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Tetraspanin CD9 modulates human lymphoma cellular proliferation via histone deacetylase activity

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ABSTRACT

Non-Hodgkin Lymphoma (NHL) is a type of hematological malignancy that affects two percent of the overall population in the United States. Tetraspanin CD9 is a cell surface protein that has been thoroughly demonstrated to be a molecular facilitator of cellular phenotype. CD9 expression varies in two human lymphoma cell lines, Raji and BJAB. In this report, we investigated the functional relationship between CD9 and cell proliferation regulated by histone deacetylase (HDAC) activity in these two cell lines. Introduction of CD9 expression in Raji cells resulted in significantly increased cell proliferation and HDAC activity compared to Mock transfected Raji cells. The increase in CD9–Raji cell proliferation was significantly inhibited by HDAC inhibitor (HDACi) treatment. Pretreatment of BJAB cells with HDAC inhibitors resulted in a significant decrease in endogenous CD9 mRNA and cell surface expression. BJAB cells also displayed decreased cell proliferation after HDACi treatment. These results suggest a significant relationship between CD9 expression and cell proliferation in human lymphoma cells that may be modulated by HDAC activity.

1. Introduction

The expression and influence of tetraspanin CD9 on the surface of numerous cell types has been thoroughly demonstrated [1–4]. Human CD9 consists of 228 amino acids with a proposed molecular weight of 24 kDa [5]. The structure of CD9 yields two intracellular amino and carboxy tails, four transmembrane domains, two differentially sized external loops, and one very short internal loop. Several studies have specifically attributed CD9 to modulate the proliferative phenotype of both normal and malignant cells [6,7]. A previous report indicates CD9 expression in Raji cells promoted motility to both fibronectin and laminin implicating a role for CD9 expression in the dissemination of B-cell lymphomas [8].

Histone deacetylase inhibitors (HDACi) have been demonstrated to regulate gene expression and resulting cell phenotypes [9,10]. These inhibitors influence chromatin structure and have been identified as promising agents for pharmacological intervention in the treatment of cancer [11,12]. HDACi induce the expression of epigenetically silenced genes resulting in growth arrest, differentiation and cell death [13,14]. Trichostatin A (TSA), a well-characterized HDACi, has significant antiproliferative effects in the treatment of leukemia [15,16]. Therefore, the regulation of HDAC activity is necessary to control the outgrowth of certain tumors.

A relationship between CD9 expression and HDAC activity was observed in an earlier study where HDAC inhibition with TSA downregulated CD9 mRNA and protein expression in primary murine macrophages [17]. CD9 expression in a cancerous melanoma cell line was also influenced by HDACi treatment; however, CD9 expression was inversely correlated with tumor progression [18]. There is an interest in the unique role CD9 has in the maturation and malignant progression of B lymphocytes and progenitor cells [19]. However, there are no reports directly linking CD9 and HDAC activity in relation to the regulation of cell proliferation.

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The purpose of this study was to investigate if CD9 expression and HDAC activity was significantly linked to CD9-promoted Burkitt's lymphoma cell proliferation. Our results demonstrate that CD9 expression increases Raji cell proliferation. Furthermore, HDACi resulted in downregulated endogenous CD9 cell surface expression and decreased cell proliferation of BJAB cell. This supports a role for epigenetic histone acetylation in regulating CD9 expression and control of cell proliferation.

2. Materials and methods

2.1. Cell culture and transfections

The human Burkitt's lymphoma cell lines Raji was purchased from the American Type Culture Collection (Manassas, VA). The human Burkitt's lymphoma cell BJAB was kindly provided by Dr. Blossom Damania (University of North Carolina, Chapel Hill, NC). The cells were maintain in RMP1640 media supplemented with 10% fetal bovine serum (FBS) and 5% penicillin–streptomycin (Invitrogen, Grand Island, NY). CD9 was stably expressed in Raji cells via electroporation of the eukaryotic expression vector pRc/CMV containing full-length human CD9 cDNA. Geneticin (G418, Gibco, St. Louis, MO) at 1 mg/ml final concentration was used for selection of positive transformants. An empty vector pRc/CMV plasmid was similarly used to generate Mock–Raji cells.

2.2. Western blotting

Whole cell lysates were prepared by lysing cells in TX-100 lysis buffer (1 M Tris, 3 M NaCl, 10% deoxycholate, 10% SDS, 10% Triton-X 100, 500 mM EDTA) with added protease inhibitor cocktails purchased from Sigma. Cell lysates were sonicated four times with 5 s bursts to release histones bound to DNA and clarified by centrifugation at 14,000*g* for 10 min. The concentration of lysates was measured using a colorimetric Bradford assay and equal concentrations of lysate were mixed with 1/3rd sample loading buffer and loaded on to a sodium dodecyl sulfate polyacrylamide gel. After electrophoresis, the proteins were transferred to a polyvinyl difluoride membrane (Millipore, Billercia, MA) and blocked for one hour in 5% BSA-Tris-buffered saline with Tween-20 (TBST). CD9 or acetylated H4 histone was detected using an anti-human monoclonal antibody to CD9 (mAb7) preciously generated in our laboratory [20,21], or anti-acetyl-histone H4 (Lys12) (Cell Signaling, Danvers, MA) followed by a sheep anti-mouse or goat anti-rabbit HRP conjugate, respectively. To verify loading, the blots were probed with anti-actin antibody purchased from Chemicon (Billerica, MA). ECL Plus purchased from GE Life Sciences (Pittsburgh, PA) was used for chemiluminescent detection.

2.3. Flow cytometric analysis

Cell surface density of CD9 was determined by flow cytometric analysis as described by Cook et al. [1]. Briefly, CD9–Raji cells were harvested by centrifugation at 800*g* for 10 min and suspended as 1×10^6 cells/ml in RPMI 1640 media containing 5% goat serum (v/v, labeling medium). CD9 expression was detected with mAb7. Non-specific antibody binding was measured using an IgG isotype matched antibody purchased from Sigma at an equal concentration. After 1 h incubation and subsequent washing by centrifugation, goat anti-mouse FITC conjugated secondary antibody (Sigma) was used to detect primary antibody binding. Data was obtained and quantified using a FACS Calibur Flow Cytometer and Cell Quest Pro data analysis software (Becton Dickinson, San Diego, CA).

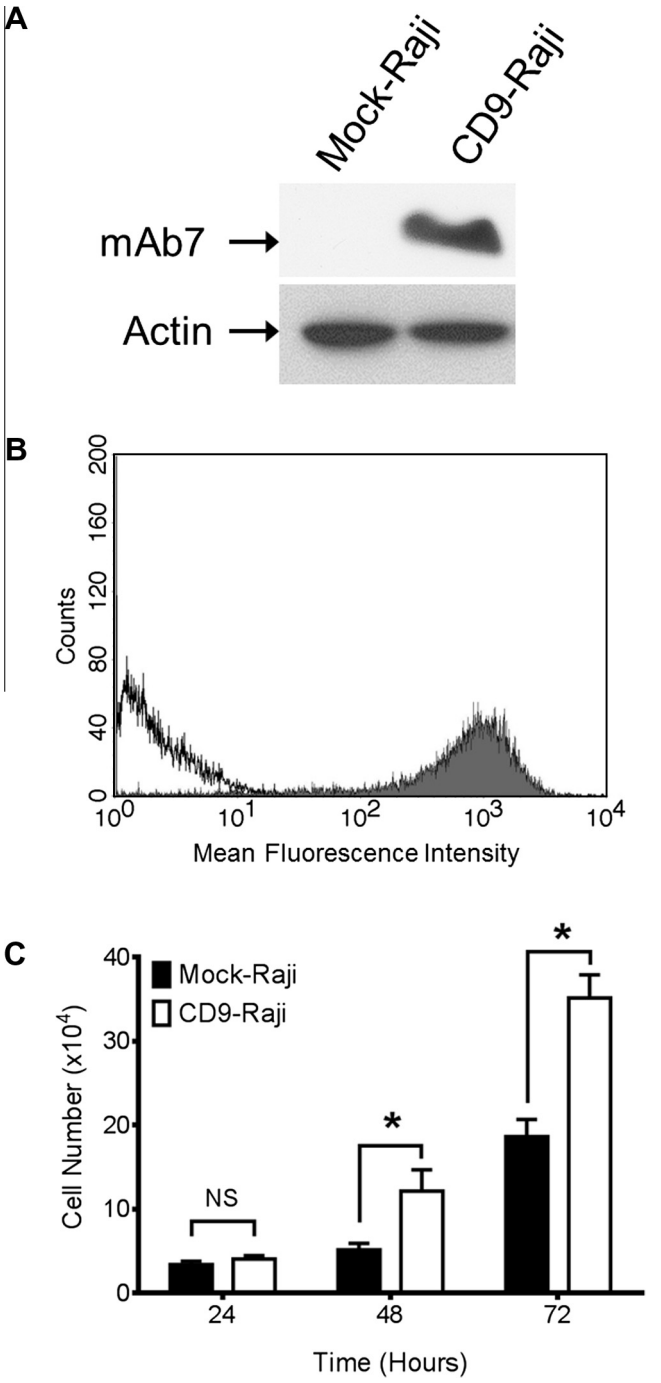

Fig. 1. Stable expression of CD9 in Raji cells increases cell proliferation. (A) CD9 expression in Raji cells was confirmed via Western blot using a monoclonal antibody specific for CD9 (mAb7). An anti-actin antibody was used to confirm equivalent loading. (B) Cell surface expression of CD9 was measured using flow cytometry. mAb7 binding to the cell surface of CD9–Raji is indicated by the shaded histogram; mAb7 binding to Mock-Raji is indicated by the unshaded histogram. (C) Cell proliferation was measured between Mock- and CD9–Raji cells at 24, 48, and 72 h by cell counting ($n = 3$, $^*p < 0.05$).

2.4. Proliferation assays

To investigate the effect of CD9 expression on cell proliferation, 2×10^5 cells were seeded in 2 ml of growth medium in six-well culture plates. Cell proliferation was monitored at 24 h intervals by harvesting the cells and counting using trypan blue exclusion and a hemocytometer to confirm cell viability.

2.5. HDAC activity assay

To investigate the HDAC activity, cells were harvested during exponential growth in growth media, and nuclear extracts were made using the Nuclear/Cytosol fraction kit purchased from MBL International Corporation (Woburn, MA). HDAC activity was assessed using a colorimetric HDAC activity assay kit that measures the deacetylation of an HDAC substrate (Boc-Lys(Ac)-pNA). Deacetylation of the HDAC substrate resulted in sensitization to a lysine-developing reagent that produced a chromophore that was quantified at 405 nm using a Versamax tunable microplate reader (Molecular Devices, Sunnyvale, CA) and was directly proportional to the HDAC activity in the sample.

2.6. qRT-PCR analysis

Total RNA was isolated using TRI reagent according to the manufacturer's protocol (Sigma, St. Louis, MO) and reverse transcribed into cDNA using the High Capacity cDNA Archive kit purchased from Roche (Indianapolis, IN). Specific gene expression assays were designed using the Roche Universal Probe Library Assay Design Center. Samples were tested in triplicate using Taqman chemistry on a Roche Lightcycler 480 and normalized to TATA box binding protein (TBP). Relative fold changes in gene expression were calculated from ΔCT values as described previously [2]. All results represent mean ± standard deviation of three independent experiments.

2.7. Statistical analysis

SigmaStat software package (Version 3.5, Systat, San Jose, CA) was utilized to perform *t*-tests and one- or two-factor ANOVA to determine if statistically significant differences existed between experimental data collected for various treatments. When significant differences were detected with ANOVA, the Student–Newman–Keuls (SNK) multiple comparison procedure was applied to identify statistically different treatment pairs. An α-level of 0.05 was accepted as significant.

3. Results

3.1. CD9 expression enhances Raji cell proliferation

The significance tetraspanin CD9 expression has on Burkitt's lymphoma cells has not been previously demonstrated except at the capacity of identifying that BJAB cells express CD9 and Raji cells do not [22]. In order to understand the effect CD9 expression has on these cells, the human Burkitt's lymphoma cell line Raji was transfected with full-length CD9 or an empty Mock plasmid. Upon transfection, whole cell protein levels of CD9 expression were detected in CD9–Raji cell lysate but were absent in the Mock–Raji cell lysate (Fig. 1A). We determined that CD9 cell surface levels were significantly greater in CD9–Raji cells compared to Mock–Raji cells as measured by flow cytometry (657.15 ± 97.71 vs. 3.34 ± 1.03) (Fig. 1B). To investigate the potential effect CD9 expression has Raji cell proliferation, we monitored cell numbers at 24, 48, and 72 h (Fig. 1C). CD9 expression did not cause a significant increase in Raji cell proliferation after 24 h; however, after 48 h there was a 58% increase in CD9–Raji cell numbers compared to their Mock counterparts. CD9–Raji cells remained more proliferative than their Mock counterparts at 72 h after which proliferation plateaued. These data suggest that the expression of CD9 results in increased Raji cell proliferation.

3.2. Enhanced cell proliferation is regulated by increased histone deacetylase activity in CD9–Raji cells

Prominent changes in cell proliferation upon CD9 expression led us to study histone modifications of gene regulation. Histone modification by histone deacetylases (HDACs) results in gene regulation, and pharmacological HDAC inhibitors have been demonstrated to induce growth arrest in cancer cells [23]. HDAC activity was measured in Mock- and CD9–Raji cells demonstrating

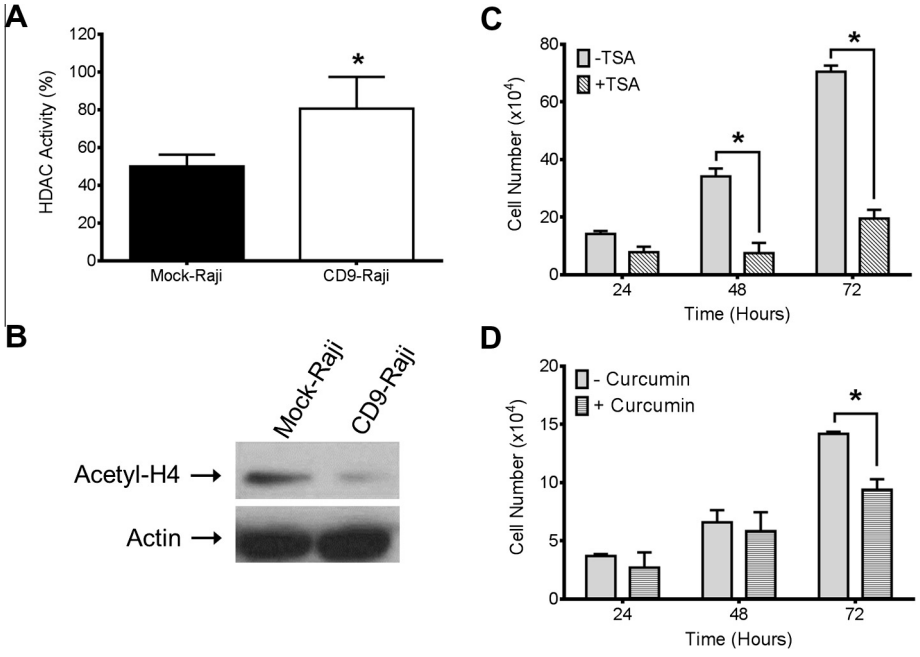

Fig. 2. CD9–Raji have increased HDAC activity and HDAC inhibition negatively effects cell proliferation. (A) HDAC activity was measured in Mock- and CD9–Raji cells as described in the material and methods section. (B) Acetylation of histone H4 was measured via Western blot analysis with an antibody specific for acetylated H4. An anti-actin antibody was used to confirm equivalent loading. (C) HDAC activity was inhibited in CD9–Raji cells using trichostatin A (TSA) and cell proliferation was monitored by cell counts at 24, 48, and 72 h post treatment. (D) The same assay was done in the presence or absence of curcumin ($n = 3$, $^*p < 0.05$).

a 30% increase in HDAC activity upon CD9 expression in Raji cells (Fig. 2A). As CD9–Raji cells exhibited increased HDAC activity, we expected these cells to have decreased histone acetylation when compared to the Mock transfectants. Acetylated histone H4 was detected in both Mock- and CD9–Raji whole-cell lysates; however, Raji cells expressing CD9 had a significantly decreased amount of acetylated H4 compared to Mock cells as demonstrated by Western blotting (Fig. 2B).

To determine whether modulation of HDAC activity in CD9–Raji cells was responsible for the enhanced proliferation observed in these cells, cell proliferation assays were performed in the presence or absence of HDAC inhibitors trichostatin A (TSA) and curcumin. TSA Treatment markedly reduced CD9–Raji cell proliferation to levels comparable to Mock-Raji cells at 48 and 72 h (Fig. 2C). Curcumin reduced CD9–Raji cell proliferation but a statistically significant difference in proliferation was not observed until 72 h (Fig. 2D).

3.3. Histone deacetylase inhibition reduces endogenous CD9 expression and cell proliferation

Due to the positive effect of CD9 expression on Raji cell proliferation and its regulation by HDAC activity, we explored endogenous CD9 expression in BJAB cells. Flow cytometric analysis of wild-type BJAB cells demonstrated that the CD9 cell surface expression levels obtained in CD9–Raji cells were comparable to endogenous CD9 expression on BJAB cells (Fig. 3A). To determine if CD9 expression may be affected by inhibiting histone deacetylase activity, we first observed any changes in CD9 mRNA after treatment with TSA or curcumin. Treatment of BJAB with TSA reduced CD9 mRNA levels 4.3-fold (Fig. 3B). Similarly, curcumin treatment reduced CD9 mRNA levels 3.6-fold in BJAB cells (Fig. 3B). Changes in CD9 cell surface expression were assessed using flow cytometry upon treatment of BJAB cells with TSA. Treatment of BJAB cells with increasing concentrations of TSA resulted in decreased CD9 levels on the cell surface (Fig. 3C). The maximal dose of TSA administered (200 μM) resulted in a 36% reduction in CD9 cell surface expression. We determined that HDAC inhibition affected the proliferation of BJAB cells by cell counting. Similar to CD9–Raji cells, treatment of BJAB cells with either TSA or curcumin significantly reduced BJAB cell proliferation at 48 and 72 h compared to control treatments (without TSA or curcumin) (Fig. 3D, E). These results indicate that CD9 expression induces HDAC activity that leads to the upregulation of gene expression and cell proliferation in human Burkitt's lymphoma cells.

4. Discussion

The progression of Non-Hodgkin Lymphoma (NHL) and other cancers begins with uncontrolled proliferation. It is well established that CD9 and other tetraspanins such as CD81 and CD53 have a significant role in modulating cell proliferative phenotypes [18,24,25]. The differential expression of CD9 in two human Burkitt's lymphoma cell lines, Raji and BJAB, fueled our curiosity to understand the role of CD9 expression in these types of NHL. We discovered that expression of CD9 in Raji cells, which do not endogenously express CD9, drove the cells to proliferate. This phenomenon was regulated by HDAC activity as demonstrated by reversal of this phenotype upon HDAC inhibition. Correspondingly, CD9 expression in BJAB cells, which endogenously express CD9, was diminished upon HDAC inhibition.

The levels obtained in expressing CD9 in Raji cells were comparable to levels of endogenous CD9 expression reported in other cancerous cell lines [7,26]. Thus, our observations in Raji cells were based on conditions that mimic typical endogenous CD9 cell surface densities. We hypothesize that epigenetic regulators involved in histone regulation by acetylation and deacetylation are modulated by CD9 expression in NHL cells. Inhibitors of HDAC stabilize the acetylation of histone proteins resulting in the inhibition of cell proliferation. Inhibition of HDAC activity has become a means to negate the proliferative phenotype of cells, particularly

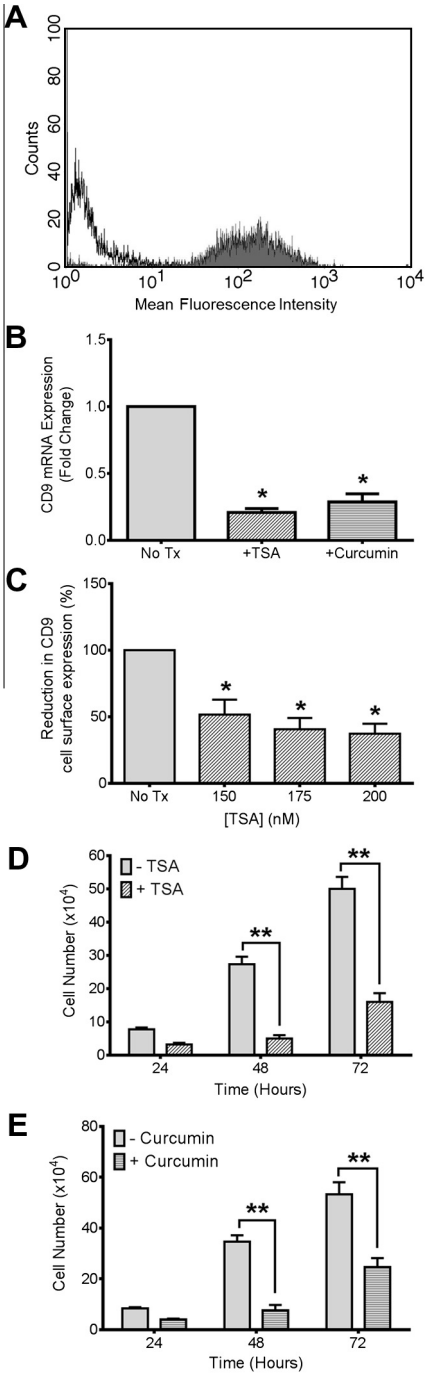

Fig. 3. HDAC inhibition results in decreases in endogenous CD9 mRNA and cell surface expression and cell proliferation. (A) CD9 cell surface expression was determined in BJAB cells using flow cytometry. mAb7 binding is represented by the shaded histogram and non-specific binding of an isotype-matched antibody (IgG) is represented by the unshaded histogram. (B) CD9 mRNA levels were measured after treatment with Trichostatin A (+TSA) or curcumin via qRT-PCR analysis. (C) Cell surface levels of CD9 were measured after incubations with increasing concentrations of TSA for 24 h. (D) Proliferation of BJAB cells was monitored at 24, 48, and 72 h in the absence or presence of TSA. (E) The proliferation assay was repeated in the absence or presence of curcumin ($n = 3$, $^{*}p < 0.05$, $^{**}p < 0.001$).

cancer cells that have highly enhanced cell proliferation [23]. These findings have led to investigation of HDACi efficacy in regulating cancer cell proliferation in clinical trials [27]. One of the best described events following HDAC inhibitor treatment is the induction of growth arrest in the G1 or G2/M phase of the cell cycle [28,29]. CD9–Raji cells had decreased acetylation of histone H4 and increased HDAC enzymatic activity when compared to Mock controls. These results suggest that histone deacetylase-related protein (HDRP) may provide multiple mechanisms for regulating the proliferative phenotype and may serve as a novel mechanism for regulating CD9 mediated cell phenotypes in these cells. The relationship of endogenous CD9 expression and HDAC activity with cell proliferative events was established by using the BJAB cells. Treatment with either TSA or curcumin significantly decreased proliferation of BJAB cells and decreased endogenous CD9 expression demonstrating for the first time that CD9 expression can be modulated by epigenetic events in this cell type. Therefore, our observations that cellular proliferation is diminished upon HDACi treatment are purely confirmatory in regards the effect HDACi has on cancer cell proliferation. However, finding that HDACi lowers the cell surface expression of CD9 in a cancerous cell line is novel and contributes to the understanding of CD9 regulation.

In summary, this report demonstrates that the CD9–HDAC axis may regulate B cell proliferative phenotypes and supports the underexplored area of CD9 expression regulation. HDRP may facilitate the observed increase in nuclear HDAC activity when CD9 is expressed on the cell surface. The functional relationship of this mechanism is under further investigation by our laboratory. It is feasible that CD9, via enhancement of PI3-K/Akt activity [3], may contribute to the capability of CD9–Raji cells to regulate HDAC-associated pathways [30]. CD9 likely serves as a feedback mechanism for certain NHL cells to strengthen communication with the extracellular environment, regulate HDAC activity, and decide to proliferate or remain dormant. Furthermore, the findings from this study hint at the possibility of using CD9 as a diagnostic marker for the efficacy of HDAC inhibition in NHL malignancies.

Acknowledgments

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