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Ephrin Receptor Expression in the Embryonic Bursa of Fabricius

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Abstract: Utilizing degenerate PCR primers complementary to conserved regions within the kinase domain of Receptor Tyrosine Kinase (RTK) genes, the goal was to compare the full complement of RTK expressed (mRNA level) between two developmental stages of bursal B-cells. We identified 21 different RTK cDNAs out of a total of 235 cDNAs sequenced between the two B-cell stages. The dataset revealed the expression of RTK genes important in developmental processes, namely the ephrin receptors (Eph). The ephrin receptors and the ephrin ligands play critical roles in embryo development by controlling the organization of cells in tissues and by inducing cellular differentiation.

Key words: Bursa of fabricius, B-cell, receptor tyrosine kinase, ephrin receptor

INTRODUCTION

In the chicken, the bursa of Fabricius is a gut-associated lymphoid tissue which functions as a primary lymphoid organ dedicated to the development of a diverse repertoire of B-cells (Glick, 1988; Ratcliffe, 1989; Cooper, 2002). In chicken B-cell development prebursal stem cells complete rearrangements of the immunoglobulin (Ig) heavy and light chain genes and express surface IgM at the time of bursal colonization between Embryonic Day (ED) 8 and ED14 (Ratcliffe *et al*., 1986; Reynaud *et al*., 1992). The prebursal stem cells colonizing the bursa are also characterized phenotypically by the surface expression of a carbohydrate epitope termed sialyl Lewis X (SLEX$^+$) [Masteller *et al*., 1995a]. The SLEX$^+$/IgM$^+$ prebursal stem cells enter the bursal mesenchyme and migrate into the double-layered epithelial lining separating the mesenchyme from the bursal lumen (Olah *et al*., 1986). During the period of ED13-15, the prebursal stem cells that have entered the epithelium undergo proliferative expansion leading to the formation of the bursal follicle as well as formation of a population of SLEX$^+$/IgM$^+$ cells, termed developing B-cells, which will undergo Ig-gene diversification (McCormack *et al*., 1989). During the period of ED15-18, there is a phenotypic switch in cell surface glycosylation from SLEX to high levels of a related carbohydrate structure termed Lewis X (LEX$^+$) [Masteller *et al*., 1995a]. The change in surface carbohydrate expression reflects the progression from one developmental B-cell stage to the next, which is coincident with the onset of Ig-gene diversification (Masteller *et al*., 1995b).

The bursal follicles provide a unique micro environment for diversification of rearranged heavy and light chain Ig-genes by a mechanism known as Ig-gene conversion (Reynaud *et al*., 1987; Thompson and Neiman, 1987). The process of Ig-gene conversion initiates in the proliferating pool of SLEX$^+$/IgM$^+$ developing B-cells between ED15 and ED18 of incubation and continues until bursal involution (Reynaud *et al*., 1987; Thompson and Neiman, 1987). The Ig-gene conversion mechanism is responsible for the development of a diverse repertoire of B-cell specificities required for maintaining humoral immunity in chickens (Masteller *et al*., 1997).

The long-term goal of our research is to understand the micro environmental signals responsible for the SLEX$^+$ to LEX$^+$ transition between ED15 and ED18. Accomplishing this goal requires a thorough understanding of the physiology of both the SLEX$^+$ and LEX$^+$ developmental B-cell stages. One approach to this goal is to conduct gene expression surveys in developing B-cells at ED15 (SLEX$^+$) and ED18 (LEX$^+$) to identify candidate genes for controlling the progression from one developmental stage to the next. To this end, a previous study to identify proteins expressed in embryonic bursal B-cells revealed the expression of proteins from the Receptor Tyrosine Kinase (RTK) gene superfamily (Felfoldi *et al*., 2008). The RTK genes are known to regulate numerous developmental processes including control of apoptosis, cell migration and differentiation (reviewed in Arighi *et al*., 2005; Andrae *et al*., 2008). Therefore, the aim of these studies was to identify the full complement of RTK genes expressed in ED15 and ED18 developing B-cells.

MATERIALS AND METHODS

Experimental animals: Fertile broiler hatching eggs were obtained from Peco Farms (Gordo, AL) and were incubated at 99.5°F with 60% relative humidity and regular rocking. At ED15 and ED18 of incubation, Ross X Ross 308 broiler embryos were decapitated for bursal tissue collection and for subsequent isolation of

developing B-cells from the bursa for RNA preparation. This project was conducted under an approved Mississippi State University Animal Care Protocol.

Preparation of total RNA: Single cell suspensions of embryonic bursal B-cells were prepared as described previously (Felfoldi *et al*., 2008). Total RNA was extracted from bursal B-cells with the Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad CA). The RNA was collected by ethanol precipitation and then adjusted to 1 mg/ml^{-1} concentration.

Preparation of cDNA: Reverse transcription reaction was performed with the Superscript III kit (Invitrogen, Carlsbad, CA) as described by Glew *et al.* (1995) to include the following treatments prior to reverse transcription; a) reagents only, no RNA; b) RNA only; c) RNA with DNase I (2 units^{-1}) and RNase A (1 ug^{-1}); d) RNA with DNase I; e) RNA with DNase I and 50 ng^{-1} bursal B-cell genomic DNA with (f) or without (g) DNase I. Samples were incubated at room temperature for 15 min and then the DNase I was inactivated at 85°C for 10 min. Samples were then incubated with (a, c and e) or without (b, d, f and g) reverse transcriptase for 1 h at 50°C. The final cDNA samples were treated with RNase H and then diluted 2-fold in 10 mM Tris and 1 mM EDTA (pH 8.0).

PCR: The cDNA samples (A-G) were evaluated for detectable levels of genomic DNA contamination with PCR primers designed to amplify the chicken GAPDH cDNA (Dugaiczyk *et al*., 1983). The GAPDH primer sequences were forward 5'-TCAATGATCCCTTCATCG ATCTG-3' and reverse 5'-AGGGTCCTGCTTCCCTAG GCAGC-3', resulting in a 954 bp cDNA product.
Degenerate primers were used to amplify cDNA from RTK genes by PCR (Iwama *et al*., 1994).

Forward primer TKI: 5'-TAGTCGACAYMGRGAYYTVGC-3'

Reverse primer TKIII: 5'-TGGAATTCCAWARSWCCASA CRTC-3' ".

NGCNMG-3' Reverse primer TKIII: 5'-TGGAATTCCAWA RSWCCASACRTC-3'

The degenerate PCR primers were synthesized where Y = C + T; M = A + C; R = A + G; V = G + A + C; W = T + A; S = G + C and N = all four nucleotides used in the synthesis.
In separate PCR reactions, primer TKI was used with primer TKII or TKIII with ED15 and ED18 bursal B-cell cDNA using a Hybaid Omn-E thermocycler (Scientific Consultants Inc, Baton Rouge, LA, USA) under the following conditions: 94°C, 3 minutes; 35 cycles of 94°C, 30 sec; 37°C, 2 min and 72°C, 3 min and a final extension at 72°C, 5 min.

The 210 bp PCR product was isolated from 1% agarose gels and then ligated into a pCR2.1-TOPO vector for transformation of *E. coli* TOP10F' (Invitrogen Corporation, Carlsbad, CA, USA). The recombinant plasmids were sequenced at the High-Throughput Genomics Unit, Seattle, WA, USA.

Data analysis: The cDNA sequences were identified with manual database BLAST search (NCBI database www.ncbi.nlm.nih.gov).

RESULTS AND DISCUSSION

The goal of this study was to compare the full complement of RTK genes expressed (mRNA level) between developmental stages of bursal B-cells at ED15 and ED18. The cDNA samples, in which contaminating genomic DNA was judged to be below our level of detection using the chicken GAPDH primers, were selected for PCR amplification with primer combinations TKI and TKII or TKI and TKIII. To this end 21 different RTK gene cDNAs out of a total of 235 cDNAs sequenced were identified between the two developmental B-cell stages (Table 1). The dataset revealed that the expression of RTK genes were important in developmental processes, namely the platelet-derived growth factor receptor chains and various ephrin receptors. In addition, five cDNAs from a Fibroblast Growth Factor Receptor (FGFR) gene were observed that are identical to the FGFR4 gene cDNA observed by Koritschoner *et al.* (1999). With relevance to genes possibly controlling bursal B-cell development, the most important finding from this analysis was the expression of cDNA from four different ephrin receptor (Eph) genes in both the ED15 and ED18 samples. To our knowledge, this represents the first demonstration of Eph expression in the bursa during the late embryonic period of development.
In previous studies, the analysis of proteins derived from developing bursal B-cells at ED15 revealed several proteins that may be associated with Eph receptor-mediated signal transduction, suggesting a possible role for Eph in bursal B-cell physiology (Felfoldi *et al*., 2008). In the present study, the previous findings were extended with the identification of cDNA from the EphA4, EphA7, EphA9 and EphB2 receptor genes in embryonic bursal B-cells (Table 1). In an earlier study, expression of the EphA9 and EphB2 genes were detected in the bursa well into the posthatch period of development, suggesting a requirement for these genes in B-cell maturation (McCarthy *et al*., 2006). In the protein profile analysis of bursal B-cells and the associated stroma, EphA9 expression was observed only in developing B-cells, whereas expression of EphB2 was found in both B-cells and bursal stromal cells (McCarthy *et al*., 2006). In the present study, bursal stromal cells contaminating the developing B-cell preparations cannot be ruled out

Table 1: Protein tyrosine kinase cDNA expressed in embryonic bursal B-cells

Protein tyrosine kinase gene	ED15 I/III	ED15 II/III	ED18 I/III	ED18 II/III
Jak1	14	5	28	1
Jak2	9	-	8	2
c-kit	24	-	4	-
Csk	1	6	7	24
Syk	1	2	5	11
Fyn	1	-	1	-
Ret	2	-	-	-
Ryk	1	4	1	1
FGFR	-	4	-	1
PDGFR α	13	29	-	3
PDGFR ß	-	-	-	2
VEGFR-2/Flk-1	1	2	-	-
EphA9	-	3	-	-
EphA4	-	5	-	-
EphA7	-	-	-	1
EphB2	-	1	-	-
MERTK	-	2	-	-
Tyk-2	-	1	-	1
ITK	-	1	-	-
FES	-	1	-	-
TrkC	-	1	-	-
Total cDNAs:	67	67	54	47

The number of protein tyrosine kinase gene cDNAs expressed in bursal B-cells at ED15 and ED18 detected with degenerate primer pairs I/III or II/III

as the source of RNA for cDNA synthesis. The developing B-cells were collected using Ficoll-Hypaque gradients which serves primarily as a lymphocyte enrichment procedure. Therefore it is possible that the developing B-cells could have been contaminated with a minor population of nonlymphoid bursal stromal cells. It is possible that the Eph cDNA detected in the developing B-cell preparations may also be expressed in the bursal stroma. Additional studies will be required to address this possibility.

The Eph and its ligands are involved in cell growth and differentiation and in mediating cell adhesion or cell repulsion events (Pasquale, 2008). The Eph receptors are divided into subclass A (EphA), which generally bind ephrin-A ligands and subclass B (EphB), that bind ephrin-B ligands (Pasquale, 2008). The interaction between Eph receptors and ephrins can generate signals through both the receptor and ligand. The RTK signals generated through the Eph receptor is termed as forward signaling, whereas signal transduction through ephrins is termed as reverse signaling (Aoto and Chen, 2007). In vertebrate development, the Eph receptors and their ligands, along with other genes play a major role in the development of the nervous system where these genes control the correct movement of ganglion cell axons in the brain (Schmitt *et al*., 2006). For example, Schmitt *et al*. (2006) demonstrated that the EphB receptors expressed on retinal cell axons bind ephrin-B ligands expressed in the dorsal tectum. In this case, the outcome of Eph-ephrin interaction resulted in cell attraction leading to the correct positioning of axons in the tectum. In other studies, Eph receptors and ephrin ligands have been found to be expressed on the same cell and can interact in a cis or trans fashion (Konstantinova *et al*., 2007). This is demonstrated in adult vertebrates in which the insulin response to blood glucose levels by pancreatic ß islet cells is mediated by EphA5 and ephrin-A5 signaling in a trans configuration. This mechanism allows communication between pancreatic ß islet cells such that forward signals through the Eph receptors prevent insulin secretion in response to low glucose levels, whereas high glucose levels suppress forward signaling but allow reverse signals through the ephrin-A5 ligand which stimulate insulin secretion (Konstantinova *et al*., 2007). Therefore, the Eph receptors and their ligands play critical roles in the development of embryonic tissues and also function in maintaining homeostasis of adult tissues.

In this study, cDNA from the EphB2 gene in bursal B-cells at ED15 was identified (Table 1). In an earlier study Santiago and Erickson (2002) found that the EphB group of receptors is important to the migration of neural crest cells in the chick embryo. In that study, neural crest cells that had differentiated into melanoblasts migrated in response to experimental preparations of soluble ephrin-B1, suggesting that ligands for EphB receptors guide melanoblasts to the appropriate micro environment for differentiation. Additional work demonstrated that melanoblasts expressed both EphB2 and the endothelin receptor B and also showed that both receptors were necessary for migration (Harris *et al*., 2008).

The EphA4 gene cDNA was detected in ED15 bursal B-cells in this study (Table 1). Also in mammals, the EphA4 receptor has been found to be critical in development of the forebrain striatum (Passante *et al*., 2008). The striatum is composed of two regions, the matrix and striosome and the neurons of each region differ biochemically (Holt *et al*., 1997). The development of the striatum requires the proper positioning of the matrix and striosome neurons and this process depends on EphA4 and ephrin-A ligands. The ephrin-A -expressing striosome neurons enter the developing striatum initially, followed by the EphA4-expressing matrix neurons. Signal transduction through EphA4 and ephrin-A ligands results in the organization of the neurons into the two regions of the striatum (Passante *et al*., 2008).

In the current study, transcripts from the recently described chicken EphA9 gene were detected in ED15 bursal B-cells (Table 1). The EphA9 receptor was first identified in a screen of RTK cDNA derived from chicken primordial germ cells (Sasaki *et al*., 2003). The EphA9

cDNA showed 78% nucleotide identity with human EphA1. However, the EphA1 gene is expressed in the epithelia of the kidney, skin and reproductive tract (Duffy *et al*., 2008), whereas the chicken EphA9 gene is expressed in the kidney, lung, testes and thymus (Sasaki *et al*., 2003).

In mammalian studies, the EphA1 receptor is expressed primarily in the epithelial cells of the kidney, skin and reproductive tract (Duffy *et al*., 2008). The lack of EphA1 expression in mice revealed defects in the ability of epithelial cells in the female reproductive tract to undergo developmentally regulated apoptosis. Moreover, the mice exhibited aberrant patterns in the organization of the caudal vertebrae, suggesting that EphA1 receptors and ephrin ligands are also critical for vertebral patterning (Duffy *et al*., 2008).

The Eph have been implicated in lymphocyte migration, and have been shown to modulate the chemokine-directed migration of developing T-cells in the mammalian thymus (Sharfe *et al*., 2002). The Eph and ephrin ligands are involved in lymphocyte development in both embryonic and adult primary lymphoid tissues in mammals (Ting and Boyd, 2008). In the mammalian thymus, Eph are expressed primarily on developing T-cells whereas various ephrin ligands are expressed on epithelial cells in different anatomical regions of the thymus (Munoz *et al*., 2002). An *in vitro* model of T-cell development, the fetal thymic organ culture, has been used to address the role of Eph and their ligands in T-cell development. The disruption of specific Eph and ephrin interactions in thymic organ cultures resulted in apoptosis in both double-positive and single-positive thymocytes, suggesting an important role for Eph in positive and negative selection of developing T-cells (Alfaro *et al*., 2007). While Eph have been shown to be expressed in human early fetal B-cells, their role in B-cell development has not been clarified (Aasheim *et al*., 1997). It is noteworthy that the EphA9 gene is expressed in the chicken primary lymphoid tissues of the thymus (Sasaki *et al*., 2003) and the bursa (this study). Given the role that Eph/ephrins play in primary lymphoid tissues (e.g. mammalian thymus) it is theorized that Eph may be involved in receptor-ligand interactions between developing B-cells and the bursal stroma.

Conclusion: The goal for this project was to identify the RTK genes expressed in developing B-cells at ED15 and ED18. It was theorized that RTK genes would be important in the differentiation of developing B-cells from the $SLEX^+$ stage at ED15 to the LEX^+ stage at ED18, concomitant with the onset of repertoire development. This hypothesis was based on published studies in mammalian B-cell development whereby self-renewing hematopoietic stem cells differentiate into common lymphoid progenitors and then differentiate along the B-cell developmental pathway under the direction of stromal cell surface proteins and cytokines (Hardy *et al*., 1991; Kondo *et al*., 1997). Eph receptors for ligands were identified in this study that, in part, controls embryonic development of various tissues by controlling the migration and organization of cells and by inducing differentiation. Therefore, it is possible that Eph receptors and ephrins represent one of the many classes of gene products that regulate the interaction of developing B-cells with the bursal stroma. Numerous studies have shown that contact with the bursal micro environment is required for survival and maturation of developing B-cells (Neiman *et al*., 1991, 1994; Funk and Thompson, 1998). However, the critical information missing in this sequence of events are the signals delivered by stromal cells that may initiate Ig-gene conversion and the survival of developing B-cells in the early embryonic period. Therefore, it is theorized that Eph receptor and ephrin ligand interactions are involved in the interaction of developing B-cells with stromal cells. Additionally, it is also predicted that the forward and reverse signals initiated by Eph-ephrin contacts may protect developing B-cells from apoptosis during a critical period of differentiation. In future studies, identification of the spectrum of Eph receptors and ephrins expressed in the embryonic bursa are planned. An examination of the timing and duration of their expression will also be conducted. This information will be important in determining the role of the Eph and ephrin gene families in differentiation from the $SLEX^+$ developmental stage to the LEX^+ stage.

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