Preliminary Study on the Role of Protein Kinase C in Cd47-Mediated Phosphatidylserine Exposure Pathway By Bric 126 In Jurkat T Cells

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ABSTRACT
Treatment failure in T-cell acute lymphoblastic leukaemia (T-ALL) occurs when leukemic blasts acquire resistance to chemotherapeutic agents. Current research efforts are focused on the search for targets for the development of more effective and less toxic anti-leukemic drugs. CD47 has been suggested to be involved in chemo-resistance and cell metastasis. Although several potential mechanisms were suggested to explain the therapeutic effect of CD47-targeting: the downstream effectors which lead to different effects by CD47 are still not well understood. In this preliminary study we assessed the role of Protein kinase C (PKC) in CD47-mediated phosphatidylserine exposure pathway in jurkat T cells. Jurkat T cells were incubated with anti-CD47 mAb (BRIC 126), anti-CD44 mAb (BRIC 235) or control in the presence or absence of Bisindolylmaleimide I, hydrochloride (PKC inhibitor). Cells were stained with annexin-V FITC. Flow cytometry analysis was used for measurement of fluorescence intensity. Cell viability was detected using trypan blue exclusion test. PKC inhibition enhanced phosphatidylserine expression in CD47 receptor mediated leukaemia cells apoptotic pathway. This indicates that PKC may be involved in CD47-mediated PS exposure pathway in jurkat T cells. The observations from this study identify CD47 and PKC as novel functional
proteins in jurkat T cells with promising therapeutic potential. This study would provide insight for targeted therapy against T-ALL disease.

**Keywords:** Jurkat T-cell, Acute lymphoblastic leukaemia, CD47, PKC, Phosphatidylserine, Apoptosis, Flow cytometry.

**INTRODUCTION**

CD47 is a 47-52kDa transmembrane glycoprotein of the immunoglobulin (Ig) super-family, with an extracellular amino-terminal IgV domain, a five times transmembrane-spanning domain and different carboxyl-terminal cytoplasmic domains generated by alternative splicing [1-4]. CD47 is heavily glycosylated [5] with a ubiquitous expression virtually by all cells in the body including lymphocytes [6]. The presence of CD47 has been shown to be crucial for immune evasion in leukemic stem cells [7-10]. CD47 exerts its anti-phagocytic role through binding to phagocytic cells that express signal regulatory protein alpha, SIRPa [7]. Upon binding, CD47 initiates a signal transduction cascade resulting in inhibition of phagocytosis [11]. CD47 activation induces apoptosis of B-cell chronic lymphocytic leukaemia cells through a caspase-independent mechanism [12]. Recently, CD47 was reported to be a marker of tumour-initiating cells in leukaemia and bladder cancer [9]. The ligation of CD47 with monoclonal antibodies, thrombospondin-1 (TSP-1) or the specific CD47-binding peptide 4N1K, derived from the C-terminal binding domain of TSP-1, induces phosphatidylserine exposure as part of apoptosis in blood cells [12,13].

Phosphatidylserine (PS), a phospholipid with a negatively charged head group, is restricted to the inner leaflet and maintained by the combined actions of ATP-dependent translocase enzymes (floppases and flippases) and scramblases between the two membrane leaflets [14,15]. CD47 and protein kinase C (PKC) have been reported to interact with these enzymes and with other chemical molecules leading to the exposure of PS on the outer leaflet thereby regulating different cellular functions [16,17]. The expression of PS on outer cell membrane is instrumental in triggering blood clotting and also serves as an "eat me" signal for the clearance of apoptotic cells [18].

PKC is a family of kinases comprising at least 11 subspecies which have been classified into three subgroups: the conventional PKCs (cPKCs) γ, βI, βII and ε; the novel PKCs (nPKCs) δ, η and θ; and the atypical PKCs (aPKCs) ζ and λ [19]. PKC subspecies differ in their Ca\(^{2+}\) dependence, sensitivity to diglyceride or phorboids, substrate specificity, substrate localization and tissue distribution [20]. Elevated cytoplasmic Ca\(^{2+}\) level has been reported to inhibit translocase and activate scramblase activities in erythrocytes and platelets, leading to the suggestion that cytoplasmic Ca\(^{2+}\) level may be playing a second messenger for PS exposure on apoptotic lymphocytes [21]. PKC isoforms are differentially localised by their association with anchoring proteins called RACK (receptors for activated C kinase) or PICK (Proteins that interact with C kinase) [22]. Report shows that direct binding of CD47 by TSP-1 readily inhibits soluble guanylylcyclase in jurkat cells, and the inhibition requires an increase in cytoplasmic Ca\(^{2+}\) levels [23].

Jurkat cells are an immortalized line of human T lymphocyte cells used to study acute T cell leukaemia, T cell signalling, and the expression of various chemokine receptors [24]. Their primary use, however, is to study or determine the mechanism of differential susceptibility of cancer cells to drugs [25] and radiation [26]. Although there are evidences of PKC
activities in jurkat cells, its function in CD47-PS exposure pathway has not been investigated in these cells. The aim of this study was to assess the role of PKC in CD47-mediated PS exposure pathway in jurkat T cells. We hypothesized that PKC may be involved in CD47-mediated PS externalization in jurkat. Our finding may help in the development of clinical strategies using PKC and CD47 as targets to further develop novel therapy for T cell leukaemia in humans.

MATERIALS AND METHODS

Cell line and antibodies

Jurkat cells were purchased from ATCC, Middlesex, UK; BRIC 235 (mouse IgG2b anti-CD44 mAb) and BRIC 126 (mouse IgG2b anti-CD47 mAb) from International Blood Group Reference Laboratory, National Blood Service, Bristol, UK.

Chemical reagents

PKC inhibitor (Bisindolylmaleimide I, hydrochloride) from Calbiochem, UK; RPMI-1640 medium (Life Technologies, UK); Hank’s balance salt solution, HBSS (Invitrogen, Paisley, UK); foetal bovine serum, FBS (Hyclone, UK) and Trypan blue stain (Sigma-Aldrich, UK); propidium iodide, annexin V-FITC, and x10 binding buffer from BD Pharmingen, UK.

Cell culture condition

Jurkat cells were routinely maintained in RPMI-1640 medium supplemented with 10% (v/v) FBS and 2mM L-glutamine as described in American type culture collection, ATCC manual. The cells were cultured in 25 cm² or 75 cm² flasks at 37°C in a humidified 95% air and 5% CO₂ incubator. The cells were manipulated aseptically in a Class 2 BSC Laminar flow hood (Thermo Electron Corporation) and were sub-cultured every 2-3 days in order to maintain a concentration between 1×10⁵ - 1×10⁶ cells/mL, depending on the experimental demands.

Cell preparation and apoptosis induction

Cells were washed three times with 1mL HBSS pH 7.4 and were spun between each wash for 3 minutes at 200g to remove any RPMI or metabolites so as to avoid any interfering protein. The counting of cells was completed with a haemocytometer (Marienfield, Germany) and standard inverted microscope (Olympus CKX31) and cells diluted in HBSS buffer at a concentration of approximately 5 ×10⁶ cells/mL. Using a flat bottom 96 well plate, jurkat cells (5 × 10⁶ cells/mL) in 200 µL of HBSS buffer were incubated with 5 µL of 1.3 mg/mL BRIC 126, 5 µL of 1.2 mg/mL BRIC 235 or control overnight in separate wells at room temperature with gentle agitation at 36 rpm on a Stuart gyro-rocker to enable even distribution and binding of antibody reagent to the cells. In separate wells, Jurkats (5 × 10⁶ cells/mL) in 200 µL were first incubated at 37°C with 1.5µM Bis I (PKC-inhibitor) for 30 minutes to allow the inhibitor to penetrate the cells. Cells were then incubated with 5 µL of 1.3mg/mL BRIC 126, 5 µL of 1.2mg/mL BRIC 235, or control overnight at room temperature with gentle agitation at 36 rpm on the Stuart gyro-rocker. Control cells were incubated with HBSS buffer only.
Flow cytometry analysis of phosphatidylserine exposure

After overnight incubation, the cells from each well were transferred to 1.5 mL Eppendorf tubes. Cells were washed three times with 1mL of HBSS, centrifuged between each wash at 200 g for 3 minutes at ambient temperature, to wash off unbound antibodies. Cells were washed with 1mL of 1x binding buffer and spun at 200 g for 3 minutes to wash off the HBSS and also provide the optimum binding condition for the next step. Cell pellets were re-suspended in 100 μL of 1 x binding buffer and stained with 5 μL annexin V-FITC for apoptosis, except for the cells in the unstained well. Cells were incubated at room temperature in the dark for 15 minutes, and after which 300 μL 1x binding buffer was added. Then 1 μL propidium iodide was added to each tube to stain only necrotic cells. Cells were immediately placed into ice bucket and kept in the dark to avoid destruction of the light sensitive Annexin V-FITC; and assayed within 1hour for PS exposure using a Flow cytometer (BD Accuri C6). Data were obtained using CellQuest software v7.5.3 (Becton Dickinson, Oxford, UK). A minimum of 10,000 cells was analysed in each flow cytometry experiment. Each experiment was repeated three times using different cell passages as to allow for means from the triplicate measurements.

Cell viability using trypan blue exclusion test

To 10 μL of treatment cell suspension (1 × 10⁵ to 2 × 10⁷ cells/mL) in Eppendorf tube was added 10 μL of 0.4% (v/v) Trypan blue stain. Then gently and thoroughly mixed, using a 10 μL Gilson pipette, to avoid lysing the cells and to permit homogenous staining or distribution. This was allowed to stand for 15 seconds at room temperature before the haemocytometer was charged for cell counting. Under the inverted microscope, non-viable (dead/stained) and viable cells (live/unstained) were counted. At least 70 cells were counted to ensure accurate count. Cell concentration/ml was calculated as: Average number of cells in one large square × dilution factor × 10⁴. Percentage viability was calculated as: (No. of viable cells counted / Total cells counted) × 100.

Statistical analysis

Statistical analysis of data were completed using GraphPad prism 6 software with statistical significance (p<0.05), determined by using the independent two-sample students t-test or ANOVA.

RESULTS

Jurkat cells incubated in HBSS medium alone (control) for 24 hours expressed a low level of PS (mean % annexin V positive cells 3.133 ± 0.186, n =3, Figure 1A). This indicated that the medium did not induce any significant increase in PS expression during the culture period. The addition of BRIC 126 was shown to induce an increase in the level of PS expression on Jurkats cells (Figure 1A) In contrast, BRIC 235 (mean % annexin V positive cells of BRIC 235 (3.000 ± 0.116, n=3) was not shown to induce any significant increase in PS expression, compared with the control (p=0.5748). BRIC 126 (mean % annexin V positive cells, 9.267 ± 1.462, n=3) induced a significant increase in PS expression in comparison with BRIC 235 (p=0.0129). BRIC 126 also induced a significant increase in PS expression when compared with control (p= 0.0141) (Figure 1A).
Percentage viable cell count showed that cells treated with BRIC 126 showed no significant decrease in mean % viable count when compared with BRIC 235 (76.67 ± 8.819 vs. 88.33 ± 1.667, n=3, p=0.3486) or Control (76.67 ± 8.819 vs. 86.67 ± 3.333, n=3, p=0.2635). It is noteworthy that PS showed an association with a concomitant loss of Jurkats viability in vitro (Figure 1B).

In order to demonstrate the role of PKC in CD47-mediated PS exposure pathway in jurkat cells, the experiment was repeated in the presence of 1.5 μM of Bisindolylmaleimide I, hydrochloride. It was observed that (BRIC 126 + Bis I) induced a significant higher PS exposure than BRIC 126 alone (11.933 ± 0.636 vs. 9.267 ± 1.462, n=3, p=0.03, Figures 2A and 3). (Control + Bis I) did not show any significant difference in PS expression when compared with Control (4.667 ± 0.882 vs. 3.133 ± 0.186, n=3, p=1.1641). In addition, (BRIC 235 + Bis I) did not show any significant difference in PS when compared with BRIC 235 alone (3.667 ± 0.882 vs. 3.000 ± 0.116, n=3, p=0.4952, Figures 2A and 3).

**Figure 1: Phosphatidylserine exposure and apoptosis.** (A) Histogram showing % annexin V positive cells following treatment with BRIC 126, BRIC 235 or control. The preliminary data shows that BRIC 126 significantly induced more PS when compare with BRIC 235 or control (P<0.05). The data are presented as means ± SD of the percentage of annexin V-positive cells of individual treatments from three different passages. (B) Histogram showing means % number of viable cells following treatment with BRIC 126, BRIC 235 or control. The preliminary data shows that BRIC 126 had more dead cells when compared with BRIC 235 or control (p>0.05). The data are presented as means ± SD of the % viable cell counts of individual counts from three different passages. See Materials and Methods for experimental details. Statistical analyses were performed using the Student’s t-test (*) indicates significance for p<0.05; n.s- non-significance for p>0.05).
Percentage viable cell count showed that cells treated in the presence of Bis I all had lower mean % viable cells than cells treated without the PKC-inhibitor. For (BRIC 126 + Bis I) vs. BRIC 126 (68.33 ± 4.410 vs. 76.67 ± 8.819, n=3, p=0.4683); (BRIC 235 + Bis I) vs. BRIC 235 (80.33 ± 5.175 vs. 88.33 ± 1.667, n=3, p=0.4469); and Control + Bis I vs. Control (80.67 ± 5.783 vs. 86.67 ± 3.333, n=3, p=0.3696). It is noteworthy that PS exposure in the presence of PKC inhibition showed an association with a concomitant but non-significant loss of Jurkats viability in vitro (Figure 2B).

Figure 2: Phosphatidlyserine exposure and apoptosis after PKC inhibition. (A) Histogram showing % annexin V+ cells following treatment with BRIC 126, BRIC 235 or control in the presence or absence of Bisindolylmaleimide I, hydrochloride (Bis I). Note that cells treatment with BRIC 126 in the presence of Bis I exposed more PS when compared with BRIC 126 alone. The data are presented as means ± SD of the percentage of annexin V+ cells of individual treatments from three independent experiments. (B) Histogram showing mean % number of viable cells following treatment with BRIC 126, BRIC 235 or control in the presence or absence of Bis I. Note that treatment with BRIC 126, BRIC 235 or control in the presence of Bis I produced higher amount of dead (non-viable) cells when compared with respective treatments alone. The data are presented as means ± SD of the % viable cell counts of individual counts from three different passages. See Materials and Methods for experimental details. Statistical analyses were performed using the Student’s t-test with p<0.05 (*).
DISCUSSION

Although several potential mechanisms were suggested to explain the therapeutic effect of CD47-targeting based on existing data and other studies [8,10], the underlying pathways or downstream effectors which leads to different effects by CD47 are still not well understood. The aim of this study was to investigate the role of PKC in CD47-mediated PS exposure pathway in Jurkat cells.
CD47 ligation with anti-CD47 mAb (BRIC 126) caused the exposure of PS, which is consistent with previous studies [1, 12, 13, 27, 28]. Cells treated with BRIC126 showed a higher PS exposure than BRIC 235 or control. This demonstrates that CD47 molecules are expressed on the cell membrane of jurkat cells and also supports previous study that ligation of CD47 with anti-CD47 mAb induce PS exposure, hence apoptosis in these cells.

Although CD47 ligation with BRIC 126 exposed of PS in jurkat cells, the level of apoptosis was small compared to some other studies [13,28]. Head et al. [13] reported a maximum level of apoptosis of (56% to 64%) after 24 hours of incubation of erythrocytes (4 × 10⁶ cells/mL) with 10 μg/ml anti-CD47 mAb (BRIC 126). Manna and Frazier [28] observed a maximum level of apoptosis of (62-65%) after 18-24 hours of incubation of jurkat cells (3 × 10⁶ cells/mL) with 5 μg/ml of anti-CD47 mAb (1F7). This present study showed maximum apoptosis of (8% to 12%) after overnight incubation of jurkat cells (5 × 10⁶ cells/mL) in 30 μg/ml of BRIC 126. These variations in PS exposure may be due to difference in experimental designs, type of cells or anti-CD47 mAbs used. Furthermore, the observed apoptosis (8% to 12%) may possibly be reflecting the actual binding affinity and epitope specificity of BRIC 126 on jurkat cells. However, more researches are needed to verify this observation.

Percentage viable cell count was done to find if the small levels of PS exposed correlate with cell death (apoptosis). Cells treated with BRIC 126 had the lowest % viable cell count when compared with BRIC 235 or control. Although PS seemed to show a concomitant association or correlation with the % viable cell count, no significant difference was observed between BRIC 126 and BRIC 235 or control. The small PS levels exposed may very well explain the high viability observed, because PS exposure is a measure of apoptosis (death) in cell.

The experiment was repeated in the presence or absence of PKC inhibitor, Bisindolylmaleimide I, hydrochloride (Bis I). (BRIC 126 + Bis I) showed a higher PS exposure than BRIC 126 alone. Although PKC inhibition caused a higher PS expression, the maximum % of apoptosis was observed not to exceed 15%. This may be due to the possible variation in Bis I inhibition of PKC isoforms, or other kinases which may be involved in the pathway or may have opposing roles [29,30]. PKC subtypes differ in their Ca²⁺- dependence, sensitivity to diglyceride or phorboids, substrate specificity, substrate localization and tissue distribution [20]. PKC δ induces DNA oxidation and ROS (Reactive oxygen species) overproduction leading to apoptosis of L-buthionine-S, R-sulfoximine-resistant neuroblastoma cancer cells and potentiates the cytotoxic effects induced by L-buthionine-S,R-sulfoximine in sensitive cells [31]. The formation of cancer stem cells (CSCs) from non-CSCs involves a shift from EGFR (epidermal growth factor receptor) to PDGFR (platelet-derived growth factor receptor) signalling and results in the PKCβ-dependent activation of FRA1 (Fos-related antigen 1), which is preferentially utilized by CSCs; thus inhibition of PKCβ specifically targets CSCs but has little effect on non-CSCs [32]. Hence, PKC sub-species may be scrambled to partake in other essential pathways at different rates which might limit or vary their involvement in CD47-PS exposure pathway.

Percentage viable cell count was done to find if PS exposure with or without PKC inhibition correlates with cell death (apoptosis). It was observed that cells treated in the presence of Bis I all had lower mean % viable cells than cells treated without the PKC-inhibitor; although not statistically significant. This again suggests that PS is a measure of cell deaths.

Results from this experiment demonstrate the involvement of PKC in CD47-PS exposure pathway in jurkat cells. This is worthy of note for future work. Therefore, further research where specific inhibitors of individual PKC isoforms are included
in the assay, would be helpful to unravel the role of PKC isoforms in this novel pathway in jurkat cells; using maybe human peripheral blood T cells as control cells.

**Limitations and Future Directions**

This study lacked the inclusion of Flow cytometric calcium flux assay which could have further strengthened our finding; there was limited scope of analysis to unravel if other molecules (PKC isoforms) played any singular or combined role in PS exposure in these cells. Several other areas have been identified that may be suitable for future work using jurkats and they include: Inhibition of other proteins (PKA, caspases, calpains etc) that have been proposed to be involved in PS exposure in other cell types; inhibition of protein phosphatases to balance this research; examine if CD47 plays role in chemo-resistance, by applying a chemotherapeutic agent to jurkat NTC (non-target control) and jurkat shCD47 and then observe their response to apoptotic induction, which may reveal CD47 as a potential target for T-cell acute lymphoblastic leukaemia.

**CONCLUSION**

Inhibition of protein kinase C pathway promoted anti-CD47 (BRIC126) induced apoptosis in jurkat T cells. There is need for identifying more effective anti-leukemic targets or drugs. The observations from this study identified CD47 and PKC as novel functional proteins in jurkat T cells with promising therapeutic potential. This study would provide insight for targeted therapy against T-ALL disease.

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