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Review Article

EPIGENETICS OF PHORBOL ESTERS, A TUMOR PROMOTER AND DIFFERENTIATION INDUCER

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Epigenetics is the study of structurally and functionally relevant molecular changes to the genome that do not constitute a change in the nucleotide sequence such as DNA methylation and histone modifications. These changes are involved in the expression and repression of different genes in a particular cell type. Phorbol esters are diterpene compound known as phorbol 12-myristate 13-acetate (PMA) or Tetradecanoylphorbol-13-acetate (TPA). They are routinely used to study the mechanism of signal transduction in tumor promotion and differentiation induction of different cell types. Recent work on the PMA has linked its function to the epigenetic changes in different cells. This review summarizes the epigenetic changes mediated by PMA and its role in the regulation of gene expression.

Keywords: Phorbol esters, PMA, DNA methylation, Histone modifications, Acetylation, Methylation

INTRODUCTION

The development of tumor involves three steps namely initiation, promotion and progression. In the initiation stage, a physical, chemical or a biological mutagen (e.g., radiation, alkylating agents and virus, respectively) causes a mutation in the cells which renders the normal cells the ability to form tumors. During the tumor promotion stage, the cell with requisite mutation further develops through the activation of oncogenes and downregulation of tumor suppressor genes. This stage is also characterized by the upregulation cell survival mechanisms (also known as transformation) and anchorage independence. The tumor progression stage is characterized by increased growth speed and invasiveness of the tumor cells. This final step causes several genetic lesions, aneuploidy and phenotypic changes (Hanahan and Weinberg, 2000 and 2011). 12-O-Tetradecanoylphorbol-13-acetate (TPA), also commonly known as tetradecanoylphorbol acetate, tetradecanoyl phorbol acetate, and phorbol 12-myristate 13-acetate (PMA), is a diester of phorbol (a diterpene compound) and is a potent tumor promoter. It has been shown that after a tumor initiation with carcinogen such as 7, 12-dimethylbenz[a]anthracene (DMBA) (with a single application of 0.2 μmol), a dose-dependent increase in the number of skin papillomas can be seen through the range of 1 to

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10 nmol of phorbol esters applied over few weeks (Verma and Boutwell, 1980). For this reason, PMA is routinely used as a biomedical research tool in the models of carcinogenesis. Because of its similarity to one of the natural activators of classic Protein Kinase C (PKC) isoforms, diacylglycerol, it is known to activate different isoforms of PKCs in different types of cells. Epigenetics is the field of study of structurally and functionally relevant molecular changes to the genome that do not involve a change in the nucleotide sequence such as DNA methylation and histone modifications (Bird, 2007). Recent work in this field has linked the actions of PMA to several epigenetic changes. This mini-review will focus the attention on the epigenetic mechanisms through which the PMA is able to exert different functions in different cells such as tumor promotion, induction of lytic phase in viral infections and dif ferentiation of multipotential precursor cells.

DNA METHYLATION AND PMA

DNA methylation is a biochemical process in which a methyl group is added to the cytosine or adenine DNA nucleotides. Such modifications are known to alter the expression of genes in cells when they divide and differentiate from embryonic stem cells or progenitor cells into specific cell types. Generally, DNA methylations are associated with gene repression while DNA demethylations are involved in the gene expression. DNA methylation typically occurs in a CpG dinucleotide stretch of the DNA but non-CpG methylation is prevalent in embryonic stem cells. DNA methylation also plays a crucial role in the development of nearly all types of cancer (Jaenisch and Bird, 2003). Given the importance of DNA methylation in chromatin remodeling, the literature was reviewed to ascertain the role of PMA to effect and regulate this process in different cell types. It has shown that in the human melanoma cell line M21, there is no substantial changes in the methylation of DNA cytosines after treatment with the PMA whereas 5-aza-2'-deoxycytidine-induced differentiation of these cells results in a 40% DNA demethylation (Steigerwald and Pfeifer, 1988 and Pfeifer *et al*., 1989). However, there is some evidence to suggest that PMA increases the level of both Sadenosylmethionine and S-adenosylhomocysteine that are the substrate and byproduct of DNA methylation event in the myeloid leukemia HL-60 cells (Borchardt, 1986). Further, the stimulation of murine CD4+ T lymphocytes with PMA and ionophore leads to reduction in the DNA methylation status (Li *et al.*, 2012). The Lyta promoter (lytic transactivator) of Kaposi's sarcoma-associated herpesvirus (strongly linked to Kaposi's sarcoma) is highly methylated during the latent phase but gets demethylated upon induction with PMA (Chen *et al*., 2001). Further, the administration of PMA appears to induce the Protein Kinase C (PKC) which decreases DNA methylation specifically at the promoter of reelin, a gene implicated in the synaptic plasticity. The activation of PKC is also accompanied by increased c-fos, an immediate early gene involved in the expression of DNA methyltransferase DNMT3a suggesting the involvement of DNA methylation in synaptic signaling cascades (Appasani, 2012). These results are taken together to suggest that DNA methylation is not a static process that occurs during DNA replication but a dynamic process involved in the regulation of chromatin remodelling and gene expression. Future work will improve our understanding of the role of PMA in the regulation of DNA methylation and demethylation of a number of genes in different cell systems.

HISTONE ACETYLATION AND PMA

Eukaryotic DNA is condensed many fold (upto 10,000) into chromatin, the basic unit of which is nucleosome that contains an octamer of core histone proteins (H2A, H2B, H3, and H4) around which 146 bp DNA is wrapped and linker histone H1 is further involved in the compaction of nucleosomes. The post-translational modifications of histones (e.g., acetylation, phosphorylation and methylation) and its variants modulate the chromatin structure and function both locally and globally to exert the changes in gene expression and repression in the cells depending on endogenous and environmental signaling events (Strahl and Allis, 2000).

Histone Acetyl Transferase (HATs) are a group of enzymes involved in the acetylation of the conserved lysine amino acids of histones by transferring an acetyl group from acetyl CoA to form ε-acetyllysine. The genes can be turned on and off by positioning the nucleosomes and linker histones on the coding and noncoding regulatory DNA sequences depending on the acetylation status of the histones (Struhl, 1998). Further, the acetylation and other posttranslational modifications of histones also provide binding sites for specific DNA-protein and protein-protein interaction domains which regulate the chromatin structure and transcription process. In general, histone acetylation increases gene expression by neutralizing the positive charges of lysine residues and thereby reducing the affinity of histones to the DNA.

It has been shown that phorbol ester treatment strongly induces expression of histone acetylases in human monocytic U937 cells and this induction mediates the interferon responsiveness of these cells (Masumi *et al.*, 1999). The induction of p300/CBP-Associated Factor (PCAF) and global transcriptional activators CBP (CREB binding protein)/p300 was detectable on day 1 and their levels reached a maximum on day 3. Further, the expression of the smaller size of hGCN5 was also increased after TPA treatment. However, during this period, there was no detectable changes in the level of histone deacetylases expressed in these cells suggesting that histone acetyltransferases induced by PMA plays an important role in the interferon responsiveness of these cells.

Interestingly, the interaction between sodium butyrate, a histone deacetylase inhibitor and PMA results in apoptosis in U937 cells and HL-60 cells, another myeloid leukemia cells. The apoptosis was associated with early procaspase 8 activation and Bid cleavage, accompanied by pronounced mitochondrial damage such as loss of mitochondrial membrane potential and cytochrome c release. Rahmani *et al.* (2002) also show that butyrate (1 mM) with low concentrations of PMA (1 nM) induce apoptosis in human myeloid leukemia cells and that this process proceeds through a PKC-/TNFalpha-dependent pathway. The concentration of PMA used to induce the HATs in U937 cells is 10 nM (Masumi *et al*., 1999) and it is 10 times higher than what is used for the induction of apoptosis along with butyrate. PMA at the concentration of 150 nM activates protein kinase C and markedly reduces the hyperphosphorylation of H2A.X which is involved in the chromatin fragmentation and nearly abolishes apoptosis of NIH 3T3 cells mediated by TNF-α (Talasz *et al*., 2002). These results may suggest that PMA functions differently in different cell types depending on the low, medium or high concentration. Further, it appears that low

concentration of PMA primes the myeloid leukemia cells towards a particular lineage whereas the butyrate by relaxing the chromatin structure via hyperacetylation make the U937 cells vulnerable to impaired differentiation and the eventual apoptosis. More work will be needed to exactly pinpoint the epigenetic mechanism that is involved in the apoptosis of U937 cells.

PMA has been shown to be involved in the expression of urokinase plasminogen activator (uPA) and its receptor (uPAR) in U937 cells which play an important role in the invasive nature of the tumor cells (Picone *et al*., 1989). It is also known that incorporation of variant histone H2A.Z within chromatin is essential for proper gene expression and genome stability. Further, H2A.Z can make the nucleosomes to adopt stable translational positions compared to H2A, which could influence the accessibility to DNA regulatory proteins (Marques *et al.,* 2010). Recent experiments with chromatin immunoprecipitation (ChiP) assays revealed that H2A.Z was indeed enriched at the promoter and downstream enhancer regions of *uPAR* and they dissociate upon activation of gene expression by phorbol ester. Further, it has been shown that expression of MMP9 gene and miR-21 microRNA which are implicated in tumor promotion are also regulated by H2A.Z variant (Chauhan and Boyd, 2012). More work in the future on PMA and its effect on epigenetic mechanisms may shed further light on tumor promotion.

Mishra *et al.* (2001) have found that when breast cancer cells expressing a low level of an oncogene HER2 were compared with HER2-overexpressing breast cancer cells, a significantly higher levels of acetylated and phosphorylated histone H3, and acetylated histone H4 are associated with the HER2 promoter. Further, PMA and okadaic acid stimulated the association of phosphorylated histone H3 on serine 10 with the HER2 promoter and also stimulated HER2 expression. However, the mechanism is not clear on how this elevated levels of the chromatin-relaxing components are embedded within the vicinity of the HER2 gene promoter.

It has been shown that when differentiation of HL-60 cells are induced by exposure to dimethylsulfoxide or phorbol esters, they did not show any alteration at the level of H4 acetylation within either the c-myc or c-fos genes or other coding regions, but did induce a transient increase in H4 acetylation within centric heterochromatin (O'Neill and Turner, 1995). The role of PMA in different modifications of histone H4 in normal cellular and carcinogenic process remains open.

The chromatin of the platelet derived growth factor B chain (PDGF-B), a gene which is induced to strong expression upon differentiation in the proliferating human K562 erythroleukemic cells by PMA is highly acetylated. This high level of acetylation was observed before gene induction and no change seen following induction suggesting that core histone acetylation is an essential precondition for transcription (Clayton *et al.*, 1993). Further, it has been shown that overexpression of histone deacetylase 1 (HDAC1) suppressed Kruppel-like factor 5 (KLF5) dependent activation of its endogenous downstream gene platelet-derived growth factor-A (PDGF-A) chain gene activated by phorbol ester. HDAC1 directly acts through its DNA binding activity and promoter activation and indirectly through the inhibition of transcriptional coactivator p300 activity suggesting that PMA also acts via HDACs (Matsumura *et al.,* 2005).

PMA as a PKC activator alone induces the transcriptional activation of p21(WAF1/Cip1) promoter, p21(WAF1/Cip1) mRNA, and protein expression without induction of the histone hyperacetylation in HeLa cells in contrast to the transcriptional activation of p21(WAF1/Cip1) by apicidin, a histone deacetylase inhibitor. Further, the PKC-mediated transcriptional activation of p21(WAF1/Cip1) by apicidin appears to be independent of the histone hyperacetylation, because apicidin-induced histone hyperacetylation was not affected by calphostin C, a PKC inhibitor. This suggests that p21(WAF1/Cip1) activation by PMA is likely to be mediated by the PKC activation and not by chromatin remodeling through the histone hyperacetylation (Han *et al.*, 2001). The above results from different cell systems suggest that the mechanism by which PMA makes use of the histone acetylation process is more complex than simple genes are on and off depending on histone acetylation status. Both genome-wide and local gene-specific studies will be needed to fully define the exact mechanisms of gene activation by PMA.

HISTONE METHYLATION AND PMA

The methylation of histones at the lysine or arginine residues are known to play an important role in the regulation of chromatin structure and function. The methylation of lysine 4 of histone H3 (H3K4) is associated with active genes and it can be mono, di or trimethylated. The di and trimethylated H3K4 is able to interact with other chromatin remodeling factors such as ISWI with high affinity but the monomethylated H3K4 is able to inhibit the binding of heterochromatic silencing factors (Lefevre *et al.*, 2005). In the undifferentiated monoblasts, the chicken lysozyme regulatory regions are marked by the presence of monomethylated histone H3 lysine 4, which becomes increasingly converted into trimethylated H3 K4 during cell differentiation. This process is shown to require stimulation with both PMA and lipopolysaccharides (LPS). Interestingly, in the lysozyme non-expressing precursor cells, linker histone H1 is already depleted at the promoter and is further depleted in a locus-wide fashion in response to PMA, but not LPS treatment (Lefevre *et al.*, 2005).

Recently, it has been shown that protein arginine (R) methyltransferase (PRMT1) methylates R3 of histone H4 whereas another cofactor associated arginine (R) methyltransferase 1 (CARM1) methylates R17 and R26 of histone H3. By using chromatin immunoprecipitation (ChiP) analysis with an antibody that is specific for methylated R17 of H3, it has been found that R17 methylation on histone H3 is dramatically upregulated when the estrogen receptor-regulated pS2 gene is activated. Further, when human breast cancer cell line MCF-7 are treated with both estradiol and PMA, they synergistically activate the pS2 gene (via R17 methylation of H3) much more than when estradiol and PMA are used for induction independently. Interestingly, an increase in acetylation of histone H3 at the pS2 promoter has also been found when both estradiol and PMA are used for the induction suggesting a cross-talk between histone acetylation and methylation (Bauer *et al.*, 2002).

HISTONE PHOSPHORYLATION AND PMA

The phosphorylation of proteins is an ubiquitous post-translational modification present in all

organisms and there is evidence to suggest that functions of different histones are also regulated through phosphorylation. The treatment of quiescent H35 rat hepatoma cells (arrested by serum starvation) with PMA resulted in maximal phosphorylation of histones H2B (14-fold) and linker H1° (11-fold) and smaller amounts of phosphorylation in H4 (2-fold). Further, insulin which is mitogenic for these cells did not cause a similar pattern of histone phosphorylation, suggesting that the effect observed was not due to a general mitogenic response in the H35 hepatoma cells (Butler *et al.*, 1986).

In the human hepatoma HepG2 cells, the histone H3 is constitutively acetylated at LDL receptor chromatin domain. The PMA treatment causes rapid hyperphosphorylation of histone H3 on serine 10 (H3-Ser10), despite global reduction in its phosphorylation levels (Huang *et al*., 2004). Further, the inhibition of protein kinase C (PKC) blocks Ser10 hyperphosphorylation and also compromises LDL receptor induction by PMA suggesting that PKC is likely involved in the H3 ser10 phosphorylation. Interestingly, the PMA mediated increase in Ser10 phosphorylation reached a peak at 1.5 h and returned to the basal level at 3 h, whereas maximal induction of the LDL receptor was observed at 4 h after stimulation, suggesting that phosphorylation is required to initiate but not to maintain stimulated transcription (Huang *et al.,* 2004).

The phosphorylation of H3T6 by protein-kinase-C beta (PKCβ) and protein-kinase-C related kinase 1 (PRK1) mediated phosphorylation of H3T11 are shown to be involved in the androgen receptor dependent gene activation. Further, apoptotic events in neutrophils were shown to involve the H3T45 phosphorylation catalyzed by protein-kinase-C delta (PKCδ) suggesting the role of histone H3 phosphorylation in programmed cell death (Sawicka and Seiser, 2012). Since PMA is known to be involved in the activation of PKCβ (Amemiya *et al*., 2005) and PKCδ (Maloney *et al*., 1998), it will be interesting to study whether PMA also causes these novel H3 phosphorylation to influence gene activation and apoptosis. Further, in MCF-7 cells, the level of transglutaminase-2 which is shown to possess histone phosphorylation activity is enhanced by PMA but reduced by 17beta-estradiol suggesting PMA may act not only through the PKC isoforms but also via other kinases (Mishra *et al.*, 2006).

Chromatin condensation and the ensuing DNA fragmentation are known to be involved in the apoptotic process. While linker histone H1 dephosphorylation is accompanied by chromatin condensation, the H2A.X hyperphosphorylation is strongly correlated to apoptotic chromatin fragmentation. PMA has been shown to inhibit nearly completely induction of apoptosis and markedly reduced hyperphosphorylation of H2A.X but not dephosphorylation of H1 linker histones in TNF-alpha induced apoptosis in NIH 3T3 cells (Talasz *et al.*, 2002). However, PMA has no effect on H2A.X hyperphosphorylation in the Fas-induced apoptosis.

When quiescent mouse fibroblast cells are stimulated with growth factors and phorbol esters, the early-response genes such as *c-fos* and *c-jun* are rapidly induced. The histone H3 is also rapidly phosphorylated on serine residues within its highly charged basic N-terminal domain during early gene induction suggesting an important role for the chromatin conformation changes in the expression of proto-oncogenes (Mahadevan *et al.*, 1991). Subsequent work using an antibody specific for phosphorylated Ser-10

of histone H3, this modification of H3 has been mapped to Ser10 residue. This H3 phosphorylation may contribute to protooncogene induction by releasing blocks in transcriptional elongation due to the destabilization of the higher order compaction of chromatin (Chadee *et al*., 1999). The tumor promoter okadaic acid activates JNK/SAPKs but not ERKs, whereas PMA activates ERKs but not JNK/SAPKs but both of them lead to increase in H3 phosphorylation in C3H10T1/*2* multipotential mesenchymal cells (Cano *et al.*, 1995). The H3 Ser10 phosphorylation is in close proximity to other modifiable amino acids of the histone H3 tail such as lysine 9 and 14. This enables the possibility of an interaction between phosphorylation of serine 10 and methylation and/ or acetylation of lysine 9 and lysine 14 (Nowak and Corces, 2004). PMA (400 ng/ml for 20 min) treatment of wild type mouse embryonic fibroblasts has been shown to induce H3 ser28 phosphorylation (Soloaga *et al.*, 2003). The H3 Ser28 modification was first identified as a site of phosphorylation on condensed chromatin and later as an inducible event upon stimulation with UV-B irradiation of JB6 mouse epidermal cells. Further, in the mitogen and stress activated protein kinase MSK1/2 double-knockout cells, PMA-stimulated phosphorylation of Ser10 on histone H3 was absent. The phosphorylation of Ser28 was also substantially reduced in the double-knockout cells suggesting the role of these kinases in the phosphorylation of histone H3 in response to PMA (Soloaga *et al.*, 2003). The recent finding that the histone H3.3 variant that has a conserved N-terminal domain can replace histone H3 at the sites of active transcription adds further complexity and possibilities to the regulation of transcription (Nowak and Corces, 2004; Sawicka and Seiser, 2012).

POLY (ADP-RIBOSYLATION) OF HISTONES AND PMA

The levels of H3K9 acetylation are high in the *FOS* promoter region relative to a neighboring intergenic region in quiescent HeLa cells and are further enhanced in response to PMA stimulation. However, this increase in acetylation levels is blocked on depletion of poly (ADP-ribose) polymerase-1 (PARP1). Based on genome-wide studies, it has been found that histone H2A.Z generally associates with nucleosomes located in and around promoter regions and at many genes, it has a role in transcriptional activation (Marques *et al*., 2010). However, in case of *FOS* promoter, the H2A.Z inhibits its promoter activity. A rapid decrease in H2A.Z enrichment at the *FOS* promoter following ERK pathway activation with a concomitant increase in H2A levels have been shown to be initiated within 5 min of PMA stimulation. This decrease in H2A.Z is mediated by PARP-1 which is required for histone variant exchange eventually leading to the transcriptional activation of the *FOS* promoter (O'Donnell *et al*., 2013). Further, there is evidence to suggest that histones H2A, H2B and H3D are likely poly ADPribosylated upon PMA stimulation in mouse embryo fibroblasts C3H10T1/2 (Singh and Cerutti, 1985).

Poly (ADP-ribosylated) histones appear to be intermediates in nuclear processes that involve DNA strand breaks. In human lymphoid cells, PMA and phytohemagglutinin (PHA) or a combination of both are known to increase the ADP-ribose units on histones from 15 upto 32 residues under *in vitro* conditions in the presence of divalent metal ions (Boulikas*et al*., 1990). Further, PMA is known to cause branching of the poly(ADP-ribose) units on histone H2B.

In addition to the above histone modifications, it has been found that a truncated 17 kDa ubiquitinated H2A(54-129) is transiently increased with the concomitant decrease in 23 kDa ubiquitinated H2A(1-129) in THP-1 cells during PMA induced differentiation suggesting a possible role of PMA in proteasomal mediated H2A degradation (Okawa *et al.*, 2003; Minami *et al.,* 2006).

CONCLUSION

PMA is not only a potent tumor promoter but also a potent differentiation inducer and it has myriad effects on different cell systems which includes proliferation, differentiation and maturation. The literature is replete with its effect on differential gene activation and repression but its role on epigenetic mechanism through which it can control these functions is very limited. Further, lots of work have been carried out with *in vitro* cell culture systems making it difficult to draw strong conclusions from them to fully understand the *in vivo* mechanisms. Given the fact that epigenetic mechanisms play an important role in tumorogenesis and differentiation, it is hoped that this mini-review will spur more research on the role of phorbol esters on epigenetic mechanisms.

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