International Journal of Advanced Research and Development
ISSN: 2455-4030
Impact Factor: RJIF 5.24
www.advancedjournal.com
Volume 3; Issue 2; March 2018; Page No. 437-440

Comparative protein profiling in normal resting and activated peripheral blood mononuclear cell (PBMCs)

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Abstract

Protein-specific information of immune cells play an important role in defining new targets and biomarkers for many diseases and an uncontrolled growth of a cell. An initial event that lead to uncontrolled growth during cancerous condition is stimulation of cells by foreign molecules or mitogens and hence it would be a great importance if new proteins markers are identified that are differentially expressed in activated lymphocytes. In this study, we have compared both qualitative and quantitative expression of proteins from normal PBMCs before and after their activation by PHA and Con A lectins. Electrophoresis of proteins from normal resting and lectin-activated PBMCs revealed that two proteins with 95 and 185 kDa are exclusively expressed on PHA-activated PBMCs. Similarly, proteins with M*r* 68, 70, and 210 kDa are only part of Con A stimulated PBMCs. On the contrary, protein bands with approx. M*r* 35, 125, 170, 200, 250 and 280 kDa were exclusively expressed in resting lymphocytes while disappeared after activation process. Further, quantification of proteins by Image J software reveled that level of protein expression has also changed drastically in activated PBMCs when compared to resting PBMCs. These results provide an insight into the fact that proteins that are expressed or altered during activation process could be the direct target for development of new drugs for blood cancer.

Keywords: PBMCs, mitogens, PHA, Con A

Introduction

Proteins are abundant in all organisms and play key roles in most biological events as catalysts, transporters and messengers. It is crucial to note that all research related to proteins increase our understanding of their levels, interactions, functions, modifications, regulations and localization in cells. Study of proteins ensures the large-scale analysis of a complete set of most significant metabolic pathways in cells or tissues (Graves *et al.*, 2002) [1]. Protein profiling an emerging independent subspecialty will definitely provide detailed insight into biological events. Qualitative and quantitative determination of protein levels can be achieved with protein profiling which shows us unique expression patterns (diseased vs. healthy, treated vs. untreated, experimental vs. control) when one cell type is compared with those of another cell type (Holland A, Ohlendieck K 2015; Aasebø E *et al*, 2016) [2, 3]. The value of protein profiling is increasing daily and there are several reasons why it is of great importance, especially as a potential tool for the early diagnosis of cancer and other diseases. The goal underlying study of proteins not only to identify all proteins in a cell, but also to identify the correlation between their expression in different cells such as normal, diseased and differentiating cells.

Protein-specific information can play an important role in defining new targets and biomarkers for a disease but is often lost when proteins are released into plasma. Several methodologies and techniques are reported to identify such proteins, but are limited to high expression (Robinson MW, Connolly B, 2005; Chevallet *et al*. 2007) [4, 5]. Hence it is important to isolate and identify proteins before their release into the plasma followed by degradation. The most affected proteins fall into this category are proteins produced by immune cells especially lymphocytes. Thus, gathering information on qualitative as well as quantitative expression of proteins in resting and activated lymphocytes will certainly help to broaden the scope for a biomarker in immune cells and their cancerous versions.

Current study provides valuable information on differential expression of proteins during mitogenic activation of lymphocytes. Also, the study provides added information that different mitogens can stimulate lymphocytes through different pathways involving expression of specific proteins which could be exploited in many ways. Therefore, monitoring changes in protein expression in blood cells is of great importance for understanding carcinogenesis, identifying diagnostic markers, and to develop new therapeutics for leukemia.

Materials and Methods

Cell culture reagents such as RPMI medium, FBS, antibiotics, Ficoll and mitogens (PHA and Con A) were obtained from Sigma Chemicals. Chemicals required for electrophoresis, silver staining were procured from Sisco Research Laboratories (SRL), India. For SDS-PAGE, a Fermentas PAGE-Ruler standard protein molecular weight markers was used and compared.

Isolation of PBMCs

Since, peripheral blood mononuclear cell (PBMCs) almost

(80-90%) comprises of lymphocytes, we performed all experiments using PBMCs isolated from normal healthy donors. Normal human venous blood was drawn in sterile syringe and the PBMCs were separated by density gradient separation using ficoll hypaque solution according to the manufacturer's instruction. In brief, heparinized blood (diluted with saline 2:1) was overlaid on ficoll hypaque solution in a ratio of 3:1 and was centrifuged at 2,000 rpm, for 30 min at room temperature, to separate three distinct layers. PBMCs form an opaque middle layer whereas RBCs settle down at the bottom. The middle layer was carefully removed and washed twice with RPMI 1640 medium. The supernatant was discarded and the cells were resuspended in complete RPMI medium. Isolated PBMCs were diluted appropriately and counted using hemocytometer.

Mitogenic stimulation of PBMCs

In order to activate PBMCs, isolated PBMCs were distributed into three T-25 flasks containing equal number of cells and maintained in RPMI medium containing 10% FBS and 1% penicillin/streptomycin for 24 h at 5% CO_2. After 24 h, cells were treated with 5 ug/ml of PHA and Con A and third flask was added with PBS to serve as untreated resting control cells (Nagre *et al*., 2010) [6].

Preparation of cell lysate and protein estimation

PBMCs were lysed with RIPA buffer for 30 mins on ice with occasional pipetting at every 5 mins and then centrifuged at 15000 RPM for 15 mins at 4 °C. After centrifugation, the supernatant was collected as protein source and residue was discarded. To determine the protein concentration, Folin and Wu based detergent compatible protein assay reagent kit (Bio-Rad $D_{C\ Cat}$ # 500-0114) was used.

Sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples from different source such as normal resting lymphocytes (untreated), PHA-activated lymphocytes and Concanavalin A-activated lymphocytes were quantified. An equal amount of protein was treated with 4x SDS buffer and boiled for 5 mins at 100 °C. Cooled and 200 ug of each sample was loaded in to the wells and electrophoresed at 100 V for 2.5 hours in 12% gel. After completion of electrophoresis, gels were subjected to silver staining as described below. A standard molecular weight protein ladder ranging from 20-250 kDa was also processed and electrophoresed as described above. To visualize, highly sensitive silver staining was performed as described by Zhao L *et al* (2012) [7].

Comparative analysis of protein bands

In order to determine the qualitative and quantitative level of protein expression in resting and activated lymphocytes, silver stained gel was scanned with very high resolution (1200 dpi) and imported to Image J software. Then the image was converted to grey scale and amount of expression was obtained by calculating the intensity of bands in the form of black pixels against white pixels. A detailed procedure for analysis of gel bands can be found at http://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j/ link (Bearer EL, 2003) [8]

Results

Isolation of lymphocytes and quantification of protein

PBMCs from were isolated by sucrose gradient centrifugation using ficoll hypaque solution and maintained in RPMI medium containing 10% FBS and 1% penicillin and streptomycin. Total 8.5 x 10^7 number of PBMCs were isolated from 50 ml of blood. These cells were activated by treating with standard mitogens PHA and Con a lectins for 72 h. Protein concentration obtained from different cells were presented in table 1.

Table 1: Comparative chart showing protein obtained from different cells

PBMCs	RestingPBMCs	PHA-activated	Con A-activated
Protein conc.(μg/μl)	4.08	4.71	6.5
Total volume(μl)	132	53	50
Total protein(μg)	539	250	325

Differential expression of proteins in resting and activated lymphocytes

To determine differential expression of proteins in resting and after their activation, SDS-PAGE was performed using cell lysates obtained from resting, PHA-activated and Con a activated cells. After 72 hour of mitogen treatment, cells were pelleted and lysed with RIPA buffer. The total protein (200 ug) was subjected to SDS-PAGE for 2 h and then stained with highly sensitive silver staining. After comparing and analysis of different proteins bands (Fig 1), it was found that various new proteins bands were appeared after activation by PHA and Con A. As expected, it was also observed that many protein bands common for all the three types of PBMCs (resting, PHA/Con an activated).

Among proteins which are only confined to PHA-activated PBMCs are likely with mol. wt of 95 kDa (#12) and a band near to 185 kDa (#9). Similarly, Con A-activated PBMCs have shown exclusive expression of three proteins at 210 kDa (#15), 70 kDa (#16) and 68 kDa (#17). It was also observed that many proteins such as mol. wt. close to 155 kDa (#10), 115 kDa (#11), 85 (#18), 60 kDa (#13), 55 kDa (#14), 68 kDa (#17) and 70 kDa (#16) were commonly expressed in activated lymphocytes irrespective of mitogens.

Level of protein concentration changes after activation by mitogens.

Table 2 summarizes differential expression of proteins in different cells along with their fold increase/decrease of expression levels. As detailed in table 2, protein bands with approx. mol wt 35 kDa (#2), 125 kDa (#4), 170 kDa (#5), 200 kDa (#6), 250 kDa (#7) and 280 kDa (#8) were exclusively expressed in resting lymphocytes while

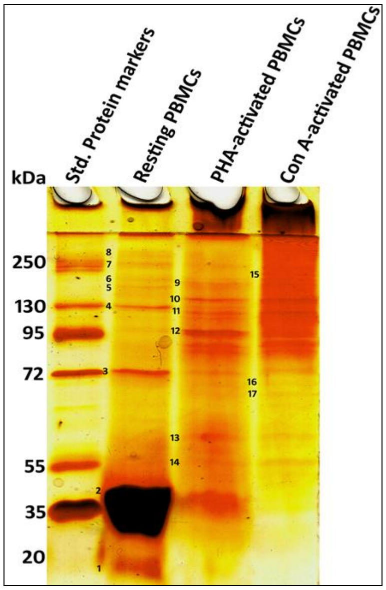

Fig 1: SDS-PAGE analysis of proteins from resting, PHA-activated and Con A-activated lymphocytes. Small numerical indicate specific bands exclusively expressed in respective lymphocytes.

Table 2: Intensity of protein bands and their fold change in resting, PHA-activated and Con A-activated lymphocytes

Band # (kDa)	Resting		PHA-activated		Con A activated	
	Intensity	Fold^a	Intensity	Fold^a	Intensity	Fold^a
1 (18)	87	1	0	-	0	-
2 (35)	1661	1	195	8.520 ↓	0	-
3 (72)	65	1	32	0.492 ↓	38	0.584 ↓
4 (125)	53	1	05	10.60 ↓	ND	-
5 (170)	15	1	ND	-	ND	-
6 (200)	13	1	ND	-	ND	-
7 (250)	24	1	ND	-	ND	-
8 (280)	18	1	ND	-	ND	-
9 (185)	ND	-	37	-	ND	-
10 (155)	ND	-	51	-	48	-
11 (115)	ND	-	22	-	31	-
12 (95)	05	1	59	11.8 ↑	12	2.4 ↑
13 (60)	ND	-	23	-	19	-
14 (55)	ND	-	14	-	26	-
15 (210)	ND	-	ND	-	08	-
16 (70)	ND	-	08	-	11	-
17 (68)	ND	-	05	-	11	-
18 (85)	ND	-	45	-	33	-

A Band intensity was arbitrarily fixed as 1 and used to express fold change in activated lymphocytes.
ND - Not detected.

Disappeared upon activation process. Interestingly, many proteins are commonly expressed in all the three lymphocytes, however their level of expression was different. For instance, a protein band with mol. wt. of 72 k Da (#3) was expressed in all the cell types but its concentration varied drastically as presented in table 2. Although it is an interesting to note that, a protein band at 35 k Da (#2) stains heavily in resting lymphocytes, it cannot be rule out the that, this could be a degradation product of other proteins.

Discussion

It is well documented in the literature that many proteins were expressed during activation of lymphocytes as a consequence of immune response (Paul 1999) [9]. However, detailed information on protein expression upon activation a prior step in leukemia is devoid of such information. Although there are many reports on activation of numerous genes during these processes but their translation into proteins from all these genes many not be the case. Hence it is essential to have comprehensive information on protein profile in activated lymphocytes which could be used as biomarkers in the early stage of blood cancers. Further, it is possible to target and develop protein as biomarkers which are commonly and exclusively expressed during the activation and leukemic condition. For instances, CD69 is the first cell surface glycoprotein detected after activation (Ziegler *et al*., 1994) [10]. Resting (unstimulated) peripheral blood lymphocytes do not usually express CD69. As such CD69 has been classified as a marker of early lymphocyte activation (Cibrián D, Sánchez-Madrid F, 2017) [11]. Once expressed on activated lymphocytes, CD69 is believed to act as a co-stimulatory molecule leading to subsequent cytokine production, proliferation and cytotoxicity (Sánchez-Madrid F, 2005) [12].

In the current study, we have made an effort to screen lymphocyte preparations from healthy donors which were activated by two different mitogens. Interestingly, we observed both qualitative and quantitative difference in protein expression pattern in mitogen activated lymphocytes compared to resting non-stimulated lymphocytes. Many prominent proteins (#10, #12, #13 and #14) which were exclusively detected in activated lymphocytes could be studied further to evaluate whether they are also part of leukemic cells and target for developing inhibitors. It was also observed that both mitogens are not similar in stimulating same kind proteins leading to activation of PBMCs suggesting pathway

Conclusion

Current study provides additional information on differential expression of proteins during mitogenic activation of lymphocytes. Also, it provides added information that different mitogens activate lymphocytes through different pathways which could be exploited for the inhibition of those pathways.

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