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Histone deacetylase inhibitor Trichostatin A induces global and gene-specific DNA demethylation in human cancer cell lines

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ABSTRACT

DNA methylation and chromatin structure are two modes of epigenetic control of genome function. Although it is now well established that chromatin silencing could lead to DNA methylation, the relation between chromatin activation and DNA demethylation is unclear. It was generally believed that expression of methylated genes could only be restored by demethylating agents, such as 5-aza-deoxycytidine (5-azaCdR), and that inhibition of histone deacetylation by Trichostatin A (TSA) only activates transcription of unmethylated genes. In this report, we show that increase of histone acetylation by TSA was associated with a significant decrease in global methylation. This global demethylation occurs even when DNA replication is blocked by hydroxyurea, supporting a replication-independent-mechanism of demethylation. TSA also induces histone acetylation, demethylation and expression of the methylated *E-CADHERIN* and *RARβ2* genes. However, the genome-wide demethylation induced by TSA does not affect all methylated tumor suppressor genes equally suggesting that induction of acetylation and demethylation by TSA shows some gene selectivity. Taken together, our data provide evidence for a reversible crosstalk between histone acetylation and DNA demethylation, which has significant implications on the use of HDAC inhibitors as therapeutic agents.

1. Introduction

The epigenome is comprised of modifiable chromatin and DNA methylation, which are two major regulatory elements that interact to direct gene expression and other biological processes in somatic cells [1]. The link between DNA methylation and chromatin structure was initially believed to be unidirectional, whereby DNA methylation leads to chromatin inactivation, which in turn causes gene repression [2]. In accordance with this hypothesis, cytosine methylation was shown to target methyl-CpG binding proteins (MBD) to methylated genes. These MBD proteins then recruit a complex of transcription repressors including histone deacetylase (HDAC) to suppress genes transcription by inactivating

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Abbreviations: TSA, Trichostatin A; 5-azaCdR, 5-aza-deoxycytidine; HDACi, histone deacetylase inhibitor; HAT, histone acetyltransferase; DNMT, DNA methyltransferase

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chromatin structure [3,4]. However, several studies have raised the possibility that the relation between chromatin and DNA methylation could be bi-directional and, a number of chromatin modifying enzymes were shown to recruit DNA methyltransferases (DNMTs) to specific genes and thus target DNA methylation [5,6].

It was previously described that inhibition of histone deacetylation is not sufficient to reactivate genes silenced by promoter hypermethylation, and that only DNA-demethylating agents, or a combination of HDAC inhibitors and DNA demethylating agents were able to restore the expression of silent genes [2]. For instance, it is commonly accepted that histone deacetylase inhibitors (HDACi), which are used extensively in both research and clinical trials target histone acetylation. Therefore, the possibility that histone acetylation could cause gene demethylation was generally not considered. Nevertheless, earlier data provided some evidence to the contrary. Sodium butyrate, a nonspecific inhibitor of HDAC was shown to induce genomic hypomethylation of Epstein Barr virus DNA in the absence of DNA replication [7]. More recently it was shown that the HDAC inhibitor Trichostatin A (TSA) could cause selective loss of DNA methylation in *Neurospora* [8].

We had previously shown that increasing histone acetylation by treating cells with TSA, brought about demethylation of non-replicating methylated DNA in HEK 293 cells, which suggested that histone acetylation could cause active DNA demethylation [9]. Furthermore, we also demonstrated that demethylation induced by TSA through histone acetylation could be blocked by ectopic expression of a histone acetyltransferase inhibitor protein INHAT [10]. However, the question remained whether these effects were limited to ectopically introduced plasmid, and whether TSA would confer a similar effect on fully chromatinized genomic DNA. The nature of the relationship between chromatin and DNA demethylation is important for understanding the programming of gene expression within the epigenome, as well as for revealing the potential impact of HDACi used in clinic on genomic methylation patterns.

We therefore addressed in this paper the issue of whether HDACi could alter global genomic methylation, as well as methylation of specific tumor suppressor genes. Furthermore, we also tested whether this effect could occur in the absence of DNA replication. Our results support the hypothesis that histone modifications could reverse the state of methylation, which suggest a true bilateral relationship between histone acetylation and DNA demethylation [8–14].

2. Materials and methods

2.1. Cell lines

Human bladder carcinoma, T24 cells were grown in McCoy 5A medium (Gibco), and human breast carcinoma, MDA-MB-231 cells were cultured in RPMI-1640 medium (Gibco), both supplemented with 10% fetal calf serum, L-glutamine, penicillin and streptomycin (Life Technologies). The cells were grown in regular culture medium in the presence of 1 μM of 5-azaCdR (Sigma), 2 mM of hydroxyurea (Sigma), or 50–300 nM of TSA (Sigma).

2.2. Reverse transcription-PCR

Total RNA was extracted using RNAeasy kit (Qiagen). The primers used for amplifying *E-CADHERIN*, sense: 5′-TACACCATCCTCAGCCAAGATCCT-3′; antisense: 5′-GTTCACTGGATTTGTGGTGACGAC-3′. For *P16*, sense: 5′-AGCCTTCGGCTGACTGGCTGG-3′; antisense: 5′-CTGCCCATCATCATGACCTGGA-3′. For *RARβ2*, sense: 5′-AGAGTTTGATG GAGTTGGGTGGAC-3′; antisense: 5′-GACGAGTTCCTCAGAGCTGGTG-3′. For *β-ACTIN*, sense: 5′-GTTGCTAGCCAGGCTGTGCT-3′; antisense: 5′-CGGATGTCCACGTCACACTT-3′, at an annealing temperature of 60 °C. A triplicate PCR was performed for each sample; the intensity of signal obtained for each message was determined by densitometry (NIH Image Software) and normalized to the intensity obtained from *β-ACTIN*.

2.3. 5-Methylcytosine quantification by nearest neighbor analysis

5-Methylcytosine level was quantified by nearest neighbor analysis as described previously [15]. The intensity of 5-methylcytosine and cytosine mononucleotide spots was measured using a phosphoimager and Image Quant image analysis program. Cytosine levels are determined as percentage of [cytosine]/[cytosine + methylcytosine].

2.4. Western blot analysis

Total protein extract was obtained by five freeze and thaw cycles in Tris-lysis buffer: (Tris–HCl 10 mM pH 7.6, $MgCl_2$ 5 mM, NaCl 300 mM, Tween-20 0.05%, glycerol 10% and complete protease inhibitors® (Roche)). The total protein yield was determined using bradford reagent (Biorad). Fifty micrograms of proteins were loaded on a 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were immunoblotted with an anti-*E-CADHERIN* antibody (BD Transduction Laboratories), followed by a secondary anti-mouse IgG antibody (Jackson ImmunoResearch). The membranes were blotted with an anti-β-ACTIN antibody as loading control (Sigma–Aldrich).

2.5. Sodium bisulfite mapping of DNA methylation and combined bisulfite restriction analysis (COBRA) of satellite 2

Sodium bisulfite mapping was performed as described previously with minor modification [16]. Two rounds of PCR were carried out to amplify the 5′ upstream sequence of *E-CADHERIN* and *RARβ2* from sodium bisulfite treated DNA. The outside primers used for *E-CADHERIN* were, sense: 5′-GAATTAGAATTGTGTAGGTTTT-3′ (nucleotide position 799–820); antisense: 5′-CTACAACAACAACAACAAC-3′ (nucleotide position 1175–1193). For *RARβ2*, sense: 5′-GAGAAGTTGGTGTTTAATGTGAGTT-3′ (nucleotide position 544–568); antisense: 5′-CATAAATTATAACAAACAAACCAAC-3′ (nucleotide position 1148–1172). The PCR product was used as a template for subsequent PCR using nested primers, for *E-CADHERIN*, sense: 5′-TTTAGTAATTTTAGGTTAGAGG-3′ (nucleotide position 836–857); antisense: 5′-ACTCCAAAAACCCATAACTAA-3′ (nucleotide position 1140–1160). For *RARβ2*, sense: 5′-GTTATTTGAAGGTTAGTAG-3′ (nucleotide position 763–790); antisense: