving rise to a population of bursal B-cells in the developing follicle at the next stage of development (ED13-15)[5]. After the initial period of cell division, bursal B-cells then will initiate the development of a broad repertoire of B-cell receptor specificities by Ig-gene diversification during the ED17-18 embryonic period[3].

The microenvironmental factors within the bursal follicles that mediate the selection and expansion of bursal B-cells as well as the initiation of Ig-gene diversification are currently unknown. The goal of our research group was to identify the local signals which instruct B-cell differentiation in the embryonic bursa. In our previous studies, transcriptomics analysis of bursal B-cells isolated at ED16 and ED19[6] revealed the expression of a multifunctional cytokine, termed myb-induced myeloid protein-1[7], which is the chicken homologue of mammalian leukocyte cell-derived chemotaxin 2 (LECT2). In chickens, the LECT2 protein is known to regulate the biological activity of peripheral blood heterophils and bone marrow osteoclasts[8,9]. The LECT2 gene is also transcribed in myeloid-type cells in the chick embryo bursa[10]. In mammals, LECT2 functions as a pleiotropic cytokine with roles in development, homeostasis and immune function in different tissues[11]. The goal of this study was to validate LECT2 gene expression in the embryonic bursa at the mRNA and protein level.

MATERIALS AND METHODS

Proteomics analysis to detect the LECT2 protein

Animal husbandry: Fertile eggs from the F_1 cross of the $15I_5$ and 7_1 highly inbred White Leghorn lines were obtained from the USDA/ARS Avian Disease and Oncology Laboratory (East Lansing, MI)[12] were used for the bursal protein profile studies. Eggs were incubated at 99.5°F and 60% relative humidity with regular turning. At embryonic day (ED) 15 embryos were decapitated for bursal tissue collection. This project was reviewed and approved by the Mississippi State University Animal Care and Use Committee.

Preparation of bursal tissue lysates: For protein profile analysis, 10 bursas were pooled as replicates as previously described[13]. Briefly, whole bursal protein lysates were prepared by mincing 10 bursas, at an average wet-weight of 23 mg bursa^{-1}, in a glass-Teflon homogenizer in the presence of radio immunoprecipitation assay buffer with protease inhibitors[14]. The lysates were stored at -80°C until mass spectrometry analysis.

Mass spectrometry analysis: Whole bursal protein lysates were evaluated with electrospray ionization tandem mass spectrometry at the Mississippi State University Institute for Genomics, Biocomputing and Biotechnology as described previously[15]. The mass spectrometry data were analyzed with the bioinformatics tools MassLynx and ProteinLynx Global Server (Waters Corporation, USA).

Evaluation of LECT2 gene mRNA expression

Animal husbandry: Fertile eggs from the Hy-Line W-36 commercial layer line was incubated as described above. At ED16 and ED19 the bursas were collected and the bursal B-cells isolated as described previously[6].

Source of total RNA: Total RNA was isolated from bursal B-cells using the Qiagen RNeasy Mini Kit as described[6].

Quantitative real-time PCR analysis: The primers specific for the LECT2 transcript[16] Lect2-1F 5′-TATGCTCCCTTTTCTGG TGAGC-3′ and Lect2-1R 5′-CACAGAAGCCTGATCCCCTG-3′ and the GAPDH control transcript[17] GAPDH-1F 5′-TGCTGCCCAGA ACATCATCC-3′ and GAPDH-1R 5′-ACGGCAGGTCAGGTCAA CAA-3′ were used in quantitative real time PCR (qPCR) analysis. Briefly, cDNAs were synthesized from about 500 ng of mRNA using SuperScript™ III Reverse Transcriptase (Invitrogen, USA), RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen, USA) and oligo dT primers. Quantitative PCR was performed in 25ul volumes in 96 well plate using TaqMan® Fast Virus 1-Step Master Mix (Thermo Fisher, USA) and Mx3005P QPCR Systems with an initial denaturation at 95°C for 30 sec followed by 35 cycles of 95°C for 20 sec and 54°C for 30 sec[18].

All samples were analyzed in three biological replicates. All LECT2 mRNA expression levels were normalized by the housekeeping gene GAPDH. The relative expression of gene LECT2 between the two groups of ED16 and ED19 was finally calculated as CT of 2- CT. The means of the fold changes and standard deviations of the gene expression were calculated to reflect experimental variation.

RESULTS AND DISCUSSION

In previous studies we analyzed the embryonic bursal B-cells' transcriptome to identify surface receptors for various chemokines, cytokines and cell-adhesion proteins predicted to be important in bursal B-cell differentiation events[6]. LECT2 gene transcripts revealed LECT2 sequence reads three-fold higher early in B-cell development (ED16) compared with the later stages of B-cell development (ED19). The qPCR with RNA samples comparing LECT2 transcript levels at ED16 and ED19 showed that the LECT2 gene had about 9-fold higher expression at ED16 versus ED19 (Fig. 1), confirming the previous transcriptomics results.

Previous studies by others evaluated LECT2 mRNA expression in chick embryo tissues using *in situ* hybridization[10]. The LECT2 mRNA was detected in regions of granulopoiesis in the embryonic spleen and pancreas. Expression of LECT2 mRNA was detected in myeloid lineage cells in the thymus and bursa prior to hatching. The total RNA samples used in this study were derived from density gradient isolated bursal B-cells[6] and therefore it is possible that myeloid cell contamination could contribute to the level of LECT2 transcripts observed in this study. Future studies using RNA from bursal B-cells purified by rigorous cell sorting will be required to exclude this possibility.

In a previous study from our lab we observed peptides from the LECT2 protein in the proteomics analysis the bursa at 3 weeks post-hatch cells[15]. However, expression of the LECT2 gene at the protein level has not been examined in the embryonic bursa. In absence of a commercially available specific antibody, we chose to conduct proteomics analysis of whole tissue extracts from an early stage of bursal development (ED15) when LECT2 gene expression would be expected based on the qPCR results. In the course of this analysis we identified 9 different peptides derived from different regions of the LECT2 protein (Table 1). The peptides were evaluated by BLAST searches and showed 100% homology to the chicken Mim-1 homolog of the LECT2 protein (accession number P08940). Taken together with the qPCR results, expression of the LECT2 gene is possibly restricted to the embryonic period of bursal development.

In previous studies, the expression of LECT2 RNA was observed in myeloid-type cells containing granules in the ED15 bursa using *in situ* hybridization[10]. However, a lack of many chicken phenotypic markers at that time precluded the complete identity of the cells transcribing the LECT2 gene. Therefore, in future studies to identify the cellular source of LECT2 it will be critical because one of this protein's functions is to induce the directional migration of mature leukocytes in

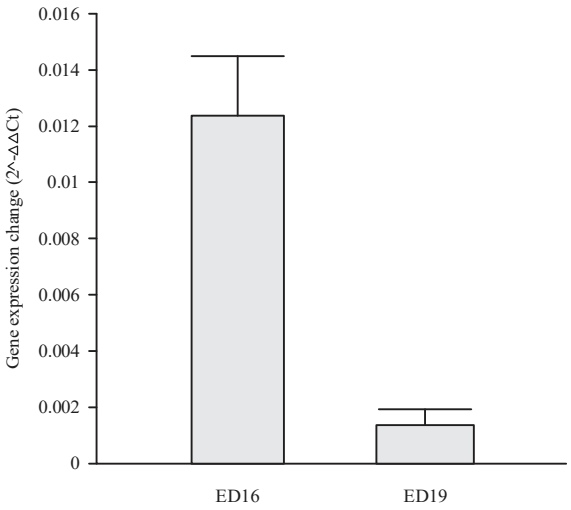

Fig. 1: mRNA expression of LECT2 in bursal B-cells at ED16 and ED19 from Hy-Line W-36 embryos
The bars denote standard deviations (n = 3)

Table 1: Peptides identified from the LECT2 protein in the ED15 bursa with mass spectrometry analysis

Peptides	Peptide sequence[1]	No. of peptides
Peptide 1	FFHNGNAIDDGVQISGSGYCVK	1
Peptide 2	YGCGNYGASR	1
Peptide 3	LLCIHPIR	1
Peptide 4	LVCIHPIR	2
Peptide 5	GVDVICTDGSIVYAPFSGQLSGPIR	2
Peptide 6	HWAQICSGNPFNR	4
Peptide 7	FFHNGNAIDDGVQIR	6
Peptide 8	VFPGIISHIHVENCDR	7
Peptide 9	GVDVICADGATVYAPFSGELSGPVK	10

[1]Single letter amino acid code

adult chickens[19]. Therefore, one possibility would be that LECT2 binds directly to bursal B-cell surface receptors. In mammals, the receptor tyrosine kinases Tie-1[20], MET and EGFR[21] and the C-type lectin CD209a[22] serve as receptors for LECT2. In our previous transcriptomics study, no transcripts from the MET or CD209 genes were found in embryonic bursal B-cells. However, low levels of transcripts from the Tie1 and EGFR genes were identified[6]. The rationale for the chemokine-induced migration of bursal B-cells towards myeloid or mesenchymal cells would be to expose the B-cells to a differentiation signals contributed by these cell types[23]. Alternatively, LECT2 may influence B-cell development in an indirect manner; meaning that LECT2 may bind to non-lymphoid bursal reticular epithelial cells (REC) and this may induce secretion of REC-derived differentiation factors. In the mouse bone marrow, LECT2 modulates cytokine secretion by bone marrow macrophages, resulting in the proliferation of hematopoietic stem cells and their migration into the bloodstream[24]. One could postulate that REC, which supports

the architecture of the bursal follicle, could be a LECT2 target in the embryonic bursa. Additional studies are needed to differentiate between these possibilities. This knowledge is required to formulate rational hypotheses concerning role of the LECT2 protein in a primary lymphoid tissue dedicated to B-cell development.

CONCLUSION

We consider LECT2 a candidate factor in the bursal microenvironment that may participate in B-cell maturation because LECT2 has a multifunctional nature and a high level of gene expression at a critical time-point in embryonic bursal B-cell development.

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