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Altered Gene Expression upon BCR Cross-Linking in Burkitt's Lymphoma B Cell Line

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Burkitt's lymphoma cell line, BL2 was stimulated by surface BCR cross-linking and altered gene expression was analyzed by RDA methodology. Consistent with previous reports, we detected up-regulated MDC, IL6R and adhesion molecule LFA1. We also detected gene expression of SIRPα, anti-apoptotic A-20, signal regulatory SLP76 and BCAR3, DNA binding proteins EGR2 and DEC1 in addition to some new genes.

Key Words: **Burkitt's lymphoma cell; BCR cross-linking; RDA; gene expression; transcription factors.**

When B cells encounter T cell dependent antigen, the antigen reactive but still low affinity antibody producing B cells migrate into lymph nodes and the spleen. The activated B cells form germinal centers (GC) and proliferate at a high rate accompanying isotype switching and hypermutation in their immunoglobulin genes. Affinity maturation takes place in the GC microenvironment. Formation of GC provides a unique microenvironment in secondary lymphoid organs which promotes interactions between B cells and surrounding T cells, Follicular Dendritic Cells (FDC), and Dendritic Cells (DC). Antigen specific B cells not only undergo signaling from surrounding cells but also actively participate in these multiple and complex interactions (1).

Surface B cell receptor (BCR) cross-linking results from interaction with antigens. However, BCR cross-linking alone could induce apoptosis or arrest of proliferation of B cells. B cell apoptosis induced by BCR cross-linking can be rescued by co-activation of CD40 by CD40 Ligand (CD40L) on T cell. T cell contact stimulation on B cell provides various signals through cell surface molecules including CD40, B7, MHC class II on B cell by CD40L, CD28, CTLA-4, and TCR on T cell.

The Burkitt's lymphoma B cells are considered as B cells isolated at a point of differentiation by transformation (2). The Burkitt's lymphoma cell line, BL2, shows cell surface marker proteins characteristics of centroblastic B cell phenotypes which suggests it is of centroblastic B cell origin (3). BCR activation is a particularly important step for late stages of differentiation of B cell. In this paper, we characterized altered gene expression in BL2 cells by BCR cross-linking as a model study of antigen stimulated germinal center centroblastic B cells by applying PCR-coupled cDNA Representational Difference Analysis (RDA). Our results may identify an important signaling pathway that exist in complex B, T, FDC, DC networks in the microenvironment of secondary lymphoid organs.

MATERIALS AND METHODS

Cells. Burkitt's lymphoma B cell line, BL2, was generously provided by Dr. George Miller at Yale Medical School. The cell line was cultured in 10% FCS containing complete RPMI1640 medium at 37°C.

Antibody cross-linking of BL2 cells. BL2 cells were incubated at a density of 0.5×10^6 cells/ml in complete RPMI 1640 medium containing 10 μg/ml anti-human IgM goat IgG $F(ab')^2$ (Jackson ImmunoResearch Lab. Inc., West Grove, PA) for 36 h prior to harvest for RNA preparations. At this concentration of anti-IgM treatment, BL2 growth was arrested after doubling their cell numbers. The anti-IgM stimulated and non-stimulated cells were processed at the

Abbreviations used: GC, germinal center; FDC, follicular dendritic cell; DC, dendritic cell; BCR, B cell receptor; RDA, Representational Difference Analysis; SIRP, signal-regulatory protein; SH2, src homology 2; SLP76, SH2 domain containing leukocyte protein of 76 kDa; MDC, macrophage derived chemokine; CD40L, CD40 Ligand; CCR4, CC chemokine receptor 4; BCAR3, breast cancer antiestrogen resistance 3; LFA, lymphocyte function-associated antigen; IL6R, interleukin 6 receptor; EGR2, early growth response protein; TCR, T cell receptor.

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same time to minimize experimental variation. Cells were resuspended in 4 ml PBS, and immediately loaded on 3 ml Ficoll/Paque (Pharmacia, Uppsala, Sweden) and centrifuged at 4000 rpm for 20 min at RT. Live cells were collected and washed with cold PBS for three times and RNA isolated.

RNA and cDNA preparations and RDA. RNA extraction was carried out by Trizol (Gibco/BRL, Grand Island, NY). About 1.0×10^6 cells were extracted with 0.5 ml of Trizol at RT for 5 min followed by 100 μl chloroform extraction. The RNA was precipitated by adding 5 μg glycogen and 300 μl isopropanol and spun at 12,000 for 20 min at 5°C. After removal of residual genomic DNA from total RNA by DNAse I (RNase free) (Boehringer Mannheim, Indianapolis, IN) followed by phenol and chloroform extractions, mRNA was prepared using an Oligotex mRNA isolation kit (Qiagen, Valencia, CA). Elution was carried out with 6 μl of 75°C preheated elution buffer twice. The remaining steps for RDA were performed as described by Hubank and Schatz (1994) (4). The final third differential products were cut with *Dpn*II and DNAs were subcloned into the *Bam*HI site in the pBlueScript II SK vector. DNA sequences of individual clones were determined with ABI PRISM 373A DNA sequencer (PE Biosystems, Foster City) using Thermo Sequenase kit (Amersham, Piscataway, NJ) with T7 and reverse sequencing primers.

RESULTS AND DISCUSSION

RDA technology is a powerful and efficient technology using PCR amplification with specific linkers as primers combined with subtraction of product of control cells from product of test cells that allows detection of differentially expressed genes (4–7). A summary of 30 clones identified by RDA analysis of activated BL2 cells is presented in Table 1. We detected gene expression of MDC, IL6R as well as SIRPα, adhesion molecule LFA1, anti-apoptotic A-20, signal regulatory SLP76, and BCAR3, DNA binding proteins EGR2 and DEC1 in BCR cross-linked Burkitt's Lymphoma BL2 cell. It is known that MDC in unstimulated B cell lineage (8) and IL6R in BL2 cell line are not expressed (9, 10). LFA1 molecule is significantly up-regulated upon B cell stimulation with anti-IgM and IL4 (11). Consistent with previous observations, we detected expression of MDC, IL6R and LFA1 in the BL2 cell stimulated by BCR cross-linking.

MDC is a recently identified member of the CC chemokine family and attracts T cells and expression is detected in macrophage, monocyte derived DC and NK. MDC interacts with CC chemokine receptor 4 (CCR4). Only in CCR4-transfected cells, but not other CCR family member receptor transfectants, a calcium flux was induced by MDC (12). CCR4 chemokine receptor is expressed on the surface of T cells, thus, MDC can provide a recognition signal to T cell (13). MDC was expressed in maturing DC. During maturation of DC, MDC expression is up-regulated *in vivo* in lymph nodes as well as *in vitro* (14, 15). Such maturing DC, supposedly due to MDC, preferentially attract antigen activated T cells more strongly than naive T cells and are thought to promote DC encounters with antigen-specific T cells (14). On the other hand, in murine and human B cells no mRNA expression is detected in unstimulated cells (8). In B cells, so far MDC was detected after CD40 stimulation (8). However, for T cells to be able to stimulate B cells through CD40, already established T-B cell contact is a prerequisite because CD40L is expressed on T cells. We detected induced MDC by BCR cross-linking in BL2 cell. In a GC environment perhaps MDC production by antigen stimulated B cells could recruit antigen activated T cells to facilitate T cell help. Upon antigen stimulation apoptosis could be induced in B cells. Activated B cells could be rescued from apoptosis by CD40 stimulation by CD40L on recruited activated T cells.

It is known that CD40 activation can induce A20 in B cells (16). However, we observed expression of anti-apoptotic A-20 gene in the B cell by BCR cross-linking. In BCR activated B cells, A-20 induction may enhance resistance to apoptosis.

We detected three src homology 2 (SH2) domain related signal molecules. Signal-regulatory proteins (SIRP) are transmembrane glycoproteins with three extracellular Ig like domains (17). Previously it has been reported that SIRP is selectively expressed by myeloid cells (macrophages, monocytes, granulocytes, DCs) and neurons (17). No SIRP expression was detected in T-cell or B-cell lines (18). A member of SIRPα

TABLE 1

Summary of cDNA Clones from RDA Analysis of Anti-IgM Activated B Cell (BL2) Products

Number of cDNA clones	Representing 11 genes	GenBank Accession No.	dbEST Id
Known sequences			
8	DEC1	NM_003670	
3	EGR2	J04076	
3	hIL6R	X12830	
2	SLP76	NM_005565	
2	homo B/K protein	AF220560	
1	SIRPalpha	Y10375	
1	MDC	U83171	
1	A20	M59465	
1	hnRNPA2	NM_002137	
1	LFA1	M29487	
1	BCAR3	NM_003567	
Total: 24			
New Genes			
3		AW755261	4215617
1		AW755262	4215618
2		AW755263	4215619
Total: 6			

Note. DEC1 refers to a transcription factor expressed in differentiated human embryo chrondrocytes. EGR2 is early growth response protein. hIL6R is IL6 receptor. SLP76 is SH2 domain containing Leukocyte Protein of 76 kDa. SIRP alpha is from a family of human proteins that inhibits signaling through tyrosine kinase receptors. MDC is human macrophage-derived chemokine precursor. A20 is a human transcription factor important in apoptosis cell signaling. hnRNP is heterogeneous nuclear ribonucleoprotein. LFA is Leukocyte adhesion glycoprotein. BCAR3 is a breast cancer anti-estrogen resistance 3 gene.

family expressed on monocytes and a sub-population of dendritic cells mediate binding to CD4 T cells (19). The SIRP cytoplasmic tail contains two immunoreceptor tyrosine-based inhibition motifs (ITIM) and is a substrate of activated protein tyrosine kinases (PTK), and its tyrosine-phosphorylated form binds SHP-2 through its SH2 domain. It also binds SHP-1 and Grb2 *in vitro* (20). SIRPs negatively regulate signaling through PTKs, however, their physiologic functions are not well characterized (17, 18). We speculate that SIRPα expressed in B cells could play important roles in B cell as a negative regulator and also could mediate interaction and signaling with CD4 T cells.

We detected expression of intracellular signaling molecules SLP76 (SH2 domain containing Leukocyte Protein of 76 kDa) in the BCR stimulated B cells. SLP76 is a crucial component in T cells. Previously, in spleen and in B cell lines SLP76 expression was detected in addition to in peripheral blood leukocytes, thymus, and T and monocyte cell lines (21). SLP76 can interact with Vav and cooperate to induce activity of the transcription factor NF-AT and IL-2 expression. SLP-76-deficient mice exhibit a profound block in T-cell development (22). SLP-76 is a substrate of the TCR activated protein tyrosine kinase pathway which associates with the adaptor protein Grb2. SLP76 could also be involved with signal regulation by SIRP. The role of SLP76 in signal transduction in B cells has not been characterized, however, it could play an important role in B cell signaling.

We also detected a recently reported breast cancer anti-estrogen resistance 3 (BCAR3) which has an SH2 domain as well as CDC48 homologous domain in its c-terminus and is involved in anti-estrogen resistance in human breast cancer cells (23). BCAR3 could be involved in signal transduction and could have important rolls in B cells.

It is interesting that we detected IL6R in BL2 cell by BCR cross-linking. IL6 together with IL2 stimulate B cell differentiation to an antibody secreting cell. Both naive and memory B cells can be induced to secrete IL-6 upon CD40 stimulation while in GC B cell stage there is no IL6 (1). On the other hand, its receptor, IL6R, is inducible at a certain stage. Induction of gp80 allows formation of an IL6R heterodimer on the cell surface together with constitutively expressed gp130 that allows IL6 responsiveness of the B cell (1, 24, 25). IL6R expression could be up-regulated upon BCR stimulation as well as previously reported anti CD40 stimulation. Thus, it is a functional characteristic of human GC B lymphocytes to lose the ability to induce IL-6 and to acquire the ability to induce IL6R in GC is parallel. This may permit better control of B cell growth and differentiation to develop to antibody secreting cells during the germinal center reaction (1).

It is noteworthy that the expression of recently identified transcription factors, EGR2 and DEC1, were also detected in the BCR stimulated BL2 cells. EGR2 was first identified as an immediate-early response gene, encoding a Cys2His2 type Zinc finger protein that binds DNA in a sequence-specific manner and acts as a transcription factor and may play a role in the regulation of cellular proliferation (26). Egr2 knockout mice display hypomyelination of the peripheral nervous system and a block of Schwann cells at an early stage of differentiation (27). DEC1 was cloned from Bt_2cAMP stimulated human chondrocytes. Its predicted sequence has a basic helix-loop-helix (bHLH) which is found in the mammalian HES family, Drosophila hairy, and Enhancer of split m7. DEC1 was expressed in various tissues including the cartilage, lung, spleen, and intestine, but not in the brain (28). These genes of DNA-binding protein are recently identified and functions are currently not well understood. Because of their significant DNA-binding motifs and induction by BCR cross-linking, these genes may have significant physiological roles in B cell signaling.

In addition, we obtained three new sequences by RDA. Their GenBank EST Accession Nos. are listed in Table 1. The AW755261 matched with other ESTs obtained from tonsil (AI567785) and lymph (AI433612). Among 500 bp of AW755261, 50 bp has 86% homology to human proto-oncogene tyrosine-protein kinase (abl) gene (GenBank Accession No. U07563.1). It is said that abl gene is the cellular homolog proto-oncogene of Abelson's murine leukemia virus and is associated with t9:22 chromosomal translocation with the BCR gene in chronic myelogenous and acute lymphoblastic leukemia.

BCR stimulation is important for late stages of B cell differentiation. These expressed genes with already known as well as unknown sequences in BCR crosslinked B cell could be potentially involved in B cell differentiation, development, and hypermutation processes. The genes detected in BL2 model in this study are from a Burkitt's lymphoma cell. Currently analysis in tissue and stage specific expression of the known genes discussed here as well as cloning the full length cDNA of the unidentified genes are in progress.

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