ISSN 1682-8356
ansinet.org/ijps

INTERNATIONAL JOURNAL OF

POULTRY SCIENCE

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International Journal of Poultry Science 7 (2): 117-124, 2008
ISSN 1682-8356

Preliminary Protein Profile Analysis of the Late Embryonic B-Cell Stages in the Chicken Bursa of Fabricius

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Abstract: The bursa of Fabricius serves as a primary lymphoid organ for the development of a diverse repertoire of B-cells. The embryonic bursa is colonized during embryonic days (ED) 8-14 by stem cells expressing the sialyl Lewis^X carbohydrate (SLEX). At ED13-15, cells with the SLEX phenotype initiate proliferation leading to the development of the bursal follicle. By ED15-17, a key differentiation event occurs resulting in the onset of repertoire development by immunoglobulin gene-conversion. This differentiation event is defined by a phenotypic transition in cell surface glycosylation from SLEX to a related carbohydrate structure termed Lewis^X (LEX). The goal of this study was to identify functional groups of genes in the two stages that might be involved in critical biological processes of proliferation, differentiation, apoptosis and cell adhesion, and explain the observed functional differences. We found that prior to the onset of immunoglobulin gene conversion B-cells express genes related to ephrin receptor signaling, epidermal growth factor receptor (EGFR) signaling and Wnt signaling. B-cells undergoing immunoglobulin gene conversion express genes from TNFR signaling and both stages were found to express members of Wnt signaling, integrin signaling and EGFR signaling pathways. The differentially expressed pathways agree with previous observations, offering explanation to signals leading to proliferation, differentiation and apoptosis in the two B-cell stages.

Key words: Bursa of fabricius, B-cell, development, mass spectrometry

Introduction

The importance of the chicken as a model for studying humoral immunity was established with early studies of a gut-associated lymphoid tissue, the bursa of Fabricius (Glick *et al*., 1956; Cooper *et al*., 1965, 1966). The bursa is known to provide a unique microenvironment for B-cell repertoire development by immunoglobulin (Ig)-gene conversion. In chicken embryos, the first hematopoietic stem cells (HSC) emerge from the para-aortic region at embryonic day 2.5 (ED2.5) (Dieterlen-Lièvre, 1975). These HSCs colonize the extraembryonic yolk sac blood islands, leading to the development of various blood cell types (Dieterlen-Lièvre and Martin, 1981). The first committed B-cell precursors, the prebursal stem cells, appear in the yolk sac from ED4 and have undergone D to J rearrangements at the heavy chain locus. The prebursal stem cells then migrate into the embryo proper and undergo a second wave of rearrangement (VH to DJH and VL to JL) at the time of bursal colonization, ED8-14 (Le Douarin *et al*., 1975; Weill *et al*., 1986; Mansikka *et al*., 1990; Reynaud *et al*., 1992). The prebursal stem cells are characterized by the expression of surface IgM (sIgM), CD45, the chicken B-cell marker Bu-1 and the SLEX carbohydrate epitope (Ratcliffe *et al*., 1986; Houssaint *et al*., 1989, Palojoki *et al*., 1995; Masteller *et al*., 1995a, Funk *et al*., 2003). The prebursal stem cells that enter the embryonic bursal mesenchyme cross the basement membrane of the double-layered epithelial lining that separates the mesenchyme from the bursal lumen (Olah *et al*., 1986) and express a surface marker recognized by the EIVE12 monoclonal antibody (Pharr *et al*., 1995). The epithelial buds represent precursors to the bursal follicles (Olah *et al*., 1986) and generally contain only 3-4 prebursal stem cells (Pink, 1986). The bursal follicle starts to form as the epithelial layers further separate when the prebursal stem cells proliferate, possibly in response to intrinsic signals from sIgM (Sayegh *et al*., 1999a), creating a large pool of candidates with productively rearranged H-chain and L-chain genes for diversification by Ig – gene conversion (McCormack *et al*., 1989). The process of Ig-gene conversion initiates in the proliferating pool of developing B-cells between ED15 and ED17 and continues until bursal involution (Reynaud *et al*., 1987; Thompson and Neiman, 1987).

By ED18 most of the bursal B-cells undergo at least one round of gene conversion, further rounds are possible until the bursa undergoes involution (16-weeks post hatch) and in splenic germinal centers upon activation by antigens (from 2 weeks post hatch) (Arakawa *et al*., 1996, 1998). The Ig-gene conversion can be monitored either by the analysis of immunoglobulin gene sequences or phenotypic changes. The markers described on developing B-cells undergoing Ig-gene

conversion are the switch from the SLEX to the LEX antigen and the expression of chB1 lectin, which is a pro-apoptotic receptor (Goitsuka *et al.*, 1997). From ED18 to the time of hatching there is a high rate of B-cell proliferation and an expansion in the sizes of follicles. Also at this time, the first wave of immigrant cells can be detected in the blood and spleen (Cooper *et al.*, 1969). After hatching a structural rearrangement takes place; some B-cells migrate out from the follicles to form the cortical area and the original embryonic follicle becomes the medullary area (Grossi *et al.*, 1974).

The second role the bursa plays is the selection of the B-cells. After hatching a large number of developing B-cells are eliminated by apoptosis (Motyka and Reynolds, 1991). Recent studies suggest that sIgM is critical for positive selection of developing B-cells by recognition of environmental antigens (Sayegh *et al.*, 1999b; Sayegh and Ratcliffe, 2000). After hatching, the bursal lumen is exposed to the microflora and yolk sac proteins from the gut, which is transported into the medulla by the follicle-associated epithelium found on the luminal side of the follicle (Bockman and Cooper, 1973; Sorvari *et al.*, 1975; Pike *et al.*, 2004; Felfoldi *et al.*, 2005). In addition, immune complexes consisting of IgG and antigens derived from the gut flora are found in the medulla of the follicle, in association with bursal secretory dendritic cells (Olah *et al.*, 1991; Yasuda *et al.*, 2002). As developing B-cells have been shown to express only IgM in the bursa, the immune-complexes must therefore consist of maternal antibody (Ekino, 1995). Therefore, it has been proposed that low affinity recognition of immune complexes by sIgM may be responsible for stimulating B-cell migration across the cortico-medullary (CM) border into the follicular cortex after hatching (Sayegh and Ratcliffe, 2000; Arakawa *et al.*, 2002). Those cells that lose expression of sIgM, or that fail to make low affinity recognition of immune complexes would presumably fail to be selected and may undergo apoptosis (Paramithiotis *et al.*, 1995; Sayegh and Ratcliffe, 2000).

Our long-term goal is to contribute a mechanistic understanding of the differentiation events occurring in the bursal follicles prior to hatching. Accomplishing this goal requires a thorough characterization of developing B-cells expressing the SLEX epitope at ED15 and the LEX epitope at ED18. The central hypothesis of this project is that cellular proteins differentially expressed between the SLEX and LEX developing B-cell populations will represent candidate proteins involved in the SLEX to LEX transition. In this manuscript, we report the proteomic comparison of the ED15 and ED18 B-cell stages in chicken bursa of Fabricius, representing the pre and post Ig-gene conversion developmental stages (Palojoki *et al.*, 1995; Masteller *et al.*, 1995b; Funk and Palmer, 2003), to further our understanding of the biological processes occurring during this period of bursal B-cell development.

Materials and Methods

Experimental animals: The embryos used were the first cross of the $15I_5$ and 7_1 highly inbred White Leghorn chicken lines (Avian Disease and Oncology Laboratory, East Lansing, MI USA). Embryos were incubated under standard conditions (100°F, 60% relative humidity, regular rocking). This project was reviewed by the Mississippi State University Animal Care and Use Committee, and assigned approval number 06-061.

Preparation of cell lysates: Single cell suspension of B-cells was prepared in RPMI cell culture medium (Mediatech Inc, Herdon VA USA) from dissected bursas of ED15 and ED18 embryos as described (Glick and Schwartz, 1975). B-cells were then purified using Histopaque (1.077, Sigma, St. Louis, MO USA) gradient centrifugation. Whole cell protein lysates were then prepared in radioimmunoprecipitation assay buffer (Ferguson *et al.*, 1994) from bursal B-cells isolated from ED15 (92 embryos) and ED18 (56 embryos). Nuclear proteins were isolated using an SDS buffer as described (McCarthy *et al.*, 2005). Equal amounts of protein (200 μg^{-1}) from each developmental stage were digested with trypsin and then the reaction products were desalted with a MacroTrap reverse-phase HPLC column for evaluation with mass spectrometry as described (McCarthy *et al.*, 2005).

Mass spectrometry: The two-dimensional liquid chromatography electrospray ionization tandem mass spectrometry was done as described elsewhere (McCarthy *et al.*, 2005; Lee *et al.*, 2006). Proteins were identified and analyzed as previously described (McCarthy *et al.*, 2006; Lee *et al.*, 2006). The search term Gallus gallus was searched against the organism field of the National Center for Biotechnology Information (NCBI) protein database to create a chicken-specific protein database. TurboSEQUEST (Bioworks Browser 3.1; ThermoElectron) was used to apply in silico trypsin digestion to the chicken database and mass changes due to cysteine carbamidomethylation and methionine oxidation were included. The chicken non-redundant protein database was used to search tandem mass spectra using a peptide (MS precursor ion) mass tolerance of 1.5 Da, and a fragment ion (MS/MS) mass tolerance of 1.0 Da. Peptide matches were considered valid if they were >7 amino acids with X correlation values of 1.5, 2.0 and 2.5 (+1, +2, and +3 ions, respectively) and Delta Cn values >0.1.

Data analysis: Identified proteins were categorized into functional groups with manual database search (www.expasy.org and the NCBI database www.ncbi.nlm.nih.gov). Proteins falling into the functional groups of proliferation, apoptosis, cell adhesion and differentiation were subjected to

additional searches to find proteins associated with canonical signal transduction pathways (www.biocarta.com).

Results

The first analysis step included combining the results from nuclear and soluble samples from the ED15 and ED18 stages, respectively. Removing duplicates and unknown proteins the ED15 sample resulted in 496 identified proteins and the ED18 sample resulted in 834 identified proteins. The proteins were sorted into functional groups, such as basic cell functions, proliferation, apoptosis, cell adhesion and differentiation, based on manual selection (Table 1). Further analysis identified functional signal transduction pathways among proteins from the ED15 and ED18 samples. The ED15 sample contained members of the following pathways: B-cell receptor pathway (data not shown), integrin signaling, ephrin receptor signaling, Wnt signaling and epidermal growth factor receptor (EGFR) signaling. The ED18 sample contained members of the following pathways: B-cell receptor pathway (data not shown), integrin signaling, tumor necrosis factor receptor (TNFR) type 1 signaling and Wnt signaling. The qualitative differences in gene expression between ED15 and ED18 bursal B-cells are presented in Tables 2 and 3.

Table 1: Number of expressed gene products identified in ED15 and ED 18 samples, categorized into functional groups

Stage	ED18	ED15
Total	834	496
Basic cell functions	589	321
Proliferation	26	18
Apoptosis	18	5
Cell adhesion	9	6

Discussion

Our results add information to explain functional changes related to one of the critical developmental steps in embryonic chicken B-cell development, the onset of repertoire development. The importance of the process is that only developing B-cells that have undergone successful Ig gene-conversion events are capable of continuing development in the bursa. This development involves giving rise to early bursal emigrant B-cells (from ED17 to hatch) (Cooper *et al.*, 1969) and differentiation into the cortical and medullary populations of developing B-cells in post hatch bursal follicle, that produce late bursal emigrant B-cells (from hatch to 16 weeks of age) (Paramithiotis and Ratcliffe, 1994).

The most important difference between the B-cells at ED15 and ED18 appeared in the apoptosis related genes. The ED18 B-cells express most of the TNFR1 pathway components, suggesting that these cells are highly susceptible to apoptosis. TNFR1 signaling is one of the best-characterized apoptosis inducer pathways (reviewed by Vermeulen *et al.*, 2005). TNFR1 can initiate apoptosis by recruiting the caspase cascade or initiate signal transduction leading to activation of NF-kβ transcription factor (reviewed by Hehlgans and Pfeffer, 2005). Ig-gene conversion results in a high number of non-functional or self-specific B-cell receptors that are eliminated from the repertoire (McCormack *et al.*, 1989). Previous observations indicate that large numbers of bursal B-cells are removed by apoptosis (Motyka and Reynolds, 1991). Based on our findings, it may be possible that bursal B-cells are susceptible to apoptosis while undergoing Ig-gene conversion. The involvement of the TNF super family in the initiation of bursal B-cell apoptosis was shown before (Abdalla *et al.*, 2004).

Ephrin receptor signaling was first described in regulation of axon guidance during nervous tissue development (Wang and Anderson, 1997). Ephrin receptors, belonging to the receptor tyrosine kinase family, were found to be expressed on different lymphocytes that have a migratory phenotype (Aasheim *et al.*, 2000, 2005). Lymphocytes were reported to express various types of both ephrins and ephrin receptors (Shimoyama *et al.*, 2002, Muñoz *et al.*, 2002). Ephrins may be important at the early stage by regulating the homing of prebursal B-cells to the bursa and possibly at the later stage of development for cell movements associated with formation of the mature bursal follicle. Our findings may be similar to the study of Aasheim *et al.* (1997), where the ephrin receptor family member Hek11 receptor tyrosine kinase (later classified as ephrin type-A receptor 7, EphA7) was reported to be expressed in pre and pro B-cells but not in more mature stages in human bone marrow. We expect similar differences in ephrin receptor expression in different developmental stages in bursal B-cell development. Further work is needed to identify chicken ephrin genes and follow their expression in B-cells.

Integrins are receptors for different extracellular matrix (ECM) components depending on different combinations of alpha and beta chains. When bound to their ligands, integrins transmit intracellular signals affecting cytoskeletal organization, cell motility and cell cycle. In B-cells integrin signaling is related to cell migration (Terol *et al.*, 1999) and cluster formation at germinal centers in spleen (Ambrose and Wagner, 2004). In bursal B-cell development, migration is needed when prebursal stem cells enter the bursal mesenchyme and cross the basement membrane to enter the developing follicles. Integrins would be important after hatch when B-cells are exported from the bursa to peripheral lymphoid organs. The bursal B-cells might utilize integrins for interaction with the ECM in the developing follicle. We found the expression of the alpha 8 and the beta 1 subunits at both ED15 and ED18 (Table 2 and 3), which could form an integrin heterodimer with specificity for the ECM (Schnapp *et al.*, 1995). Another possible heterodimeric combination could involve the

Table 2: Proteins involved in cellular processes in ED15 developing B-cells

Symbol	Gene Name	GI number
--------- Ephrin receptor signaling pathway members in ED15 sample ----------		
ABL1	Abelson murine leukemia viral oncogene homolog 1	50751126
GNAQ	Guanine nucleotide binding protein q polypeptide	22651974
ITGA11	Integrin, alpha 11	50753262
ITSN1	Intersectin 1 (SH3 domain protein)	50745113
JAK2	Janus kinase 2	45382379
RAC1	Ras-related C3 botulinum toxin substrate 1	45384330
---------- Integrin signaling pathway members in ED15 sample ----------		
ABL1	Abelson murine leukemia viral oncogene homolog 1	50751126
ACTN2	Actinin, alpha 2	46048687
ITGA8	Integrin, alpha 8	124950
RAC1	Ras-related C3 botulinum toxin substrate 1	45384330
RAC3	Ras-related C3 botulinum toxin substrate 3	45384328
TSPAN7	Tetraspanin 7	50752761
ITGA11	Integrin alpha 11	50753262
---------- Other cell adhesion molecules in ED15 sample ----------		
PCDH7	Protocadherin 7	50747110
PCDH12	Protocadherin 12	50754824
DSCAM	Down syndrome cell adhesion molecule 5	0729979
---------- Wnt signaling pathway members in ED15 sample ----------		
WNT10A	Wingless type 10A	54260406
JAK1	Janus kinase 1	45382379
ARD30A	Ankyrin Repeat Domain 30A	50728154
---------- EGFR signaling pathway members in ED15 sample ----------		
Jak1	Janus kinase 1	45382379
MAPK3	Mitogen-activated protein kinase kinase kinase 3	50754317
EPS15R	Epidermal growth factor receptor substrate EPS15R	50761168

alpha 11 and beta 1 chains forming a receptor for collagen (Humphries *et al.*, 2006). Most of the integrin adhesion molecules are shared on ED15 and ED18 B-cells, there was no major difference found in integrins and other cell adhesion molecules. The expression of similar integrin chains in the ED15 and ED18 samples suggests that the ECM microenvironment in the bursal follicle does not change during this period of embryonic bursal development. Significant changes in cell adhesion proteins would therefore be expected at earlier time-points during the homing of prebursal stem cells to the bursa, and later, with export of B-cells to peripheral lymphoid organs begins.

Other cell adhesion molecules, belonging to the cadherin superfamily showed differences between ED15 and ED18 B-cells (Table 2 and 3). As cadherins mediate monophylic cell-to-cell type interactions, we speculate that in the bursal system cadherins play a role in cluster formation of B-cells. The different cadherin expression pattern in different stages suggests that B-cells prefer to interconnect with cells that are in the same developmental stage.

Members of the wingless (Wnt) signaling pathway are expressed in diverse genera ranging from Drosophila to human. The members of this pathway are highly conserved due to the important role they play in development (reviewed by Clevers, 2006). The different combinations of Wnt growth factors and Frizzled receptor subtypes lead to two major pathways: the canonical pathway, through beta-catenin and the non-canonical pathway, through Janus kinase (Clevers, 2006). In mature T lymphocytes Wnt signaling induces transmigration, increasing malignancy in lymphomas where it is activated (Wu *et al.*, 2007). In B-cell development, Wnt is considered to provide a proliferation signal to immature B-cell stages in bone marrow (Reya *et al.*, 2000). Other studies found Wnt signaling important in regulation of developmental processes including proliferation, differentiation and apoptosis (Døsen *et al.*, 2006; Ranheim *et al.*, 2005). The Wnt signaling pathway was found to be active in both ED15 and ED18 B-cells; as both cell stages undergo differentiation and proliferation (Ratcliffe, 1989). The components identified in ED15 stage are related to the non-canonic Wnt pathway, while the components found at ED18 are related to the canonic type. The difference between the two pathways is not well characterized, so we cannot make conclusions about the differences between ED15 and ED18 differentiation based on the Wnt signaling pathway.

Epidermal Growth Factor Receptors belong to the receptor tyrosine kinase protein super family. EGFR signaling is essential in all multicellular organisms and regulates a high number of cellular functions, as well as organ development and pattern formation (reviewed by Edwin *et al.*, 2006). In mammalian B-cells, the EGFR signal was found to provide a survival and proliferation signal in the bone marrow microenvironment

Table 3: Proteins involved in cellular processes in ED18 developing B-cells

Symbol	Gene Name	GI number
	---------- TNFR signaling members in ED18 sample ----------	
P33	Arginine-specific ADP-ribosyltransferase	258401
ARHGDIB	D4 GDP dissociation inhibitor	50728568
TNFRAP1	TNFR associated protein1	57525126
TRADD1	TNFRSF1A-associated via death domain	50753615
TNFR8	Tumor necrosis factor receptor superfamily member	45383273
MKP1	MAP kinase phosphatase-1	50764356
	---------- Integrin signaling pathway members in ED18 sample ----------	
ACTN2	Actinin, alpha 2	46048687
ITGA8	Integrin, alpha 8	124950
RAC1	Ras-related C3 botulinum toxin substrate 1	45384330
ITGA11	Integrin, alpha 11	50753262
ITGB2	Integrin, beta 2	46048728
ITGB1	Integrin, beta 1	86129418
	---------- Other cell adhesion molecules in ED18 sample ----------	
PCDHB20	Protocadherin-beta20 (Protocadherin-beta T)	50762730
TCAD-2	T-Cadherin 2	386363
CAD13	Cadherin 13 (H-cadherin)	48976117
FLM1	Flamingo 1	40287630
DSG4	Desmoglein 4	50737406
	---------- Wnt signaling pathway members in ED18 sample ---------	
FLM1	Flamingo 1	40287630
WNT5A	Wingless type MMTW integration site protein 5A	45382433
sFRP-2	Secreted frizzled-related protein 2 precursor	61216846
	---------- EGFR signaling pathway members in ED18 sample ---------	
STAT3	Signal Transducer and Activator of Transcription 3	71896343
PI3K	Phophatidylinositol 3 kinase	50761547
MEK2	Dual specificity mitogen-activated protein kinase	22499630
MK2	MAP kinase-activated protein kinase 2	50760337

(Spengeman *et al.*, 2005). Both ED15 and ED18 B-cells express components of the pathway, in agreement with reports that both stages proliferate at a high rate (Ratcliffe, 1989). Therefore, it may be possible that proliferative signals are provided to the B-cells through EGFR. The role of EGFR signaling was investigated in chicken ovarian follicle development (Volentine *et al.*, 1998; Wang *et al.*, 2007) and feather pattern development, as examples of epithelial-mesenchymal interaction (Atit *et al.*, 2003). In the bursal system, EGF signaling might play two roles: it can provide a differentiation signal, regulating the epithelial-mesenchymal interaction at follicle formation as seen in other systems; and EGF signaling might be one source of proliferation signal for both ED15 and ED18 B-cells.

Conclusion: This is the first study to evaluate protein expression at the ED15 and ED18 B-cell stages in the chicken. The proteins revealed signal transduction pathways that differ between the two stages and refer to observed functional differences. The information derived from this work should give insight into the kinds of microenvironmental signals that developing B-cells receive in the bursal follicles. The reported pathways help to explain possible sources of developmental (Wnt, ephrin receptor), proliferative (Wnt, EGFR), apoptotic (TNFR) signals and interactions with the microenvironment (integrins, cadherins) in the B-cell stages. Future studies will confirm and extend MS data using alternative methods and identify specific receptor-ligand pairs in the bursal system associated with the reported signal transduction pathways.

Acknowledgments

This project was supported in part by a Mississippi Agricultural and Forestry Experiment Station Special Research Initiative Grant awarded to Dr. J. Paul Thaxton. The authors are grateful to Ms. Sarah W. Anderson (Department of Poultry Science, MSU) for excellent assistance in the research and Dr. Tibor Pechan (MSU Life Sciences and Biotechnology Institute) for mass spectrometry analysis. We also thank Dr. Michael Kidd (Department of Poultry Science, MSU), Dr. Bindu Nanduri (College of Veterinary Medicine, MSU), and Dr. Stephen Pruett (College of Veterinary Medicine, MSU) for their helpful suggestions and critical review of the manuscript. This article is contribution No. J-11209 from the Mississippi Agriculture and Forestry Experiment Station.

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