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Research Article

Monocyte/Macrophage - like cell differentiation induced by TPA in HL60 cells leads to loss of Histone H4 Lysine 16 Acetylation

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ABSTRACT

12-O-Tetradecanoylphorbol-13-acetate (TPA) is a phorbol ester and induces monocyte/macrophage like cell differentiation in HL60 cells. The levels and patterns of gene expression differ greatly between differentiated and undifferentiated HL60 cells. Epigenetic histone modifications play a crucial role in transcriptional activation and alterations in the modification levels greatly affect the gene expression pattern. Acetylation of Histone H4 Lysine 16 (H4K16ac) is one such modification and has a significant role in transcriptional activation. Changes in its acetylation level either due to a physiological or pathological effect will have a dramatic effect on cellular gene expression. Here, a study was done to see the effect of TPA induced differentiation on H4K16ac levels in HL60 cells. Results obtained from flow cytometric analysis showed expression of macrophage cell surface marker CD11b on TPA differentiated HL60 cells and the western blots revealed a drastic downregulation of H4K16ac in differentiated cells. Immunoblotting and co-immunoprecipitation assay revealed DNA damage dependent enhancement of H4K16 acetylation and its co-localization with γH2AX in undifferentiated cells. Whereas, TPA differentiated cells (CD11B+ve) didn't show any such enhancement in H4K16acetylation levels in the presence of DNA damage. The present study shows that TPA induced differentiation of HL60 cells into macrophages leads to the downregulation of H4K16 acetylation.

Keywords: 12-O-Tetradecanoylphorbol-13-Acetate (TPA); Differentiation, DNA damage; Acetylation; Downregulation; Gene expression

INTRODUCTION

12-O-Tetradecanoylphorbol-13-Acetate (TPA) induces monocyte/macrophage like terminal differentiation in promyelocytic leukemia cell line HL60 and causes an irreversible growth arrest of cells in G1 phase of cell cycle. HL60 cells are derived from a 36 year old female patient with acute myeloid leukemia and they can differentiate into granulocytes in the presence of DMSO and into monocytes in the presence of TPA [1-3]. TPA differentiated cells show phenotypic and genotypic alterations which are comparatively different from undifferentiated cells. Adhesiveness with fibroblastoid morphology, expression of specific cell surface antigens (CD11c, CD9, CD11b, CD54, etc.), increase in Lysozyme activity, decrease in myeloperoxidase enzyme levels, presence of nonspecific esterase activity, rapid reduction of proteins involved in cell cycle progression and DNA replication, changes in miRNA expression profile, and differentiation specific gene expression with relatively altered mRNA abundance, are some of the unique features observed in differentiated cells [4-10]. Post translational histone modifications, specifically acetylation, play an important role in the regulation of gene expression [11-14]. Histone H4 N-terminal Lysine residues K5, K8, K12 & K16 are potential sites for acetylation and among them, acetylation of Lys 16 has a strong impact on chromatin relaxation and favors gene expression [15-18]. Acetyl transferase, hMOF (Males absent On the First), is mainly responsible for the acetylation of H4K16 and siRNA mediated silencing of this enzyme causes considerable decrease in H4K16 acetylation [19-21]. So far, a huge amount of work was done to understand the role of H4K16 acetylation in nuclear activities like replication, transcription and DNA repair in undifferentiated cells [22-24]. But, the status and the functional importance of K16 acetylation in terminally differentiated cells are not known. In the present work, I tried to analyze the effect of TPA induced differentiation on Histone H4 Lys 16 acetylation in HL60 cells.

MATERIALS AND METHODS

Cell culture and Treatment with TPA

HL 60 cells were grown in RPMI-1640 medium supplemented

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with 10% FCS at 37^{0}C with 5% CO_2. Approximately $5×10^5$ cells were treated or untreated with TPA (10 ng/ml) for 72 h. (A distinct macrophage like morphology was observed after 72 h, hence this time point was used for the induction of differentiation).

DNA damage induction

HL60 cells were grown as mentioned above and approximately $5x10^5$ cells were either treated with etoposide or left untreated. Cells treated with etoposide (final conc. 10 μM) were incubated for about 3 h at 37^{0}C with 5% CO_2. After 3 h, cells were washed thrice with PBS and then removed from the culture dish by scrapping.

Preparation of whole cell extracts and western blotting

Both treated and untreated cells were lysed in RIPA buffer containing protease inhibitor mix and centrifuged at 14000 rpm for 10 min at 4^{0}C. Protein concentration was estimated by using BCA protein assay kit (Pierce, Product #:23225) following manufacturer instructions. Cell extracts were prepared in RIPA buffer containing protease inhibitor mix at 4oC. Equal concentration of protein from treated and untreated samples were loaded on to 12% PAGE and western blotting was performed with specific antibodies, anti-acetyl H4K16 (ab109463, abcam), anti-acetyl H4K5 (ab51007, abcam) anti-acetylH4K8 (ab15823, abcam) and anti-γH2AX antibodies (ab11174, abcam). β Actin was used as a loading control.

Flow cytometric analysis for CD11b marker

Cells were pelleted down at 1400 rpm for 5 min after TPA treatment and washed thrice with PBS. 20 μl of phycoerythrin (PE) labeled CD11b antibody (1:100 dilution in PBS) was added to the cells and incubated for 30 min at 4^{0}C. Cells were washed with PBS and spun down at 1400 rpm for 5 min. Cells were resuspended in 500 μl of fresh PBS, vortexed and analyzed by Flow cytometer (BD Biosciences) for CD11b cell surface antigen.

Co-immuno precipitation

Co-immuno precipitation was done with immunoprecipitation kit (Dyna beads-protein G, Invitrogen cat#:100. 07D) and the protocol was followed according to manufacturer's instructions. Briefly, 50 μl of Protein-G coated beads were transferred into a microfuge tube and supernatant was removed by placing on a magnet. Beads were resuspended in 200 μl of antibody binding buffer containing either anti acetyl H4K16 antibody or anti γ H2AX antibody. Mixture was incubated for 10 min with rotation at room temperature. The supernatant was removed by placing tube on a magnet and beads were washed by resuspending them in 200 μl of antibody washing buffer. Etoposide treated and untreated cell extracts were added to the antibody bound beads and incubated for 10 min at room temperature with rotation. Supernatant was removed and beads containing Antigen-Antibody complex were washed with 200 μl of washing buffer. After thorough washing, buffer was removed and beads were resuspended in 20 μl elution buffer and then in 10 μl of SDS PAGE sample buffer. After incubating at 70^{0}C for 10 min, supernatant was separated from beads and loaded on to 12% PAGE.

RESULTS

HL 60 cells treated or untreated with TPA for 72 h were subjected to flow cytometric analysis for observing macrophage cell surface marker CD11b, to confirm the induction of differentiation. In untreated or undifferentiated cells, only 6.49% were found to be CD11b positive. Whereas in treated cells, about 83.76% were CD11b positive, indicating the induction of differentiation (Figures 1A & 1B). Part of the cells from both untreated and treated was used to perform western blots to check the acetylation levels of Histone H4 Lys (K) 5, 8 and 16. A significant decrease in the acetylation levels of all these three lysine residues was observed after TPA induced differentiation (Figure 1C). Histone H4 N-terminal Lysine residues K5 and K8 were also found to be involved in transcriptional activation in yeast. Since Histone H4 Lys 16 acetylation has a predominant role in the chromatin organization and gene expression, further experiments were done to understand and validate its status during TPA induced differentiation. In our previous study [25], it was identified that Histone H4K16 is hyper acetylated in response to DNA damage induced by genotoxic agents like methyl methane sulfonate, camptothecin and hydroxyurea in proliferating cells. Consistent with the previous result, when HL60 cells were treated with etoposide, a topoisomerase I inhibitor and DNA damage inducer [26], hyperacetylation of Histone H4K16 was observed, after 3hr of etoposide treatment (Figure 2A). γH2AX, a known biomarker for DNA damage confirmed the induction of damage by etoposide. Further, co-immunoprecipitation with anti H4K16ac and anti γ. H2AX antibodies showed coexistence of both H4K16 acetylation and γ H2AX modifications at the site of DNA damage (Figure 2B). After observing the DNA damage induced enhancement of H4K16 acetylation in undifferentiated HL60 cells, experiments were done to see the effect of DNA damage in TPA differentiated cells.

For this, four different conditions were considered: 1) Cells neither treated with TPA nor with etoposide, 2) Cells treated with etoposide, 3) Cells treated with TPA, 4) Cells treated with TPA and Etoposide. Flow cytometric analysis showed that, in conditions where there is no TPA (1 & 2) only small number of cells were CD11b positive (Figures 3 (i), a & b). In Conditions where cells were treated with TPA (3 & 4), majority of cells were found to be CD11B positive (Figures 3 (i), c & d), confirming they were

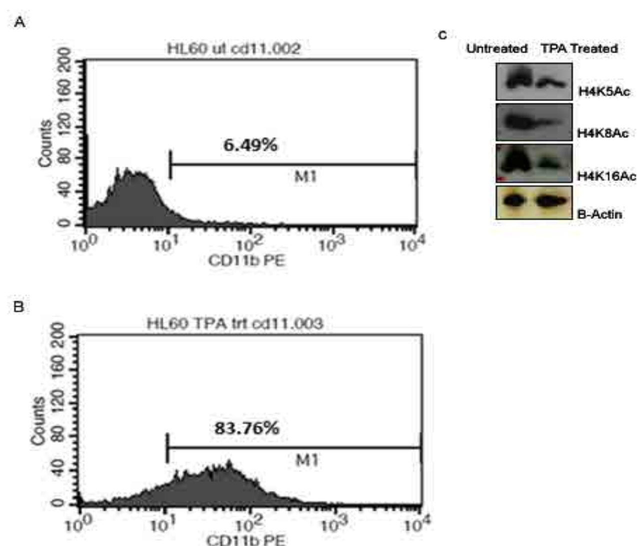

Figure 1: TPA induced differentiation leads to loss of Histone H4-K5,K8 & K16 acetylation in HL60 cells: HL60 cells treated or untreated with TPA were subjected to flow cytometric analysis for macrophage cell surface marker CD11b. (A) Only 6.49% were CD11b positive in untreated cells, (B) Majority of the cells, about 83.76% were CD11b positive in TPA treated cells, (C) Whole cell extracts of untreated and TPA treated HL60 cells were probed with anti-acetyl H4K5, anti-acetyl H4K8, anti-acetyl H4K16, and anti β actin (loading control) antibodies.

differentiated cells. Western blots done with cell extracts of the above four different conditions gave a surprising result. As expected, cells treated with only etoposide alone showed a significant increase in H4K16 acetylation, when compared to cells that are not treated with etoposide, and treated with only TPA but not with etoposide (compare lanes 2 with 1 & 3 of Figure 3 (ii)). Interestingly, cells treated with TPA and then with etoposide didn't show any increase in H4K16 acetylation in response to etoposide induced DNA damage (compare lanes 2 with 4 of Figure 3 (ii) & (iii)). This result was counter intuitive since etoposide induced DNA damage was expected to increase H4K16ac levels as it does in undifferentiated HL60 cells. Unexpectedly, γ H2AX, used as a biomarker for DNA damage, also showed a trend similar to H4K16acetylation (Figure 3 (ii)). Down regulation of γ H2AX may impair DNA

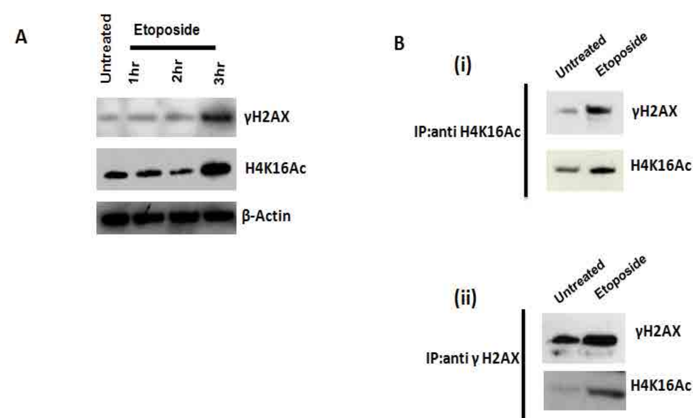

Figure2: DNA damage induced by etoposide enhances H4K16 Acetylation levels. (A) Cell extracts of HL60 cells treated/untreated with etoposide for 1 to 3 h were probed with γH2AX and anti-acetyl H4K16 antibodies. Treated HL60 cells showed DNA damage dependent elevation of H4K16 acetylation levels. (B) Coimmunoprecipitation with antibodies against acetyl H4K16 and γH2AX showed increased levels of H2AX ser 139 phosphorylation (γH2AX) and H4K16 acetylation levels in etoposide treated cells compared to untreated cells.

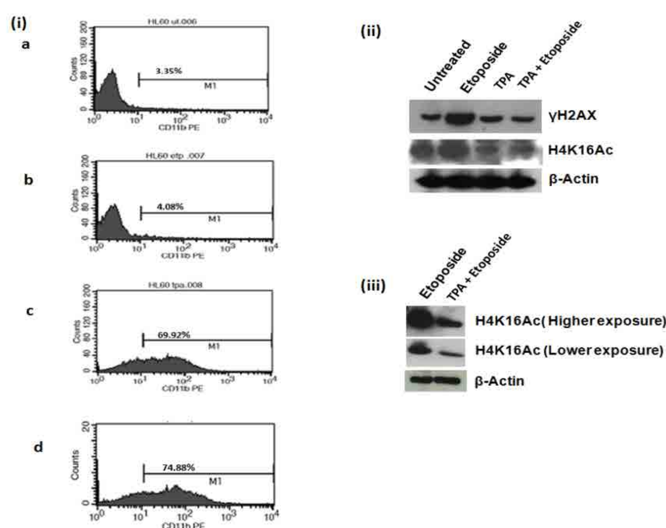

Figure 3: DNA damage induced by etoposide was not able to enhance H4K16ac levels in TPA differentiated cells (i) FACS analysis was done with HL60 cells for CD11b surface antigen after treating with TPA and Etoposide. (a) Untreated Cells, (b) Etoposide treated cells, (c) TPA treated cells, (d) TPA and Etoposide treated cells (ii) Part of the cells from each condition are subjected to western blot with anti γ H2AX and anti-acetyl H4K16 antibodies (iii) HL 60 cells incubated with TPA for one week and then treated with Etoposide for 3 h to observe the effect of genotoxin.

repair pathways and hence, it will be interesting to know how TPA differentiated cells cope with DNA damage. In the present study, the obtained data indicate that TPA induced macrophage like differentiation in HL60 cells leads to loss of Histone H4 Lys 16 acetylation.

DISCUSSION

The HL60 cell line is an established model for studying various cellular and biochemical pathways involved in drug-induced differentiation. TPA induces Macrophage/monocyte like differentiation in HL60 cells and the differentiated cells have specific gene expression pattern [27]. Histone acetylation is predominantly involved in the regulation of gene expression. Epigenetic Histone modification, Histone H4 Lys16 acetylation, has been shown to have a precise role in chromatin organization and transcriptional activation. In the present study, an attempt was made to understand the status of Histone H4 Lys 16 acetylation in TPA differentiated cells. HL60 cells treated with TPA showed a significant decrease in H4K16 acetylation when compared to undifferentiated cells. Along with K16, other N-terminal Lysine residues K5 and K8 also showed hypo acetylation after differentiation (Figure 1). Acetylation of N-terminal lysine residues in Histone H4 was shown to be involved in transcription initiation in both lower and higher organisms. Hence, loss of acetylation on these residues implicates their effect on gene expression. H4K16 acetylation was found to be specifically involved in the relaxation of higher-order chromatin structure [16,28]. A specific reduction in the acetylation of this lysine residue causes chromatin condensation and makes DNA inaccessible for transcription. Etoposide is a genotoxic agent and induces DNA strand breaks causing the activation of DNA damage response. Previous studies have shown that chemical genotoxins and ionizing radiation elevates H4K16 acetylation in response to DNA damage [25,29]. Consistent with the previous reports, etoposide induced DNA damage showed a discernable enhancement of H4K16 acetylation in undifferentiated HL60 cells and found to coexist with γH2AX, a known biomarker for DNA damage (Figure 2). But upon TPA induced differentiation, etoposide mediated DNA damage was not able to show any effect on H4K16 acetylation (Figure 3). This observation gives scope for a thought that H4K16 acetylation might be irreversibly reduced or maintained in hypo acetylated phase in TPA differentiated cells. It has been reported previously that loss of histone acetylation leads to chromatin condensation [30]. Since H4K16 acetylation is highly essential for chromatin relaxation, hypoacetylation of this residue may also cause compaction of chromatin, restricting the access for transcriptional machinery. Previous work has shown that H4K16acetylation is regulated in a biphasic manner by Histone deacetylases, SIRT 1 and SIRT2. SIRT 1 is mainly involved in gene locus specific deacetylation of H4K16 and SIRT2 deacetylates H4K16 at global level [31]. It is yet to be determined whether the loss of H4K16 acetylation in TPA differentiated cells is locus specific or global reduction.

CONCLUSION

The present study shows TPA induced differentiation of HL60 cells leads to the downregulation of histone H4 Lysine 16 acetylation. H4K16 is hyperacetylated in response to DNA damage in proliferating and undifferentiated HL60 cells. Whereas, similar DNA damage dependent hyperacetylation was not observed in differentiated cells indicating a permanent loss of K16 acetylation.

My future work will be able to give more insights on possible regulatory mechanisms involved in the downregulation of H4K16 acetylation in TPA differentiated cells.

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