CD44 induced enhancement of phosphatase activity and calcium influx: Modifications of EGR-1 expression and cell proliferation

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ABSTRACT

The purpose of this study was to investigate how CD44 impaired Akt phosphorylation, EGR-1 expression and cell proliferation. E6.1 Jurkat cells, which lack endogenous CD44 expression, were engineered to express CD44. Previously we showed that Akt is hypophosphorylated, EGR-1 expression is reduced and proliferation is impaired in CD44 expressing E6.1 Jurkat cells. The cell cycle was studied using flow cytometry and the role of calcium (Ca^{2+}) in Akt phosphorylation and EGR-1 expression was investigated using Western blotting. Phosphatase activity was assessed using a commercially available kit. CD44 expressing cells showed disruption at the G1 to S transition. Chelation of Ca^{2+} from the culture media impaired Akt phosphorylation and EGR-1 expression in both CD44 expressing cells and the open vector control. Moreover, Ni^{2+} disrupted cell proliferation in both cell types suggesting Ca^{2+} import through calcium release activated calcium channels (CRAC). Staining of cells with fura-2 AM showed significantly higher Ca^{2+} in CD44 expressing cells as compared with the vehicle control. Finally, non-calcium mediated phosphatase activity was significantly greater in CD44 expressing cells. We propose that the enhanced phosphatase activity in the CD44 cells increased the dephosphorylation rate of Akt; at the same time, the increased intracellular concentration of Ca^{2+} in the CD44 cells ensured that the phosphorylation of Akt remains intact albeit at lower concentrations as compared with the vector control. Reduced Akt phosphorylation resulted in lowered expression of EGR-1 and hence, reduced the cell proliferation rate.

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1. Introduction

CD44 is a type I transmembrane glycoprotein. Although classically considered to mediate trafficking via its adhesive interactions with hyaluronan [1–3], CD44 has also been shown to regulate cell signaling and cell proliferation. CD44 lacks a signaling domain, however it can facilitate signal transduction by serving as a platform for molecular recruitment and assembly [4].

Several reports in a variety of cell types have shown that CD44 can augment cell proliferation responses. For example, CD44 can trigger mobilization of Ca^{2+} in aortic endothelial cells which in turn leads to their proliferation [5]. On the other hand, negative regulation of cell proliferation by CD44 has been reported only rarely. Gadhoum et al. showed that CD44 inhibited the proliferation of NB4 cells [6]. More recently, we have evaluated the proliferation of E6.1 Jurkat cells engineered to express the standard isoform of CD44 [7]. Importantly, E6.1 Jurkat cells do not endogenously express CD44. We found that CD44 significantly reduced cell proliferation and down-regulated early growth response-1 (EGR-1) protein. EGR-1 is a zinc finger transcription factor that can activate the promoters of many genes whose products can influence cell proliferation [8,9]. Transfection of control E6.1 Jurkat cells with EGR-1 siRNA also inhibited cell proliferation, confirming its role. Disruption of the PI3K/Akt pathway with pharmacological inhibitors reduced EGR-1 expression and cell proliferation, mirroring the properties of CD44 expressing E6.1 Jurkat cells. Finally, Akt was hypophosphorylated in cells expressing CD44 showing its potential role in negatively regulating Akt activation. Results from these studies suggested a novel pathway in which CD44 can negatively regulate cell proliferation by Akt...
inactivation and down-regulated EGR-1 expression [7]. On the other hand, the mechanism of Akt inactivation was not determined.

Protein phosphatase 2A (PP2A) is known to be an important regulator of the cell cycle [10]. Moreover, Akt is one of its molecular targets [11]. Because PP2A has been shown to associate with CD44 in T cell leukemia [12] we hypothesized that Akt was being inactivated in CD44 expressing E6.1 Jurkat cells via phosphatase activity. Indeed, we found that calcium independent phosphatase activity was significantly enhanced in the CD44 expressing cells, but not the vector control cells. At the same time, Ca$^{2+}$ from the culture environment ensured that the phosphorylation of Akt remains intact albeit at lower concentrations as compared with the vector control. Interestingly, Yasuoka et al. has previously reported that Ca$^{2+}$ may also increase the expression of PP2A [13]. This may further suggest a role for Ca$^{2+}$ in both inductive and suppressive proliferative functions in our system. In conclusion, our results suggest that reduced Akt phosphorylation due to phosphatase activity resulted in lowered expression of EGR-1 and hence, reduced the cell proliferation rate in CD44 expressing E6.1 Jurkat cells.

2. Materials and methods

2.1. Cell lines and tissue culture

E6.1 Jurkat cells were engineered to express CD44 or an open vector control as described previously [7]. Expression of CD44 was confirmed by flow cytometry (Supplementary Fig. 1). Cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B, and 800 μg/mL Hygromycin B. Calcium was removed from media via ethylene glycol tetraacetic acid (EGTA) chelation at a concentration of 2.5 mM. LY294002 (Sigma-Aldrich #L9908) was used at a concentration of 10 μM for 24 h. Wortmannin (Sigma-Aldrich #W1628) was used at 5 μM for 24 h. Cantharidic acid (Enzo Life Sciences #BML-PR106-0010) was used at either 100 nM, 250 nM, or 500 nM as indicated for 24 h in Western blotting and 48 h in proliferation experiments.

2.2. Cell cycle analysis

Cells were synchronized by serum starvation for 24 h before each experiment. One million cells per mL were suspended in 70% ethanol and incubated at 20°C for 2 h. Cells were centrifuged and washed in PBS twice before being incubated in 60 μg/mL propidium iodide (Sigma-Aldrich #P4170) and 10 μg/mL RNase A (Sigma-Aldrich #R6513) for 1 h at 37°C. Cells were then analyzed via flow cytometry utilizing a Beckman Coulter Cytomics FC500.

2.3. BrdUrd incorporation

Cells were synchronized in the G1 phase by serum starvation for 24 h prior to experiments. 5-Bromo-2’-deoxyuridine (BrdUrd) was added to media at 10 μM and cells were cultured under normal conditions for 30 min. Samples were stained for BrdUrd according to kit manufacturer instructions (BD Biosciences #557891) before analysis via flow cytometry.

2.4. Proliferation assays

One hundred thousand cells per group were plated into 96 well plates in 200 μL of media. Cells were allowed to proliferate for 24 h or 48 h. Proliferation was assessed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay (Promega #G5421). To rule out the possibility that an observed decrease in proliferation was due to a difference in viability, trypan blue staining was performed before and after incubation to assure that viability was maintained throughout the incubation period. Inducible calcium release activated channel
incubated for 15 min at 37 °C. The suspension was diluted a further one fourth with PBS and incubated for 15 min at 37 °C. After washing, cells were suspended in a buffer containing 140 mM NaCl, 5 mM KCl, 1 mM MgSO$_4$, 1 mM CaCl$_2$, 20 mM HEPES, 1 mM Na$_2$HPO$_4$, and 5.5 mM glucose at pH 7.4. Cells were placed on coverslips coated in poly-L-lysine and incubated for 10 min at 37 °C before imaging. Intracellular calcium was measured at 37 °C by the ratiometric technique using fura-2-AM (excitation at 340 nm and 380 nm, emission at 510 nm) (Invitrogen, Carlsbad, CA) as described by utilizing a Nikon Eclipse TE2000-S microscope and NIS-Elements AR3.2 software (Nikon Instruments, Melville, NY) [14]. The Grynkiewicz equation was used to convert the 340 nm/380 nm ratio to internal levels of calcium in nanomolar concentrations [15].

2.7. Phosphatase assay

Phosphatase activity in the cells was measured using the kit from Enzo Life Sciences (#BML-AK804-0001). Briefly, cell protein extracts were chilled on ice in protease inhibitor lysis buffer. Extracts were then desalted using a resin column and the amount of recovered protein was estimated by the Bradford method. Equal concentrations of proteins were added to wells containing RII phosphopeptide substrate. Wells containing EGTA were used to determine calcineurin activity. Results were expressed as nmol of phosphate per well after development with BIOMOL Green and absorption at 655 nm.

2.8. JC-1 mitochondrial staining

One million cells per sample were stained with 5μM JC-1 dye from Invitrogen (#J3168) by incubating at 37 °C for 15 min. Cells were spun down and washed once in warm PBS. An ISS PCl spectrophuorometer was used to measure the fluorescence intensity of each cell sample at an ex/em of 485 nm/530 nm for the monomeric form of JC-1 and an ex/em of 535 nm /590 nm for the aggregate form of JC-1.

2.9. Statistical analysis

Two groups were compared by the 2-tailed Student’s t-test and more than two groups by the ANOVA with Tukey’s multiple comparisons test. A p-value of less than 0.05 was considered significant. All results are shown as the mean ± SD. Each experiment was repeated thrice with similar results in the experiments.

3. Results

3.1. CD44 expression causes cell cycle arrest

Our laboratory has previously shown that EGR-1 regulates the proliferation of E6.1 Jurkat cells and that CD44 expression decreases the expression of EGR-1 [7]. The doubling time for CD44 expressing E6.1 Jurkat cells is 34.67 ± 0.58 h as compared with a doubling time of 23.00 ± 1.00 h for E6.1 Jurkat cells lacking CD44 expression (values are presented as the means ± standard deviations). This significant increase in doubling time (P < 0.01) is evidence that CD44 significantly perturbs E6.1 Jurkat cell proliferation. Due to EGR-1’s importance in regulating the cell cycle [16–19] we wanted to examine potential dysregulations of the cell cycle due to CD44 expression. E6.1 Jurkat cells expressing CD44 were found to have a higher accumulation of cells in the G1/G0 phase of the cell cycle as compared with the vector control (Fig. 1A). To determine if there was a deficiency in CD44 expressing cells when progressing through the G1 to S phase checkpoint we directly
measured the rate at which the cells transitioned between phases. Vector control and CD44 expressing E6.1 Jurkat cells were synchronized in the G1 phase of the cell cycle and then allowed to proliferate for 30 min in the presence of BrdUrd. CD44 expressing cells showed a slower transition from the G1 phase into the S phase when compared with the vector control cells (Fig. 1B). These data show that CD44 expression triggers a disruption in cell cycle at the G1 to S phase transition, which is consistent with a decrease in EGR-1 expression which is a transcription factor responsible for the expression of early genes within the cell cycle.

3.2. CD44 expression induces calcium influx

Cells were loaded with Fura-2 on poly-L-lysine coated slides in order to measure their intracellular calcium concentrations. E6.1

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**Fig. 4.** EGR-1 expression is regulated by Ca\(^{2+}\). (A) Western blot for EGR-1 expression with and without calcium. A representative blot is shown as the left panel and cumulative densitometry values are shown as the right panel. (B) Cell proliferation before, during, and after a 24 h period without extracellular calcium. Results are normalized to percent of control cell line with calcium. There was no difference between Vector and CD44 cell lines without calcium (p > 0.05). (C) Western blotting for EGR-1 expression before removal of extracellular calcium, after one day of rest following 24 h without extracellular calcium, and after four days of rest following 24 h without extracellular calcium. A representative blot is shown as the left panel and cumulative densitometry values are shown as the right panel. Densitometry values from three different Western blots were used to calculate the means ± SD. Asterisks indicated statistical significance using the 2-tailed Student’s t-test (A and B) or the ANOVA with Turkey’s multiple comparisons test (C) (**p < 0.05; ***p < 0.01).
Jurkat cells expressing CD44 were found to have significantly more intracellular calcium as compared with the vector control cells (Fig. 2, Supplementary Fig. 2). These results showed that CD44 expression disrupts calcium homeostasis.

3.3. CD44 expression induced Ca\(^{2+}\) influx uses CRAC channels to regulate proliferation and Ca\(^{2+}\) is required for proper cell cycling

To determine if E6.1 Jurkat cells were utilizing Ca\(^{2+}\) release activated calcium channels (CRAC) for their calcium influx we cultured cells with the inhibitor, Ni\(^{2+}\) [20–22]. While both CD44 expressing and vector control E6.1 Jurkat cells were both inhibited by Ni\(^{2+}\) the vector control cells proliferated twice as much as the CD44 expressing cells. These results show that E6.1 Jurkat cells require Ca\(^{2+}\) for their proliferation (Fig. 3A). To determine if excess calcium influx was responsible for the delay in the cell cycle seen in Fig. 1 we synchronized CD44 expressing E6.1 Jurkat cells and cultured them with and without extracellular calcium. CD44 expressing E6.1 Jurkat cells required Ca\(^{2+}\) for their proliferation and showed significantly greater death rates without Ca\(^{2+}\) (i.e., the increased sub-G1 phase of the cell cycle; Fig. 3B).

3.4. EGR-1 expression is regulated by Ca\(^{2+}\)

EGR-1 expression is regulated in part by Ca\(^{2+}\) signaling [23,24]. In order to determine if EGR-1 is regulated by calcium in E6.1 Jurkat cells, we cultured them and the vector control cells with and without Ca\(^{2+}\). EGR-1 expression was reduced in both vector control cells and CD44 expressing cells (Fig. 4A), indicating that Ca\(^{2+}\) is required for EGR-1 expression. Upon culture in media without Ca\(^{2+}\) for 24 h, both CD44 expressing and vector control cells proliferated less than those cultured in the presence of Ca\(^{2+}\) (Fig. 4B). In order to determine if this decrease in proliferation due to Ca\(^{2+}\) deprivation was a permanent or transient effect, we restored Ca\(^{2+}\) to cells which had been deprived of extracellular Ca\(^{2+}\). After one day of restored Ca\(^{2+}\) in the media, both the CD44 expressing and vector control E6.1 Jurkat cells showed proliferation equivalent to cells without available Ca\(^{2+}\) (Fig. 4C). These results indicate that Ca\(^{2+}\) is required for both the vector control and CD44 expressing E6.1 Jurkat cells. After four days of restored Ca\(^{2+}\), EGR-1 expression proliferation returned to its baseline in both CD44 expressing and vector control cells (Fig. 4D). These data show that Ca\(^{2+}\) is required for EGR-1 expression and proliferation.

3.5. CD44 regulates EGR-1 through Ca\(^{2+}\) signaling and the Akt pathway

To investigate the possibility that CD44 expression regulates Akt phosphorylation through Ca\(^{2+}\) signaling, we cultured cells with and without Ca\(^{2+}\) and measured the phosphorylation of Akt. As we have previously shown, Akt was hypophosphorylated in cells expressing CD44 under normal conditions [7]. When Ca\(^{2+}\) signaling is removed through chelation with EGTA, both the vector control cells and CD44 expressing Jurkat T cells have hypophosphorylated Akt (Fig. 5A). The observed decrease due to the removal of calcium was proportional in each cell line, with both control and CD44 expressing cells having approximately half as much phosphorylated Akt.

To determine if Akt regulation of EGR-1 expression occurs upstream or downstream of Ca\(^{2+}\) based EGR-1 expression, we cultured
cells with Akt inhibitor LY294002 in addition to the removal of extracellular calcium. Importantly, LY294002 was non-toxic to cells. Moreover, Wortmannin provided similar results as previously reported [7]. EGR-1 expression was reduced in control and CD44 expressing Jurkat cells in response to lack of extracellular Ca\(^{2+}\), Akt inhibition, and both the lack of extracellular Ca\(^{2+}\) and Akt inhibition (Fig. 5B). We interpret these data to suggest that CD44 expression results in decreased Ca\(^{2+}\) mediated Akt phosphorylation through another mechanism, potentially increased phosphatase activity.

### 3.6 CD44 increases phosphatase activity in E6.1 Jurkat cells

In the above experiments, Ca\(^{2+}\) was shown to play a role in both EGR-1 expression and Akt phosphorylation. Because CD44 expressing cells showed significantly higher concentrations of Ca\(^{2+}\), how can we explain Akt hypophosphorylation and reduced EGR-1 expression? Rajasagi et al. has shown that CD44 is associated with the expression of phosphatase enzymes [12]. We therefore hypothesized that CD44 expressing E6.1 Jurkat cells could potentially express higher activities of phosphatases. As shown in Fig. 6A, total phosphatase activity was significantly higher in CD44 expressing cells as compared with the vector control. Calcineurin, which is a Ca\(^{2+}\) dependent phosphatase did not have significantly different activities between the CD44 expressing and non-expressing cells. Yasuoka et al. showed that elevated Ca\(^{2+}\) levels in myocardic H9c2 cells decreases the activation of Akt by inducing PP2A expression and activity [13]. Similarly, as shown in Fig. 6B, we found that use of the PP2A inhibitor cantharidin increased phosphorylation of Akt after 24 h in CD44 expressing cells, but not the vector control cells. Inhibition of PP2A with cantharidic acid also rescued the proliferation of CD44 expressing E6.1 Jurkat cells, and the increase in proliferation over the control baseline is not observed in similarly treated vector control cells (Fig. 6C). Finally, the manuscript by Rajasagi et al. also shows that CD44 association with PP2A in EL4 T lymphoma cells results in apoptosis via mitochondrial depolarization [12]. By contrast, we found that CD44 expression in Jurkat E6.1 cells does not induce apoptosis [7] nor does it impact mitochondria polarization [12] (Fig. 6D). Thus, CD44 appears to have different effects based on the genetic background of the cell type under investigation.

### 4. Discussion

We previously have shown that expression of CD44 in Jurkat E6.1 cells significantly impaired cell proliferation by reducing Akt activation and EGR-1 expression. The purpose of these current studies was to determine the mechanism whereby CD44 inactivates Akt, down-regulates EGR-1 and impairs cell proliferation. Here, we advance our previous studies by showing that CD44 expression enhances the uptake of Ca\(^{2+}\) and increases the activity of phosphatase enzymes. Chelation of Ca\(^{2+}\) significantly reduced Akt phosphorylation and EGR-1 expression in both CD44 expressing and control vector cells. Although it is also possible that EGR-2 and EGR-3, which can serve as negative regulators for EGR-1 expression, our findings that LY294002 impairs EGR-1 expression strongly suggests that the PI3K/Akt pathway is the major pathway for EGR-1 expression in E6.1 Jurkat cells. CD44 expressing E6.1 Jurkat cells showed an increase in the S phase of the cell cycle when the extracellular calcium was removed and increased cell death. This result provides further support to Fig. 3A, reinforcing the notion that extracellular Ca\(^{2+}\) is required for proliferation in CD44 expressing E6.1 Jurkat cells. Finally, cell proliferation was significantly impaired by
Ni²⁺, which is an inhibitor of CRAC channels. Therefore, we reasoned that Ca²⁺ is responsible for the Akt mediated expression of EGR-1 in both CD44 expressing and vector control Jurkat E6.1 cells and for their proliferation.

Analyses showed that CD44 dysregulated Ca²⁺ homeostasis as compared with the vector control cells. The concentration of intracellular Ca²⁺ in CD44 expressing cells was nearly three times higher than the intracellular concentration of Ca²⁺ in vector control cells. Because Ca²⁺ was shown to be requisite for Akt phosphorylation and EGR-1 expression it would seem reasonable to postulate that CD44 expressing cells should have higher concentrations of activated Akt and EGR-1 and proliferate at a greater rate than vector control cells. On the contrary, we have observed that Akt was hypophosphorylated and EGR-1 expression was reduced in CD44 expressing cells. At the same time, we also found a significant increase in the activities of phosphatases in the CD44 expressing cells. The phosphatase PP2A has been immunoprecipitated with CD44 in EL4 T lymphoma cells and cross-linkage of CD44 has been shown to activate PP2A in EL4 T lymphoma cells [12]. Increased intracellular calcium has been shown in H9c2 myocardic cells to increase the expression and activity of PP2A, resulting in decreased Akt phosphorylation [13]. We found that inhibition of PP2A with cantharidic acid resulted in increased phosphorylation of Akt similar to the vector control cells. Cantharid acid had no overall effect on the phosphorylation of Akt in the control cells, suggesting a CD44 specific effect on PP2A activity. We propose that the increased phosphatase activity in the CD44 cells increases the dephosphorylation rate of Akt. Previous investigations have shown that phosphatases can directly dephosphorylate Akt [2,26]. At the same time, the increased intracellular concentration of Ca²⁺ in the CD44 cells ensures that the phosphorylation of Akt remains intact albeit at lower concentrations as compared with the vector control cells. We propose that the lower concentration of active Akt in the CD44 expressing cells results in lower concentrations of EGR-1, hence reduced proliferation rates.

E6.1 Jurkat cells are a T cell lymphoblastic leukemia line. Previous investigations have shown that there is a link between CD44 expression and T cell lymphoblastic leukemia. Cavalcanti et al. found that CD44 expression was detected in 77% of patients with disease and was correlated with mediastinal mass, adenomegaly, infiltration of the central nervous system and other organs. This group proposed that CD44 could be regarded as a marker for tissue infiltration in T cell leukemia [27]. Similarly, Viskova et al. found that high levels of CD44 expression were linked to a highly aggressive subtype of acute lymphoblastic leukemia [28]. Khan et al. found that CD44 surface expression predicted relapse in children with acute lymphoblastic leukemia [29]. More recently Kamazani et al. found that CD44 expression in acute lymphoblastic leukemia was correlated with higher white blood cell counts, a higher percentage of blast cells in the bone marrow, and that CD44 expression could serve as predictor of responsiveness to therapy [27]. Although we currently do not know the impact of CD44 expression on the in vivo lethality of E6.1 Jurkat cells, current experiments in mice are planned in our laboratory to determine its effect.

In conclusion, we have reported a unique functional role for CD44 in regulating cell proliferation. Results and experimental systems described in this study provide the technical and conceptual foundation for further investigating the role of CD44 in cell proliferation. Future experiments showing the effect of CD44 expression in vivo may elucidate the mechanistic relationship between CD44 expression in acute lymphoblastic leukemia and the negative outcomes associated with CD44 in clinical studies.

Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.03.016.

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