Extreme skewing of annexin II and S100A6 expression identified by proteomic analysis of peritoneal B-1 cells

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Keywords: annexin, B cells, calcium, proteomics, rodent

Abstract

B-1 cells differ phenotypically, biochemically and functionally from conventional B-2 cells. The origin of these differences remains uncertain. We hypothesized that unbiased analysis of differences in protein expression between B-1 and B-2 cells might provide information not otherwise available, and thus undertook 1-dimensional (1D) gel analysis combined with mass spectrometry. We identified annexin II and S100A6 in peritoneal B-1 cells (B-1P) but not in splenic B-2 cells (B-2S); these results were confirmed by western blot analysis and reflected in mRNA expression. Further analysis of mRNA indicated that elevated expression levels of annexin II and S100A6 were unique to B-1P among several naive lymphoid populations. However, expression of annexin II and S100A6 protein was up-regulated in mitogenically stimulated B-2S. In both naive B-1 cells and stimulated B-2 cells, annexin II and S100A6 formed Ca++-sensitive complexes. These results confirm that the emerging field of proteomics detects differentially expressed molecules independently of RNA screening methods. These results identify two proteins (annexin II and S100A6) that are unexpectedly differentially expressed in B-1 cells and, although members of larger families, may fulfill unique, subset-specific functions. These results also validate 1D GE/LC-MS/MS as a reliable screening tool in identifying final protein product expression differences between B-1P and B-2S.

Introduction

B-1 cells constitute a unique subset of B cells, initially distinguished from the more abundant conventional B (B-2) cells by expression of the pan-T cell surface glycoprotein, CD5. Additional identifying phenotypic characteristics include surface Ig (σ2) Mhi, slgDlo, B220lo, CD23neg and CD43pos. Beyond phenotype, B-1 cells are characterized by a number of unusual ontogenetic, biochemical and functional features (1–4). Among the latter in mice are predominant localization to the peritoneal cavity, indolence to BCR-triggered mitogenesis, hyperresponsiveness to phorbol ester treatment, autologous self-replenishment and spontaneous secretion of IgM. The spontaneous production of non-immune (natural) Ig is of particular importance because of its critical and non-redundant role in host immune defense against viral and bacterial infection (5–9). This and other unconventional characteristics of B-1 cells have stimulated efforts to elucidate the physiology of this B cell population. In comparison to B-2 cells, B-1 cells have been found in previous work to express activated forms, and/or unexpected levels, of a number of signaling molecules and transcription factors, including increased Lck (although this has been questioned), increased protein kinase C-α, decreased vav, phosphorylated extracellular signal-regulated kinase, activated STAT3, up-regulated NF-AT and down-regulated Bcl-6, Pax-5 and Notch-2 (10–17). None of these observations, however, seem capable of explaining the unique features of B-1 cells. Because the molecular
mechanisms that might account for differences between B-1 and B-2 cells have not been determined, unbiased approaches to compositional analysis have been pursued, in particular, evaluation of gene expression by DNA microarray (18). More recently, these efforts have led to proteomic analysis based on 2-dimensional (2D) gel electrophoresis followed by mass spectrometry of differentially expressed protein spots, which resulted in the identification in B-1 cells of the smooth muscle-specific protein, transgelin 2 (19).

Two-dimensional gel electrophoresis is labor intensive and does not lend itself to rapid analysis. An alternate approach involves protein inventory by 1D gel electrophoresis followed by capillary liquid chromatography, nanospray ionization and tandem mass spectroscopy (LC-MS/MS) (20). Using 1D GE/LC-MS/MS, we analyzed lysates of highly purified peritoneal B-1 cells (B-1P) and splenic B-2 cells (B-2S). To determine the utility of this approach in comparing and contrasting B cell subsets, we selected two proteins, annexin II and S100A6, which are both members of Ca++-binding/sensitive protein families, and B-1 cells have been reported to contain elevated levels of Ca++ in comparison to B-2 cells (21).

Methods

Animals

Male BALB/cByJ mice at 8–14 weeks of age were obtained from Jackson Laboratory (Bar Harbor, ME, USA). Mice were cared for and handled in accordance with National Institutes of Health and institutional guidelines.

B cell purification and cell culture

Unseparated cells were obtained by peritoneal washout and splenic disruption, were stained with immunofluorescent antibodies directed against B220 and CD5 and were subjected to FACS at 4°C using a MoFlo instrument (Dako-Cytomation, Fort Collins, CO, USA) to yield purified B-1P (B220+CD5+) and B-2S (B220+CD5−) (sort-purified B-1 and B-2 cells), as previously described, including the use of an anti-CD8 ‘dump’ channel for B cell purification (14). Sort-purified B-1 and B-2 cell populations were re-analyzed by immunofluorescent staining with antibodies directed against CD3, Mac-1, CD14 and sIgM, and were >97% pure. Splenic B-1 cells (B-1S) (B220+CD5+), germinal center (GC) B cells (B220+CD95+) and marginal zone (MZ) B cells (CD21hiCD23lo) were also sort purified. Purified B cells were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 10 mM HEPES at pH 7.25, 2 mM L-glutamine, 50 mg/ml streptomycin, 100 U/ml penicillin and 100 µg/ml streptomycin.

LC-MS/MS protein inventory following 1D PAGE

Protein samples (10 µg) were electrophoresed in one dimension on 4–20% gradient polyacrylamide gels (Invitrogen). Gels stained with Coomassie brilliant blue were divided into 42–44 slices of 1 mm width; gel slices were individually digested with trypsin (Promega, Madison, WI, USA) and analyzed by capillary liquid chromatography, nanospray ionization and tandem mass spectroscopy using an LTQ 2D linear ion trap mass spectrometer (Thermo Finnigan) at the Joslin Diabetes Center, Proteomics Core (Boston, MA, USA). Data acquisition was carried out using a sequence of full-scan MS (range 400–1200 m/z) followed by 10 data-dependent MS2 events. Assignment of MS2 data was performed using the non-redundant protein database (nr) from the National Center for Biotechnology Information and TurboSEQUEST (BioWorks 3.1, Thermo Finnigan). Peptide identifications were based on the following criteria: Cross-correlation Score (XCorr) >1.5, >2.0 and >2.5 for charge states +1, +2 and +3, respectively; Delta Correlation (dCN) >0.1; Primary Score (Sp) >200; Ranking of the Primary Score (Rsp) <3 and percent ions >30%. Protein identifications were made when two or more unique peptides contributing to a protein match were obtained from a single gel slice or adjacent slices.

Protein expression

Cell pellets were extracted and immunoblotted as previously described (22). Membranes were developed using the ECL Western Blotting Analysis System from Amersham Biosciences (Piscataway, NJ, USA). As a protein-loading control, blots were stripped and reprobed with anti-β-actin antibody (Sigma–Aldrich). Polyclonal anti-annexin II and anti-S100A6 antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Immunoprecipitation

Whole-cell lysates were pre-cleared with control IgG and protein A/G agarose for 30 min. Pre-cleared lysates were then incubated with anti-annexin II or anti-S100A6 antibodies as indicated for 2 h at 4°C, followed by further incubation with protein A/G agarose and a lysis buffer wash. Immunoprecipitates were collected by centrifugation, washed three times with PBS and subjected to SDS-PAGE and western blotted as described above.

Gene expression

RNA was prepared from B cells using Ultraspec reagent (Biotecx, Houston, TX, USA) and was DNase treated as previously described (23). cDNA was prepared using AMV reverse transcriptase (Roche Applied Sciences, Indianapolis, IN, USA), and was normalized by PCR for β2-microglobulin expression. Gene expression was then assessed by real-time PCR using a MX3000P qPCR machine (Stratagene, La Jolla, CA, USA) and the following primers (forward/reverse): β2-microglobulin (CCCCGCTCACATTGAAATCCGCATATGTATCAGTCCTAGTGG); annexin II (CCAAGTTGA-CAGTCTCAGTGG); annexin II (ACCGTTGAGTACCTCCCG/ACCCACACTGGATTGACC).

Reagents

Fluorescence-labeled anti-B220, anti-CD5, anti-Mac-1, anti-CD23, anti-CD3, anti-CD14, anti-lgM, anti-CD95, anti-CD21, and anti-CD8 antibodies for fluorescent staining were obtained from BD PharMingen (San Diego, CA, USA). F(ab’)2 fragments of anti-lgM were obtained from Jackson Immunoresearch (West Grove, PA, USA) and used at 15 µg ml−1. LPS from Salmonella typhimurium was obtained from Sigma–Aldrich and used at 25 µg ml−1.
Results

Identification of B-1P annexin II and S100A6 protein expression

B-1P- and B-2S-solubilized proteins were subjected to electrophoresis in one dimension on a gradient polyacrylamide gel that was sliced and subjected to LC-MS/MS analysis. This resulted in identification of 812 proteins in the B-1P sample and 441 proteins in the B-2S sample. We noted within these groups the differential expression of annexin II and S100A6, members of two well-known families of Ca++-binding/-sensitive proteins. Inasmuch as B-1P have been reported to contain elevated levels of Ca++ (21) in comparison with B-2S, we focused on these members as suitable and representative examples (within hundreds of proteins) to determine the utility of this proteomic approach to the pursuit of new B cell subset-specific markers. Assignment of peptide masses following trypsin cleavage to these proteins is shown in Table 1.

To confirm the validity of the differential expression identified by 1D GE/LC-MS/MS analysis, we carried out western blotting on whole-cell lysates obtained from B-1P and B-2S. In three separate experiments, B-1 cells expressed much more annexin II and S100A6 than B-2 cells, confirming quantitative extrapolation from the qualitative data provided by mass spectrometry (Fig. 1).

The very small number of macrophages present in sorted B-1 cells (though typically not >1%) but not sorted B-2 cells raised the theoretical possibility that preferential expression of annexin II and S100A6 in B-1 cell samples might be due to extremely high expression by this contaminating population. To address this possibility, lysates from peritoneal macrophages were examined by western blotting side by side with B-1P and B-2S lysates. Although macrophage expression of both proteins was detected, the levels were less than those found in B-1P lysates, and therefore could not account for the latter (Fig. 1C).

Table 1. Annexin II and S100A6 proteins were identified in B-1 cells by 1D GE/LC-MS/MS

<table>
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<th>Peptide mass</th>
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<td>1844.9</td>
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(A) Peptide masses obtained by 1D GE/LC-MS/MS were identified as annexin II through the National Center for Biotechnology Information (NCBI) database. (B) Peptide masses obtained by 1D GE/LC-MS/MS were identified as S100A6 through the NCBI database.

B-1P annexin II and S100A6 RNA expression

In order to determine whether differential annexin and S100 protein expression was reflected in relative levels of gene expression, we carried out real-time PCR subsequent to reverse transcription (RT) on RNA samples obtained from sort-purified B-1P and B-2S. B-1P and B-2S differed dramatically in the levels of annexin II and S100A6 RNA expressed in keeping with results obtained by western blotting and mass spectrometry (Fig. 2A).

The extremely high relative levels of annexin II and S100A6 expression on the part of B-1P raised the question of whether lymphoid populations might be similarly affected. Because some populations can only be recovered in small numbers, we pursued this with additional RT/real-time PCR assays on RNA obtained from sorted lymphoid populations and macrophages. With regard to annexin II, only macrophages approximated the expression level of B-1P and no population exceeded it, although B-1 cells obtained from splenic tissue (B-1S) and GC B cells expressed moderate amounts of annexin II, whereas MZ B cells and T cells expressed very little, as did B-2S (Fig. 2B and C). With regard to S100A6, B-1S and macrophages expressed very small amounts which were still an order of magnitude less than...
the amount expressed by B-1P, and other populations expressed little or none. Thus, the differentially high expression of annexin II and S100A6 on the part of B-1P is unique to this population (and again cannot be explained by macrophage contamination because, as with protein, annexin II and S100A6 RNA levels in purified macrophages did not exceed the levels present in purified B-1P).

**Annexin II and S100A6 complexes**

Annexin and S100 proteins typically form complexes. In order to determine whether complexes form between annexin II and S100A6 in B-1 cells, where expression of these proteins is elevated, immunoprecipitation and western blotting were carried out with B-1P and B-2S lysates. Immunoprecipitation of annexin II yielded S100A6 detected by western blotting; conversely, immunoprecipitation of S100A6 yielded annexin II detected by western blotting, only in B-1P and not in B-2P (Fig. 3A). Thus, annexin II and S100A6 exist as complexes in B-1 cells, as has been reported for other cell types (24, 25).

Annexin–S100 complexes are disrupted in the absence of Ca++ (25); in order to determine whether B-1P annexin II and S100A6 complexes behave similarly, BAPTA was added for 1 h before preparation of lysates and subsequent analysis by immunoprecipitation and western blotting. Similar amounts of annexin II were immunoprecipitated in all samples (data not shown). However, in contrast to the situation in naive B-1P, after treatment with BAPTA, immunoprecipitation of annexin II failed to yield S100A6 on western blotting (Fig. 3B). Thus, annexin II and S100A6 complexes in B-1 cells are Ca++ sensitive.

Many features of the naive B-1 cell phenotype have been found to be inducible in B-2 cells. To determine whether this is true of the uniquely elevated B-1 cell expression of...
Annexin II and S100A6, we stimulated naive B-2S with LPS and with anti-Ig for a period of 48 h, after which lysates were western blotted. Both annexin II and S100A6 proteins were strongly up-regulated in stimulated B-2S, approximating the levels present in naive B-1P (Fig. 3C). We then questioned whether these molecules exist in complexes that depend on Ca++ by immunoprecipitating annexin II and western blotting for S100A6, with and without prior treatment of stimulated B-2S with BAPTA. Similar amounts of annexin II were immunoprecipitated in all samples (data not shown). Immunoprecipitation of annexin II from LPS-stimulated B-2S yielded S100A6 on western blotting, but only in the absence of BAPTA (Fig. 3D). Thus, high-level expression of annexin II and S100A6 Ca++-sensitive complexes in B-1P is unique among naive lymphoid populations but is reproduced in B-2S after mitogenic stimulation.

Fig. 3. Annexin II and S100A6 form complexes and are induced in mitogenically stimulated B-2S. (A) Sorted populations of B-1P and B-2S were isolated and whole-cell extracts were immunoprecipitated with anti-annexin II and then western blotted with anti-S100A6; extracts were also immunoprecipitated with anti-S100A6 and western blotted with anti-annexin II. One of three experiments with similar results is shown. (B) Sorted B-1P were exposed to BAPTA for 1 h before preparation of whole-cell extracts, as indicated; extracts were immunoprecipitated with anti-annexin II and then western blotted with anti-S100A6. One of three experiments with similar results is shown. (C) Sorted B-1P and B-2S were examined for protein expression of annexin II and S100A6 by western blotting as described in the legend to Fig. 2. B-2S were examined initially (naive), after culture in medium for 48 h (media), and after stimulation with either LPS or F(ab′)2 fragments of goat anti-mouse IgM (αIgM) for 48 h. One of four experiments with similar results is shown. (D) Sorted B-2S were stimulated with LPS for 48 h and then exposed to BAPTA for 1 h before preparation of whole-cell extracts, as indicated; extracts were immunoprecipitated with anti-annexin II and western blotted with anti-S100A6. Similar amounts of annexin II were immunoprecipitated in unstimulated and BAPTA-treated samples. One of three experiments with similar results is shown.

Discussion

The present study relied on proteomic analysis of B cell subsets, particularly B-1 cells, by 1D GE/LC-MS/MS, to identify differences in protein composition that might not be detected through other means. In this way, we found marked relative over-expression of annexin II and S100A6 by B-1 cells in comparison to B-2 cells, and we confirmed B-1 cell over-expression of annexin II and S100A6 proteins by western blotting. Although proteome analysis has confirmed its ability to find molecular differences between closely related cell populations that could not be detected by RNA-based methods (19), we also found over-expression of annexin II and S100A6 RNAs by B-1 cells in comparison to B-2 cells, suggesting that this feature of B-1 cells, which appears to be unique among lymphoid populations, would be identifiable through RNA-based detection methods. However, these results confirm that the emerging field of analysis by 1D GE/LC-MS/MS proteomics is at least as valid and reliable as RNA-based techniques in identifying differences between B-1P and B-2S, and as an exclusive feature among current screening tools, the former identifies differences in final protein product expression which the latter fails to provide.

In a survey of lymphoid populations at the RNA level, S100A6 was uniquely expressed by B-1P and not by B-1S, GC cells, MZ B cells or T cells. Although B-1 cells share some characteristics with MZ B cells, S100A6 expression differentiates these populations. At the same time, the lack of S100A6 expression by B-1S suggests that over-expression of S100A6 is not an intrinsic B-1 cell characteristic, but may be a feature of just those B-1 cells that migrate to the peritoneal cavity, or may be induced by the peritoneal environment in those B-1 cells that exist there. Multiple phenotypic, biochemical and functional criteria distinguish B-1P and B-1S, including surface Mac-1 expression, constitutively activated STAT3, IL-10 gene expression, enhanced in vitro viability, spontaneous IgM secretion, mitogenic stimulation by phorbol myristate acetate and regulation by CXCL13, all of which are present in B-1P but not present, or present to a much lesser degree, in B-1S (4, 14, 26, 27). As a result of the work described herein, S100A6 expression joins this list. These same considerations apply to annexin II, although to a somewhat lesser extent, as B-1S and GC cells expressed up to one-third of the RNA level present in B-1P. These new results again suggest the distinctiveness of the peritoneal environment as a target for B-1 cell subset migration or as an inducer of B-1 cell gene expression.

Many characteristics of B-1 cells are inducible in B-2 cells, and expression levels of annexin II and S100A6 were up-regulated in B-2 cells by stimulation with LPS and with anti-Ig. Further, in both B-1 cells and stimulated B-2 cells, annexin II and S100A6 existed as Ca++-dependent complexes. This raises the question of the role of annexin II–S100A6 complexes in B cells. Annexin II is an inducible, calcium-dependent phospholipid-binding protein (reviewed in 28, 29) whose expression is increased in a variety of human malignancies (30–33). It binds plasminogen and plasminogen activator and is responsible for excessive plasmin generation by acute pro-myelocytic leukemia cells (34, 35). In addition to other activities, annexin II is secreted by osteoclasts...
and acts in an autocrine fashion as an osteoclast stimulatory factor (36, 37). S100A6, also known as calcyclin, is an inducible, small, calcium-binding protein (reviewed in 38) whose expression, like that of annexin II, is increased in many human tumors (39–42). Further, it appears to function in regulating cytoskeletal organization and may be involved in the regulation of beta-catenin (43, 44). Inasmuch as B-1 cells are self-replenishing, the growth-related activities of these molecules may well be causally related to their differential expression in B-1 cells. This potential role is emphasized by induction of annexin II and S100A6 expression in B-2 cells by two different mitogens with the formation of Ca++-sensitive complexes similar to those observed in naive B-1 cells. However, the actual effects of annexin II and S100A6 in naive B-1 cells or stimulated B-2 cells remain unknown (although, at a minimum, they appear to represent biomarkers for proliferation in these two situations as they do in malignant cells). The present results point to a previously unrecognized role for these proteins in B-1 and B-2 cell physiology, and emphasize the importance of proteomic analysis in identifying previously unrecognized molecular distinctions worthy of further investigation.

Acknowledgements
This work was supported by United States Public Health Service grants AI29690 and AI60896 awarded by the National Institutes of Health. R.F. is the recipient of a grant awarded by Oficina de Ciencia y Tecnología, Generalitat Valenciana and The Spanish Liver Association, Spain.

Abbreviations

B-1P peritoneal B-1 cells
B-1S splenic B-1 cells
B-2S splenic B-2 cells
1D 1-dimensional
GC germinal center B cells
MZ marginal zone B cells
RT reverse transcription
slg surface Ig

References


