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Histone Modification in Acute Leukemia: Arginine Methylation and Deimination

ABSTRACT:

Fusion proteins observed in acute leukemias are created by chromosomal translocations between genes that encode transcriptional regulators such as RUNX1 (AML1) and ETO (1,2). The fusion proteins aberrantly recruit histone modifying activities that alter gene expression patterns and block hematopoietic differentiation. AML1-ETO fusions, for example, recruit histone deacetylase (HDAC) activities to repress genes that are normally activated upon histone acetyltransferase (HAT) recruitment by AML1. Clinical trials are currently in place to test effectiveness of small molecule inhibitors of histone deacetylases in treatment of leukemia and other cancers (3). However, acetylation/deacetylation of histones represents only a small fraction of chromatin regulatory potential that might be altered in leukemia. Histones are also subject to phosphorylation, ubiquitylation, ADP-ribosylation, and methylation of both lysine and arginine residues (4). Histone methylation is particularly interesting because reversal of this modification may require special mechanisms (5). No demethylase enzymes have yet been found. Histone replacement by ATP-driven machineries or proteolysis of histone tails has been proposed to remove methyl-lysines from histones. Deimination of methylated arginine residues to create citrulline might provide a means for methylarginine removal (5,6).

Interestingly, a novel peptidylarginine deiminase (PAD) has been observed in human myeloid leukemia HL60 cells (7). This activity, PAD V, was induced upon differentiation of these cells to granulocytes in the presence of retinoic acid (RA) and DMSO, or differentiation to monocytes by treatment with 1, 25-dihroxyvitaminD3. Induction of PAD V in HL60 cells treated with the calcium ionophore A23187 was linked subsequently to conversion of arginine residues to citrulline in histones H3, H4, and H2A, as well as nucleophosmin/B23 (8). The specific arginine residues in these proteins affected by PAD V were not identified, and the importance of these effects to HL60 differentiation is not known.

HYPOTHESIS:

We hypothesize that histone arginine methylation and arginine conversion to citrulline are linked to changes in gene expression mediated by RAR. If so, then activation or inhibition of PAD enzymes may provide a novel means of augmenting or fine-tuning RA-based therapies for acute leukemias.

SPECIFIC AIMS:

We propose to test our hypothesis through the following Specific Aims:

1. **Define patterns of arginine methylation in histones H3 (R2, 17, 26) and H4 (R3) before and after HL60 cell differentiation.** Antibodies specific for the methyl-arginine isoforms of these histones are commercially available and will be used in immunoblots and in chromatin immunoprecipitation (ChIP) experiments. In particular we are interested in defining methyl-arginine histone isoforms associated with activation or specific genes before and during HL60 differentiation by RA.

2. **Define which arginines in H3, H4 or H2A are converted to citrulline upon HL60 differentiation.** Histones will be isolated from HL60 cells following induction of differentiation and treatment with the calcium ionophore to activate PAD. Histones containing citrulline will be enriched by immunoprecipitation using an available anti-citrulline antibodies and the sites of arginine conversion to citrulline will be determined by mass spect analysis. We will then raise antibodies to peptides corresponding to these sites so that we can monitor the kinetics of arginine deimination more readily in vivo.
3. **Determine whether arginine deimination is associated with arginine demethylation and changes in gene expression upon HL60 differentiation.** ChIP experiments will be done to define the distribution and kinetics of arginine methylation and deimination at specific target genes during RA induction.

BACKGROUND AND SIGNIFICANCE

The importance of chromatin in gene regulation has become increasingly clear over the past several years as histone modifying enzymes and ATP-dependent remodeling complexes have been identified (9). Chromatin is composed of nucleosomal subunits that can be folded into progressively higher order structures. Each nucleosome consists of an octamer of histone proteins and two turns (146 bp) of DNA spooled around the exterior of the histone octamer (10,11). Individual nucleosomes as well as more compacted structures are refractory to transcription. These barriers can be overcome by chromatin remodeling either by an ATP-dependent complex, such as Swi/Snf, or by alterations in the levels, sites, and types of post-translational histone modifications. Each histone contains a number of conserved residues that are subject to modifications including acetylation, methylation, phosphorylation, and ubiquitylation, among others (12) . These covalent modifications alter the charge and/or structure of the histones and thereby trigger changes in the degree of chromatin folding. Particular modifications can also enhance or prevent binding of non-histone regulatory proteins (4).

Several connections between histone modifications and leukemia have also become clear in the last few years (13). Aberrant recruitment of HDACs by oncogenic fusion proteins contributes to blockage of hematopoietic differentiation and formation of leukemia. Both AML1-ETO and PML-RAR_{α} fusion proteins recruit corepressor complexes that interact with HDACs, thereby blocking expression of genes required for myeloid differentiation (2,14). AML1 normally interacts with CBP or p300, two highly related histone acetyltransferases that promote gene activation (15). Loss of CBP in mice causes a block in hematopoiesis, demonstrating the importance of this HAT to normal blood development (16). Loss AML1-CBP interactions, then, likely also contributes to development of human leukemias. Abnormal gene silencing by DNA methylation is observed in a number of cancer types, including leukemias, (17) and is accompanied by histone deacetylation, increased methylation of histone H3 at lysine 9 and decreased methylation of H3 at lysine 4 (18). Such findings illustrate the potential for regulatory interplay between different epigenetic marks that control transcriptional activity.

HDAC inhibitors are already in clinical trials for treatment of leukemia and other cancers (14,19-21). Combination of HDAC inhibitors with decitabine, to inhibit DNA methylation, offers

further promise of therapeutic response (18,22). However, drugs that target other histone modifications are currently lacking. Reversal of improper alterations in histone methylation levels or patterns offers much potential for 'resetting' the histone code in cancer cells. Unlike acetylation, though, histone methylation is not easily reversed. No demethylase enzymes have been found in any organisms, suggesting methylation may be reversed in a less direct manner than acetylation.

One very recent report indicates that histone arginine methylation may be reversed by deimination, which converts arginine to citrulline (6). Although somewhat less studied than lysine methylation, methylation of specific arginine residues in histones is associated with gene activation. In particular, two arginine methylatransferases, CARM1 and PRMT1, act as cofactors for gene regulation by nuclear hormone receptors (23-25). The role of these enzymes in hematopoiesis is not known, but CARM1 acts synergistically with two histone acetyltransferases, CBP and ACTR, in RAR_{α}-mediated activation of gene expression in response to retinoic acid in transient transfection experiments (25). Interestingly, citrulline-containing histones have been detected in HL60 promyeloid leukemia cells following retinoic acid (RA) treatment (8). These results suggest that arginine methylation occurs in histones H3, H2A, and H4 in these cells, and that these methylation events are influenced by RA-induced differentiation. Deimination of histones and other proteins is mediated by protein arginine deiminases (PADs; also called PADIs), which are activated by calcium. Expression of a specific PAD, PAD V, is induced upon stimulation of differentiation of HL60 cells to granulocytes by retinoic acid or dimethyl sulfoxide (7,26), suggesting that deimination of histones or other proteins may be important for differentiation. CARM1 is recruited to promoters in response to estrogen induction, and subsequent recruitment of PAD4 is associated with down regulation of estrogen-induced transcription (6). These findings raise the possibility that PAD recruitment may also modulate retinoic acid responses, perhaps through deimination of methylated-arginines in the histones. Our proposed experiments will test these ideas, setting the stage for novel therapies based on PAD regulation.

PLAN OF ATTACK:

Specific Aim 1: **Define patterns of arginine methylation in histones H3 (at R2, 17, 26) and H4 (R3) before and during HL60 cell differentiation.** Published studies have established that the citulline content of histones H3, H4 and H2A is increased upon PAD activation in granulocytes (8). These findings suggest that specific arginines, and arginine methylation, may be important in HL60 differentiation. As a first step in determining whether PAD activation reflects alterations in arginine methylation patterns important to changes in gene expression patterns, we will perform immunoblots on bulk histones isolated from undifferentiated HL60 cells and at specific time points following RA induction. PAD V expression is induced for two days during RA treatment (6), so we will examine histones on days 0, 1, 2, and 3. We are expert in the analysis of histone modifications, and antibodies specific for H3 or H4 isoforms methylated at specific arginines are commercially available from Abcam. The information gained from these experiments will provide a baseline for comparison to arginine resides in H3 and H4 that are converted to citrulline in response to PAD activation (Specific Aim 2).

Specific Aim 2: **Define which arginines in H3, H4 or H2A are converted to citrulline upon HL60 differentiation.** Although citrulline was detected in histones H3, H4 and H2A in HL60 cells following RA treatment, the locations of the citrulline residues were not defined. Since citrulline is, in essence, a post-translational histone modification, the locations of the citrulline residues may help to constitute a 'histone code' for RA response and granulocyte differentiation. To test this idea, histones will be isolated from HL60 cells after treatment with RA for 0, 1, 2, and 3 days. Just prior to harvest, cells will incubated with the calcium ionophore A23187 to activate PAD V as described by Hagiwara et al (24). Histones will be further purified by size exclusion chromatography and affinity purification using antibodies immobilized on agarose beads. Histones containing citrulline will be enriched by immunoprecipitation using available anti-citrulline antibodies (Upstate Biochemicals) and the sites of arginine conversion to citrulline will be determined by mass spect analysis as described by Cuthbert et al (6). We will then raise antibodies to peptides corresponding to these sites (as we have done previously for specific acetylated histone isoforms) so that we can monitor the kinetics of arginine conversion to citrulline more readily in vivo. Together with the experiments in Specific Aim 1, these experiments will define the relationship between arginine methylation at specific sites and conversion of these residues to citulline by PAD V. These experiments will also relate the timing of these events to RA response and HL60 cell differentiation.

Specific Aim 3: **Determine whether arginine deimination is associated with arginine demethylation and changes in gene expression upon HL60 differentiation.** To determine more directly if arginine methylation and arginine conversion to citrulline are important to mediating or modulating RA response, we will do chromatin immunoprecipitations to monitor these events before and during RA treatment of HL60 cells. We will use commercially available antibodies to the methylated histones or antibodies generated above for specific citrulline modification sites. We will examine the distribution and levels of arginine methylation and citrulline at genes that are induced rapidly in response to RA (e.g. C/EBP, Fgf protein tyrosine kinases, and defensin) (27). These experiments will determine whether deimination of histones at these promoters antagonizes arginine methylation upon RA induction, as seen for estrogen-responsive genes (6). If so, these studies will define an important new aspect of the mechanism by which RAR regulates gene expression.

CLINICAL POTENTIAL: Retinoic acid based therapies enhance differentiation of leukemic cells (28). However, some AML patients are resistant to these therapies. Already HDAC inhibitors show promise in augmenting RA responses (3,19), providing precedence for the development of new therapies based on chromatin modifications. Our studies will determine whether methylation of arginines within histones accompanies RA response, and whether conversion of arginine to citrulline modulates RA response. If so, then RA responses might be fine-tuned through the use of PAD inhibitors (to prolong arginine methylation and RA response) or calcium and calcium ionophores (to dampen these responses). Longer term studies will test this possibility. Interestingly, rheumatoid arthritis is often characterized by citrullination of autoantigens (29). Indeed, autoantibodies that recognize these citrullinated proteins have proven to be powerful in early diagnosis of arthritis and other autoimmune diseases. Such studies raise the possibility that citrullinated histones may also provide new avenues for diagnosis of leukemia.

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