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Review

Proteomic changes during the B cell development

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Abstract

An antibody-secreting B cell is derived from a lymphoid stem cell through a series of developmental stages: progenitor B cell (pro-B cell), precursor B cell (pre-B) cell, immature B cell and mature B cell stage. The gene rearrangement of antigen receptor genes and their expression on the cell surface are crucial regulation steps cells to develop to the next stage. This control mechanism occurs at the sequential manner during the B cell development. Proteomic approach using two-dimensional (2-D) gel electrophoresis and mass spectrometry makes possible to analyze the expression pattern of total proteins at each developmental stage and isolate proteins differentially expressed in B cells. Some transcriptional factors such E2A and Pax5 are expressed at the earlier stages of B cell development and repressed at later developmental stages. RAD52-related protein and chromatin assembly factor 1 are dominantly expressed at early B cells undergoing DNA rearrangement. These comparative analyses of total proteins in each B cell can provide some crucial information to understand the molecular basis of B cell ontogeny.

Keywords: Proteomics; B cell development; Gene rearrangement

Contents

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1. Introduction

With T lymphocytes, B lymphocytes are major cells responding to cellular defenses in our immune systems, specially in adaptive immunity capable of recognizing and selectively eliminating specific foreign antigens (e.g. microorganisms, virus, or molecules). T cells are mainly involved in cellular immune-responses through the cytotoxic mechanism and production of cytokines. In contrast, B cells play a key role in humoral immune-responses by the production of soluble components, named immunoglobulins (Ig) (or antibodies). These antibody molecules can directly recognize to foreign invaders and trigger to defeat them. Both B and T lymphocytes encounter antigens through specific antigen receptors expressed on the surface of cells and become immunocompetent cells.

B cell development begins in bone marrow as common lymphoid stem cells differentiate into several distinctive intermediate B cells; progenitor B cell (pro-B cell), precursor B cell (pre-B cell), immature B cell, and mature B cell. This B cell differentiation is ordered by stepwise re-arrangements of immunoglobulin genes (heavy chain and light chain genes) and expression of specific surface marker proteins: B220, c-kit, CD25 and IgM [1,2]. Overall scheme about B cell development is depicted in Fig. 1. First, lymphoid stem cells begin to differentiate into pro-B cells. This developmental stage can be sub-divided into two different stages: early pro-B cell stage and late pro-B cell stage. During the transition from lymphoid stem cells to early pro-B cells, D-J joining of Ig heavy chain gene segments takes place by expression of two recombination activating gene (RAG1 and RAG2) at the lymphoid-specific manner upon the commitment to the B cell lineage [3]. Development of early pro-B cells to late pro-B cells induces V-DJ joining of Ig heavy chain gene segments. Upon completion of rearrangement process of Ig heavy chain genes, the re-arranged heavy chain gene (μ) is expressed together with two surrogate light chains (VpreB and λ5) on the surface of B cells [4]. These B cell groups are called Pre-B cells, and they can be sub-divided into two groups. One pre-B cell group appears at the earlier stage, and its cell size is large (called large pre-B cell). This large pre-B cell then undergoes further differentiation into the small size cell of pre-B cell (called small pre-B cell). During this developmental process, the V-J joining of Ig light chain gene segments is induced. Small pre-B cells become immature B cells by a further differentiation process. These immature B cells express complete IgM molecules as a antigen receptor on the cell surface [5]. These B cells differentiated in bone marrow move to periphery tissues and undertake one more developmental step to become naïve mature B cells. Upon encountering antigens, naïve B cells are activated, proliferated and differentiated into mature B cells in the periphery. Mature B cells express both IgM and IgD as antigen receptor molecules. This antigen-driven activation and clonal selection of naïve B cells leads to generation of plasma cells producing soluble antibodies and memory B cells.

Cell surface markers are specifically expressed on the cell surface at distinctive B cell lineages. Commitment to the B cell lineage induces the expression of a protein tyrosine kinase c-kit [6]. Thus, earlier B cells such as pro-B cells pre-

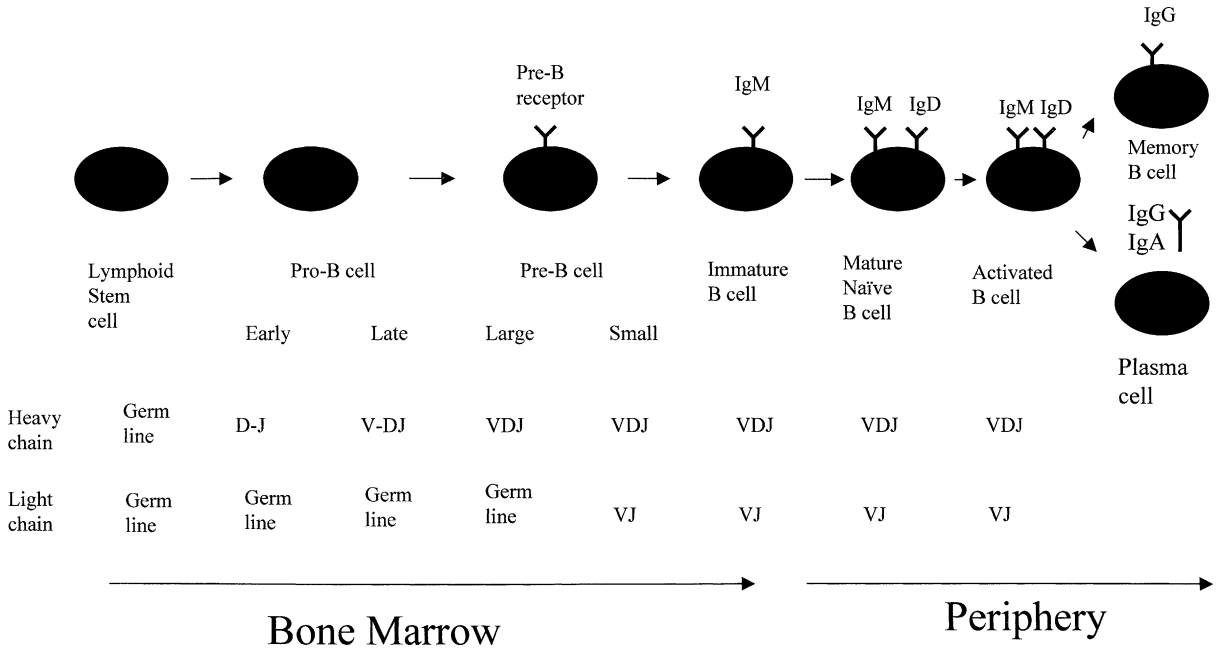

Fig. 1. The developmental stages of B lymphocyte. B cell begins to develop from lymphoid stem cells in the bone marrow at the antigen-independent manner and gets to mature in the periphery upon contact with antigens. Pre-B cells express pre-B cell receptor consisting of immunoglobulin (Ig) heavy chain and two surrogate light chains (Vpre-B and λ5). Immature or mature B cells express IgG or/and IgD as antigen receptors. The variable region of heavy chain genes (V, D, J, and C segments) or light chain genes (V, J, and C segments) is produced from gene rearrangement specifically occurring at developmental stages.

dominantly express this membrane-bound protein tyrosine kinase on the surface. In addition, pro-B cells begin to express many other surface marker proteins such as CD45R (B220), CD19, CD24 (HSA), CD43, IL-7R and two accessory molecules of antigen receptor Ig-α/Ig-β [1]. CD45R, CD19, HSA and Ig-α/Ig-β are continuously expressed on other B cells in later developmental stages. IL-7R and CD43 molecules appear on the cell surface until the large pre-B cell stage. Pro-B cells also begin to express two surrogate light chains VpreB and λ5 [4]. These two surrogate light chains associate with Ig μH chain and assembly into a pre-B cell receptor at the pre-B cell stage (Fig. 1). After the small pre-B cell stage, these surrogate light chains are replaced by true light chains. Immature B cells therefore begin to expose antigen receptors assembled with μ heavy chain and light chains on the cell surface. During the Pre-B cell developmental stage, cells transiently express CD25 molecules on the surface.

Besides these cell surface markers, many proteins are specifically expressed during the B cell developmental process. They include not only proteins involving in the rearrangement process of Ig gene segments but also transcriptional factors such as PU.1, GATA factor, ikaros, E2A, EBF (early B cell factor), and Pax5 [7,8]. Recombination activating genes (RAG1 and RAG2) are essential proteins for a lymphoid-specific gene rearrangement process, named V(D)J recombination. They are predominantly expressed in the earlier stages of B cell development where V(D)J recombination takes place [9,10]. Terminal deoxynucleotidyl transferase (TdT), another protein involving in V(D)J recombination, is also specifically expressed in the pro-B cell stage [11]. The B cell development stage-specific expression of transcriptional proteins has revealed their crucial roles in regulating B cell differentiation. For examples, PU.1, GATA and ikaros play an important role in the commitment of pluripotent hematopoietic stem cells to the B cell lineage [12-14]. Three other transcription factors, early B cell factor (EBF), E2A and B cell-specific activator protein (BSAP, called Pax5), have been also studied by gene targeting for their roles at the B cell development [7,8]. In particular, they are essential for the early antigen-independent stages of B cell development in bone marrow: pro-B cells to pre-B cells.

The variable region of Ig heavy chains is composed of three different gene segments: variable (V), diversity (D) and joining (J) [2,15]. These three gene segments undergo the rearrangement process to form a functional heavy chain during the B cell development. This recombination process specifically and sequentially occurs at the distinctive stages during the B cell development. The D-J arrangement undergoes first and then the V-DJ joining follows when the D-J joining is successfully completed. If a D-J joining fails, B cells induce another joining of D and J gene segments. The variable region of Ig light chains also takes place at a specific stage of B cell differentiation but only undertakes V-J joining because D segments in light chain genes are absent. Because this fatal DNA rearrangement (DNA double strand break and repair) has to occur at right time and right place in the fast developing B cells, it is strictly and specifically regulated in B cells. To date, it is very unclear what factors and mechanisms are involved in this regulation.

2. Proteome changes during the B cell development

B lymphocytes in different developmental stages are distinguishable as shown in Fig. 1. Specific protein expression at each stage might determine the commitment to move on each developmental stage and cellular functions (e.g. B cell receptor gene rearrangement). Thus, determination of overall protein expression pattern in each developmental stage can provide information about not only B cell ontogeny but also regulation of gene rearrangement process. In fact, RNA expression profiles at consecutive developmental stages have been determined in some previous studies [16–19]. However, the level of mRNA in cells does not always reflect the amount of functional proteins. Thus, the determination of proteome changes in each B cell developmental stage might be more informative.

Many technologies have been used for analysis of gene products uniquely or differentially expressed in a certain cell or tissue. One of them, proteomic analysis, a direct comparison of normal and malignant cell total proteins, make a possible to isolate some disease-specific proteins. These isolated proteins can be used as target proteins for diagnosis or drug development. In addition, regulators in many biological mechanisms (e.g. cell cycle, cell development or differentiation, etc.) can be isolated from these comparative analyses of proteins [20,21]. The recent great progress in proteomics is due to advances of both protein separation techniques (e.g. 2-dimensional gel (2-D) electrophoresis, high pressure liquid chromatography or capillary electrophoresis) and protein identification by mass spectrometry.

2.1. Methods for proteomic analysis

2.1.1. Sample preparation and nuclei isolation

Cultured B cells were washed with 1× PBS twice, and total proteins were solubilized in lysis buffer (8 M urea, 4% Chaps, 40 mM Tris, 100 mM DTT, and 0.5% ampholyte) for 1 h. Soluble proteins were separated by a centrifugation at 15,000 rpm for 10 min at 15 °C. The protein concentration of the supernatant was determined using a standard Bradford method. For nuclei isolation, cultured cells were added with one volume of hypotonic solution (10 mM HEPES–KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF). Cells were homogenized with douncing homogenizer 10 times and nuclei were separated at 14,000 rpm for 10 min at 4 °C. Nuclear total proteins were solubilized in the lysis buffer mentioned above.

2.1.2. 2-Dimensional gel electrophoresis

IPG gel strips (Pharmacia) were rehydrated in a swelling solution (7 M urea, 2% Chaps, 100 mM DTT, 0.5% IPG buffer (Pharmacia), and bromophenol blue) containing 50 μg (for silver staining) or 500 μg (for Coomassie staining) proteins for 12 h at 20 °C. Isoelectric focusing was performed at 20 °C at three steps: 250 V (15 min), 10,000 V (3 h), and 40,000 V h^{-1}. IPG gel strips were equilibrated in an equilibration buffer I (50 mM Tris–HCl, 6 M urea, 30% glycerol, 2% SDS, 10 mg/ml DTT) for 15 min and re-equilibrated in equilibration buffer II (50 mM Tris–HCl, 6 M urea, 30% glycerol, 2% SDS, and 25 mg/ml iodoacetamide) for 15 min. For SDS gel electrophoresis, a 7.5–17.5% gradient SDS gel was prepared, and the equilibrated IPG gel strip was laid on the top of the gel filled with 0.5% agarose solution. Gel electrophoresis was carried out at 16 °C at 5 mA/cm (constant current) for 1 h and 10 mA/cm until bromophenol blue reached the bottom of gel. For silver staining, the gel was fixed in a solution (50% methanol and 12% acetic acid) for 1.5 h, washed in 50% ethanol for 30 min twice, then treated with 0.2% sodium sulfoxide for 1 min. After washing with deionized water for 1 min three times, the gel was impregnated in a solution (0.2% silver nitrate and 0.75 mL/L formaldehyde) for 20 min and washed in water twice. The gel was developed in a solution (2% sodium bicarbonate, 0.0004% sodium sulfoxide, 0.5 mL/L formaldehyde) and stopped by adding 1% acetic acid at a desired time point. For Coomassie blue staining, the gel was soaked in a fixation solution (30% ethanol and 2% phosphoric acid) for 30 min then in 2% phosphoric acid for 20 min, and equilibrated in a solution (2% (v/v) phosphoric acid, 18% ethanol, 15% ammonium sulfate) for 30 min. The gel was stained in the equilibration solution containing 1% Coomassie brillant G blue for 24–72 h.

2.1.3. Image analysis

The scanned gel image was analyzed using a standard protocol of PDQuest software (Biorad).

2.1.4. Protein identification

The Voyager TM-delayed extraction (DE) STR biospectrometry workstation was used for matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry. The desired gel pieces were excised carefully and washed in water for 15 min twice followed by additional wash in water/acetonitrile (1:1) for 15 min twice. After removing all liquid, acetonitrile was added to cover the gel pieces. Acetonitrile was removed when the gel pieces have shrunk. The gel pieces were rehydrated in 0.1 M ammonium bicarbonate for 5 min, then incubated for 15 min by addition of an equal volume of acetonitrile. After removing all liquid, gel pieces were dried in a vacuum centrifuge for 20 min. The gel pieces were swollen in 10 mM DTT/0.1 M ammonium bicarbonate and incubated for 45 min at 56 °C then chilled to room temperature. After removing excess liquid, the same volume of freshly prepared 55 mM iodoacetamide in 0.1 M ammonium bicarbonate was added and incubated in the dark for 30 min room temperature. The iodoacetamide solution was removed, and the gel pieces were incubated in 30 μL of 0.1 M ammonium bicarbonate for 5 min then additionally incubated for 15 min by adding an equal volume of acetonitrile. After incubation with ammonium carbonate/acetonitrile once more, the gel pieces were dried in a vacuum centrifuge for 20 min. The gel pieces were rehydrated in trypsin digestion buffer and placed on ice for 45 min. The buffer was replaced by 20 μL of digestion buffer with trypsin (12.5 ng/μL). After overnight digestion at 37 °C, a sufficient volume of 25 mM ammonium bicarbonate to cover the gel pieces was added and incubated for 15 min. Then, the same volume of acetonitrile was added and incubated for 15 min. Five percent formic acid/acetonitrile (1:1) was added to the recovered supernatant and incubated for 30 min. After repeating this step, all the extracts were dried in a vacuum centrifuge for 6–7 h. The dried peptide was dissolved in 20 μL 5% formic acid and sonicated for 5 min with water bath sonicator. The peptide sample (2 μL) with standard calibrant (1 μL) was mixed in 2 μL matrix mixture (matrix solution (20 mg a-cyano-4-hydroxycinnamic acid/1 mL acetone):nitrocellulose solution (20 mg nitrocellulose/1 mL acetone):2-propanol = 2:1:1). MALDI plate deposited with 2 μL of sample was dried for 30 min room temperature and rinsed with 5 μL of 5% formic acid followed by an additional wash with 5 μL of water. After drying at room temperature, the plate probe was inserted into the MALDI mass spectrometry. Mass search was performed in sequence database (Swiss-prot or NCBI) after MS-fit (http://prospector.ucsf.edu/ucsfhtm13.4/msfit.htm).

2.2. Total cellular proteome changes

2.2.1. Pro-B cell to pre-B cell

The B lymphocyte development from pro-B cell to pre-B cell occurs in bone marrow and is characterized by expression of cell surface markers and Ig gene rearrangement. Pre-B cell expresses CD25 on its surface whereas pro-B cell does not. c-kit is expressed only in pro-B cell [6]. During these stages of development, the rearrangement machinery genes such as RAG1, RAG2 and TdT are specifically expressed, and expression of two surrogate light chain genes VpreB and λ5 are induced [9-11]. Comparison of protein expression in two cell types can give some insights into how proteome is changed from pro-B cell to pre-B cell. According to the our results using 2-D gel at the range of pH 4–7, 79 among total cellular proteins are expressed only in pro-B cells, and 28 total cellular proteins are exclusively expressed in pre-B cells (Table 1). In addition, 40 proteins and 21 proteins are over three-fold up-regulated in pro-B cell and pre-B cell, respectively. Some uniquely expressed, up-regulated or down-regulated proteins between pro-B and pre-B cells are showing in Fig. 2. Chromatin assembly factor 1 (CAF1) is uniquely expressed in pro-B cell. This protein has been known as a chromatin assembler that plays a role in changing chromatin structure [23]. Modulation of the chromatin structure is a crucial part in transcriptional control of many genes and gene rearrangement

Table 1
Comparison of pro-B cell and pre-B cell proteins in 2-D gel

	Unique proteins in pro-B cell	Unique proteins in pre-B cell	Proteins up-regulated in pro-B cells	Proteins up-regulated in pre-B cells
Cellular proteins (pH 4–7)	79	28	40	21
Nuclear proteins (pH 4–7)	119	37	30	28

process [24–27]. Thus, some chromatin-related proteins are involved in the differentiation of B cell. Previous studies using DNA chip have shown that many chromatin proteins are differentially expressed at the specific developmental stage during the B cell ontogeny [17,18]. For examples, expression of high mobility group proteins such HMG-17 and HMG1 is largely increased in pre-B cells. In particular, HMG1 have been known as enhancer or modulator in V(D)J recombination [28]. The chromatin remodeling complex SWI/SNF facilitates not only transcription in many cells but also V(D)J recombination in lymphocytes [29].

Microtubule proteins (tubulin α-6 and tubulin α-1) and heat shock protein 60 are up-regulated in pro-B cells. Heat shock proteins (HSP) are involved in many cellular reactions by modulating other proteins. According to our unpublished result, a HSP 70 participates in V(D)J recombination by remodeling the protein/DNA complex. In addition, expression of some signal proteins such as Ayk1, LCK and Soc-2 is more increased in pre-B cells than in pro-B cells [17,18]. Tyrosine protein kinase LCK has been suggested as having many roles in lymphocyte development and other cellular functions [30,31]. Several serine/threonine kinases including Ayk1 were also dominantly expressed in pre-B cells in previous studies [17,18]. However, the cellular function of Ayk1 is unclear. Because pre-B cells start to display antigen receptors assembled with two surrogate light chains during the B cell ontogeny, the Ig μ heavy chain is largely expressed in pre-B cells.

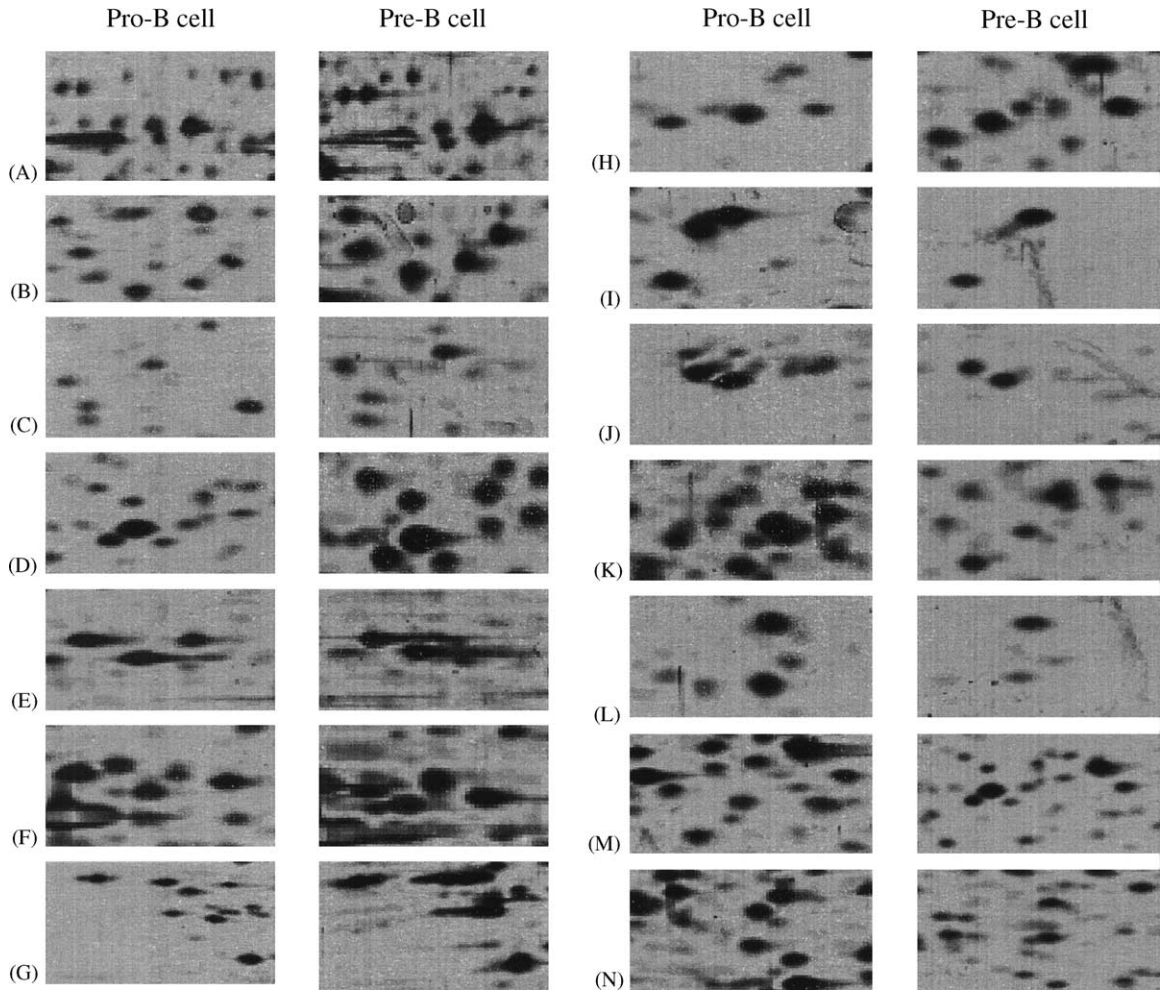

Fig. 2. 2-D gel analysis of pro-B and pre-B total cellular proteins. Total cellular proteins of pro-B or pre-B cell were prepared and analyzed in two-dimensional gel electrophoresis as described in text. Protein spots of some regions (A-N) in the gel were compared each other.

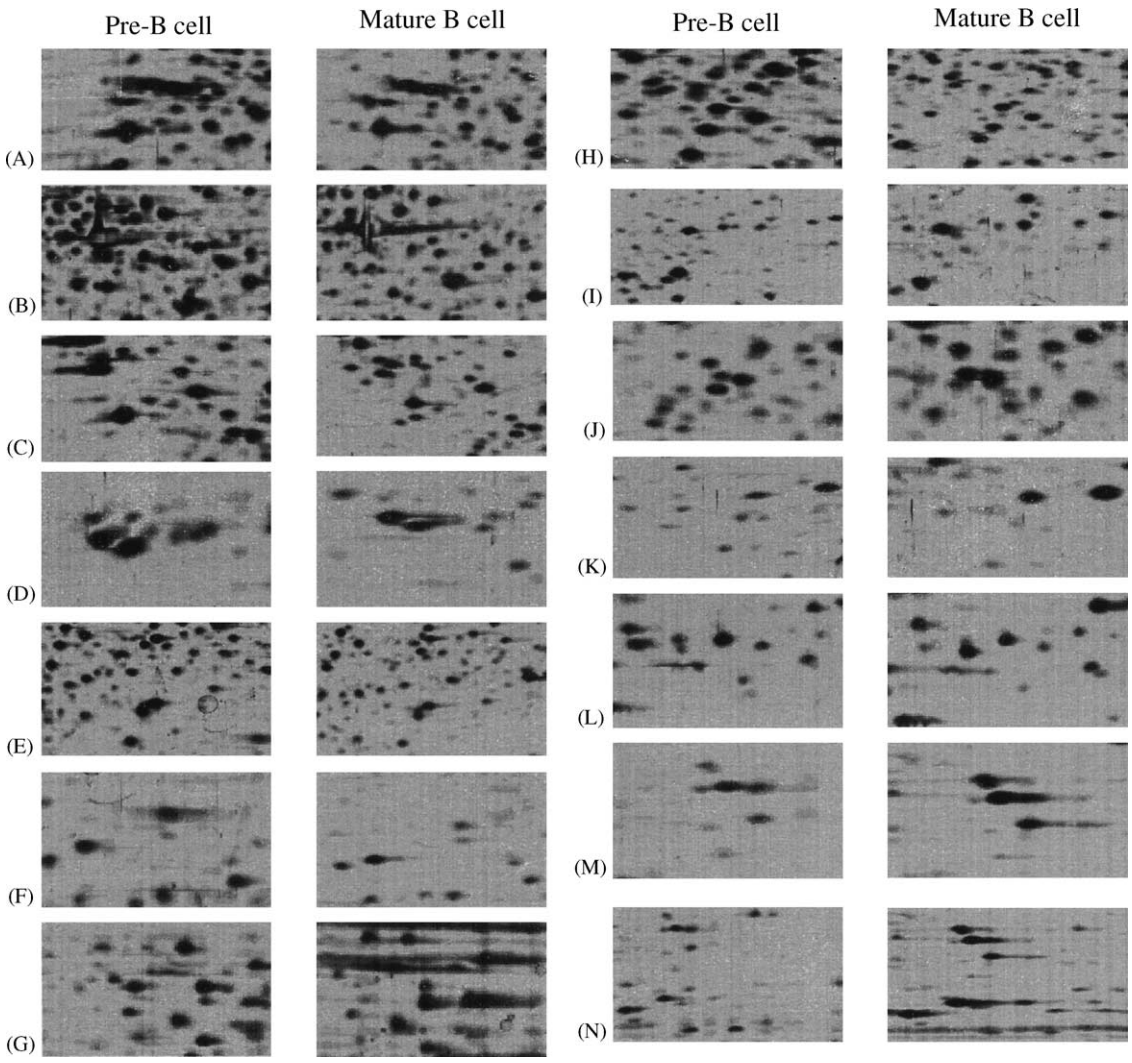

Fig. 3. 2-D gel analysis of pre-B and mature B total cellular proteins. Total cellular proteins of pre-B or mature B cell were prepared and analyzed in two-dimensional gel electrophoresis as described in text. Protein spots of some regions (A–N) in the gel were compared each other.

2.2.2. *Pre-B cell to mature B cell*

When pre-B cells have a functional rearranged light chain gene, they are committed to develop into immature B cells and express IgM on the cell surface. Immature B cells undergo a further maturation process and become mature B cells expressing not only IgM but also IgD on the surface. Comparison of total cell proteins of pre-B cell and mature B cell indicates how these two B cells differentially express genes involved in B cell development and maturation. While mature B cells uniquely express about 130 cellular proteins, only 18 cellular proteins are exclusively expressed in pre-B cells. At the mature B cell, 55 genes are up-regulated and 24 genes are down-regulated compared to pre-B cells (Table 2, Fig. 3). Once B cells become mature, they proliferate in response to antigen stimulation and move to the periphery tissues for the further maturation. Thus, proteins involved in cell survival or cell migration or adhesion are largely expressed in mature B cells. An anti-apoptotic protein bcl-2 has its increase of expression in mature B cells [10,32]. Also, many proteins in the MAP kinase pathway are up-regulated in mature B cells [18].

Table 2
Comparison of pre-B cell and mature B cell proteins in 2-D gel

	Unique proteins in pre-B cell	Unique proteins in mature B cell	Proteins up-regulated in pre-B cells	Proteins up-regulated in mature B cells
Cellular proteins (pH 4–7)	18	132	24	55
Nuclear proteins (pH 4–7)	114	60	10	21

2.3. Total nuclear proteome changes

During the B cell development, many transcriptional factors in the nucleus have been implicated for their crucial roles in the cell differentiation [33,34]. The ets family protein PU.1 is required for all myeloid and lymphocytic lineages from multipotent progenitors and further development to B cells [12,35]. The zinc finger protein Ikaros is essential for the earlier development of T or B cells, but not for myeloid cells. It associates with chromatin remodeling proteins such as SWI/SNF or NURD and enhances to expression of Flk-2, c-kit, L-selectin and IL-2Rβ [14,36,37]. Two basic helix–loop–helix proteins E2A and EBF are required for both B and T cells development by inducing expression of RAG1/2, VpreB, λ5, TdT and Igα/β [38,39]. The Pax5 is induced at the earlier stage of B cell development. LEF-1, a family of high mobility group transcription factor, acts as an important regulator during the lymphocyte development and proliferation [40]. Together, transcription factors are a key component to control the B cell development. In addition to nuclear transcriptional factors, V(D)J recombination, a key process to produce antigen receptors in the B lymphoid development, takes place in the nucleus. This process controls the progression of stepwise B cell development. If the heavy chain genes fail to be rearranged at the pre-B cell stage, progression of cells to the immature cells is blocked. Thus, analysis of total nuclear proteins of B cells in the different developmental stages can give some insights into the understanding about the B cell development.

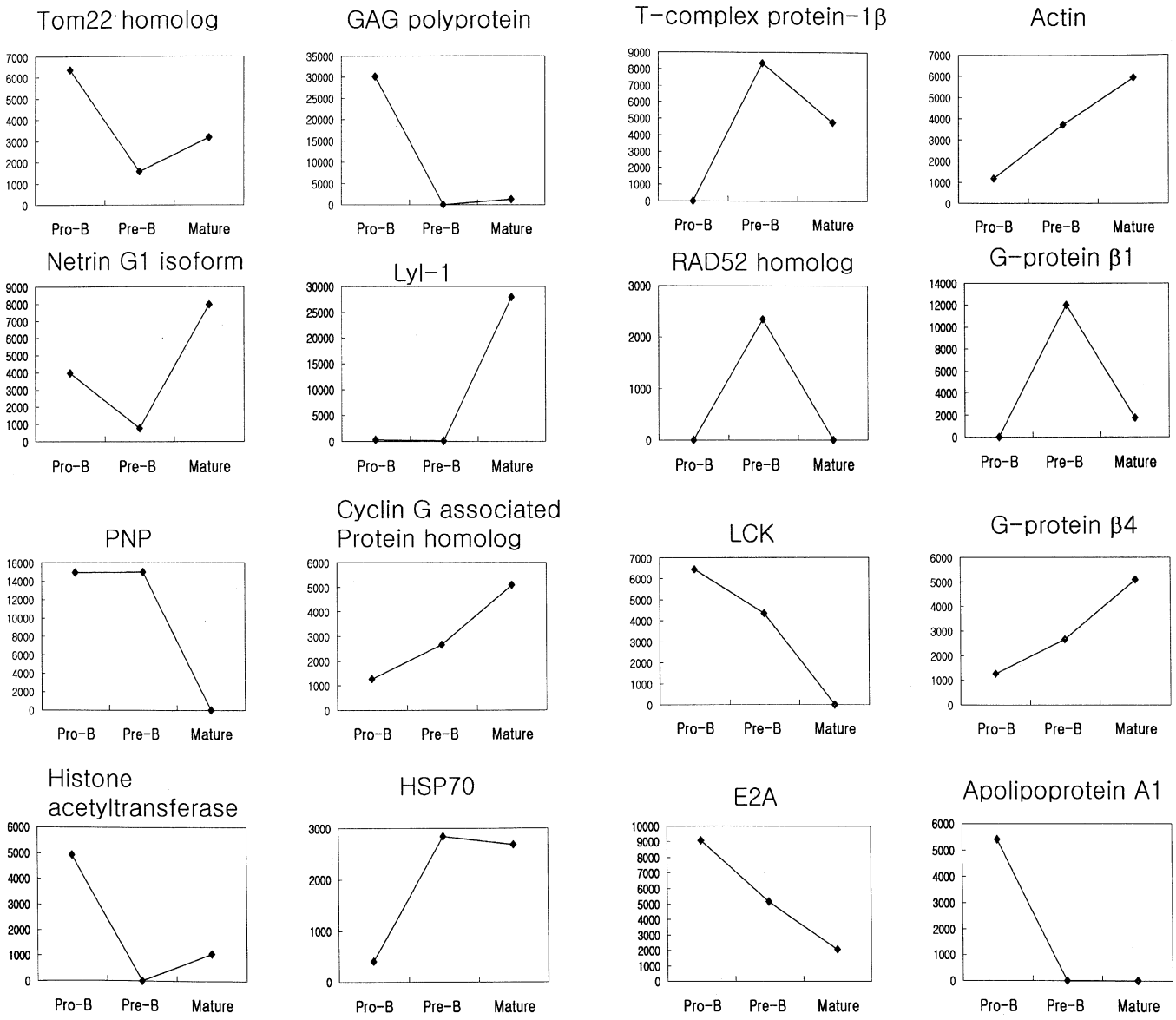

Fig. 4. Expression profiles of differentially expressed nuclear proteins in the B cell development. The expression level at three developmental stages (pro-B cell, pre-B cell and mature B cell) of each protein was analyzed by PDquest software (Bio-Rad) and indicated on the *y*-axis as some arbitrary numbers.

Many nuclear proteins are uniquely expressed at each developmental stage (Tables 1 and 2). One hundred and nineteen and 37 proteins at the range of pH 4–7 are exclusively expressed in pro-B cells and pre-B cells, respectively. Expression of 30 pro-B cell proteins and 28 pre-B cell proteins is up-regulated. In the mature B cell, about 60 proteins are uniquely expressed, and many proteins are three-fold more up- and down-regulated (Table 2). Among them, expression of Tom 22 homolog, histone acetyltransferase, GAG polyprotein and apolipoprotein A1 is inhibited with respect to the progression of cell development after their elevated expression at the pro-B cell (Fig. 4). Tom 22 is originally identified as a translocase at the mitochondrial out membrane [41]. It acts as a receptor or organizer for proteins. Histone acetyltransferase, a major chromatin structure modifier, plays a role in transcription regulation of many genes [42]. The expression of LCK and E2A proteins is gradually decreased by B cell differentiation progression (Fig. 4). E2A has been known a specific transcription factor dominantly expressed at the early stage during the B cell development [43]. On the contrary, cyclin G associated protein homolog, actin and guanidine nucleotide binding protein β4 (G-protein β4) show their elevated expression according to the B cell development (Fig. 4). In addition, RAD52 homolog, T-complex protein 1β and G-protein β1 are strongly expressed in pre-B cells, instead their expression at other developmental stages is blocked (Fig. 4). Conversely, while Netrin G1 isoform is down-regulated in pre-B cells, its expression in both pro-B and mature B cells is largely increased. RAD52 is a DNA damage responding protein [44]. Its elevated expression in pre-B cells might explain the large requirement of DNA repair proteins in this particular developmental stage where the active DNA rearrangement process is undergoing to generate the antibody diversity.

3. Conclusions

The B lymphocyte development occurs at several distinctive stages. Each developmental stage represents expression of different cell surface markers and also a specific step of rearrangement of antigen receptor genes. Thus, many proteins might be very specifically expressed at each developmental step to regulate the cell differentiation and gene rearrangement process. To date, several genes have individually been studied their roles in control of B cell lineage. Recently, the development of massive screening methods makes it possible to isolate genes or proteins differentially expressed in malignant tissues or functionally distinguishable cells such as cell lineage or chemical-induced cells. Previously, some researches using DNA chip techniques isolated many B cell lineage-specific genes. Because these techniques are based on the mRNA level in cells, the results using them do not always reflect the level of proteins directly participating in cellular functions. Proteomic approaches using two-dimensional gel electrophoresis allow one to identify proteins differentially expressed at each B lymphocyte developmental stage. According to the previous study [22], not only some proteins known as B cell lineage-specific factors such as E2A and LCK, but also some other proteins have been newly identified. Effort to isolate and identify proteins differentially expressed in the different stage during the B cell development is undergoing. Those results might provide some clues to more clearly understand the control mechanism of the commitment into B cell lineage and differentiation.

Acknowledgement

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