Epigenetic Control during Lymphoid Development and Immune Responses

Aberrant Regulation, Viruses, and Cancer

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ABSTRACT: Methylation of cytosines controls a number of biologic processes such as imprinting and X chromosomal inactivation. DNA hypermethylation is closely associated with transcriptional silencing, while DNA hypomethylation is associated with transcriptional activation. Hypoacetylation of histones leads to compact chromatin with reduced accessibility to the transcriptional machinery. Methyl-CpG binding proteins can recruit corepressors and histone deacetylases; thus, the interplay between these epigenetic mechanisms regulates gene activation. Methylation has been implicated as an important mechanism during immune development, controlling VDJ recombination, lineage-specific expression of cell surface antigens, and transcriptional regulation of cytokine genes during immune responses. Aberrations in epigenetic machinery, either by genetic mutations or by somatic changes such as viral infections, are associated with early alterations in chronic diseases such as immunodeficiency and cancer.

KEYWORDS: methylation; acetylation; lymphopoiesis; immune response; viruses

CONTROL OF VDJ RECOMBINATION BY CHROMATIN ACCESSIBILITY

The diversity of the immunoreceptor repertoire is dependent on unique, lymphoid-specific processes that involve double-strand DNA breaks in order to create novel immunoreceptor genes. VDJ recombination is a site-specific recombination process that allows for cutting and new assembly of gene segments that encode for the T cell receptor (TCR) or immunoglobulin genes (Ig).[1–4] During the initial step, the gene segments are specifically recognized and cleaved by the putative VDJ recombinase, which comprises the recombination activating gene (RAG)-1 and -2 proteins. The

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RAG dimer binds to the recombination signal sequences (RSS), highly conserved DNA motifs flanking the gene segments undergoing rearrangement. After cleavage and specific end modification the ends are joined together via an ubiquitously expressed DNA repair pathway.

A failure to recombine the immune receptor genes leads to immune deficiency. Conversely, aberrant recombination may result in chromosomal translocations and development of leukemia. Thus, the process of VDJ recombination must be tightly regulated. One level of control is imposed by strict regulation of RAG gene expression.[5] However, another mechanism must account for lineage and locus specificity and for the temporal order of recombination. For example, TCR genes are completely assembled only in T cells and Ig genes in B cells using the identical RAG and RSS motifs system. Within a locus there also exists a strict temporal order: for example recombination of the TCR-γ variable 3 region precedes that of the variable 2 region. A chromatin accessibility model has been proposed to explain these phenomena in which RSS sequences reside in inaccessible chromatin until proper signals during development lead to opening of the chromatin structure, allowing free access of the VDJ recombinase to specific target sites.[6] Direct evidence that lineage specificity and temporal order of recombination is controlled by chromatin structure was first demonstrated in an *in vitro* system. Chromatin derived from recombining lymphocytes was supplemented *in vitro* with RAG recombinase activity. Endogenous RSSs packaged in their "natural" chromatin configuration were differentially accessible to the recombinase depending upon the developmental stage of the chromatin source. Thus, alterations in chromatin structure during development determine successful targeting of the recombinase to the appropriate loci.[7]

What determines the molecular changes that modulate accessibility for the VDJ recombinase to its chromatin templates? The mechanism responsible for chromatin modification was studied in a mouse model in which induction of VDJ recombination depends on an external signal.[8–12] The cytokine interleukin-7 (IL- 7) is crucial for lymphoid development.[13,14] A defect in the IL-7 signaling pathway results in severe immune deficiency and a lack of γδT cells. This deficiency is due in part to absence of recombination at the TCR-γ locus.[15] Thymocytes with a deletion of the IL-7 receptor do not initiate cleavage of the TCR-γ locus and show a suppression of germ line transcripts. Furthermore, nuclei derived from IL-7R$^{-/-}$ thymocytes are inaccessible to RAG-mediated cleavage *in vitro*, suggesting that IL-7 signaling specifically controls access of the VDJ recombinase to the TCR-γ locus.[16,17] Since the TCR-γ locus is hypermethylated in the absence of IL-7 signaling and methylated DNA can recruit histone deacetylases via methyl DNA binding proteins (rendering histones hypoacetylated), IL-7 signaling may specifically regulate histone acetylation levels within the TCR-γ locus. In concordance with this model, the histone deacetylase inhibitor trichostatin A (TSA) induces VDJ recombination in the absence of IL-7 signaling.[16] Thymocytes with a targeted deletion of the TCR-α enhancer failed to recombine and showed reduced levels of histone 3 acetylation.[18] Thus, there exists a striking correlation of enhancer activity, histone acetylation levels, and recombination at specific loci. The histone deacetylase inhibitor TSA can overcome the reduced acetylation due to the lack of a specific enhancer and open the site for VDJ recombination.[19] In the IL-7 model, hyperacetylation of histones at regulatory sites within the TCR-γ locus is reduced in IL-7R$^{-/-}$ thymocytes, suggesting that locus-specific acetylation is dependent on IL-7 signaling.[14] Treatment of IL-7$^{-/-}$ Rag$^{-/-}$ thy-

mocytes *in vitro* with IL-7 had direct effects on acetylation. Within five hours of stimulation with IL-7, an increase of histone 3 and 4 acetylation was observed at the TCR-γ locus, and this led to induction of germ ine transcripts, indicating an "open" form of chromatin.

How does IL-7 signaling increase histone acetylation and thus enhance chromatin accessibility? The transcription factor Stat5, previously implicated in IL-7 signal transduction, specifically binds to regulatory sites within the TCR-γ locus. Introduction of Stat5 cDNA substituted for the IL-7 signal and resulted in specific histone acetylation and restoration of VDJ recombination at the TCR-γ locus.[20,21] Opening of chromatin may facilitate association of other *trans*-acting factors, further histone modifications, and recruitment of chromatin remodeling complexes such as SWI/SNF complexes, leading to nucleosomal sliding and repositioning of nucleosomes, thus facilitating binding of the RAG recombinase. In this regard, it has been demonstrated that histone acetylation cooperates with SWI/SNF chromatin remodeling complexes *in vitro* by enhancing the accessibility to RAG-mediated cleavage.[22]

In concert with increased histone acetylation, demethylation of the immunoreceptor loci occurs prior to recombination. Recombination substrates that have been hypermethylated *in vitro* are unable to undergo rearrangement.[23,24] Methylation may regulate recombination by modulating regional chromatin accessibility to the recombinase. In addition, methylation may directly block recognition or cleavage of the RSS sequence.[25] Although methylation can impede rearrangement, hypomethylation is not sufficient to induce recombination. Recombinationally active B cells that carry a targeted deletion of DNA methyltransferase 1 show a hypomethylated genome.[26] Despite demethylation at the Ig-κ locus, recombination was not induced at this locus. Thus, demethylation may be necessary but not sufficient for recombination. Differential methylation has also been implicated in the process of allelic exclusion.[27] The unrearranged allele remains methylated, whereas the other allele becomes demethylated prior to rearrangement. The epigenetic difference in the two alleles is represented in replication timing. Thus, the delay in replication may direct the choice for monoalleleic demethylation and subsequent accessibility for RAG cleavage.[28]

The second step of VDJ recombination after cleavage involves processing and the repair of double-stranded DNA breaks as mentioned above. Specific chromatin modifications appear at the site of breakage, in the form of phosphorylation of an H2A variant. Mice with a deletion of the H2A variant suffer from genomic instability. Thus alteration of chromatin structure not only directs the targeting of the RAG recombinase but may also influence the efficiency of rejoining broken DNA ends and ultimately participate in the prevention of genomic instability and tumorigenesis.[29,30] It remains uncertain if and how changes in chromatin structure required for VDJ recombination differ from those required for transcriptional regulation since both processes are closely correlated.

An aberrant recombination process has been inferred as one of the molecular mechanisms for chromosomal translocations. Translocations are genetic hallmarks in lymphoid malignancies and frequently juxtapose a protooncogene next to the strong enhancer elements of immune receptor genes, leading to dysregulation of the protooncogene.[31,32] Several murine models support a role for VDJ recombination in lymphoma development,[33,34] and the absence of the RAG recombinase can lower the risk for chromosomal translocations and malignant transformation.[35,36] Recently, a

hidden transposase activity of the RAG recombinase was revealed.[2,3] It has been suggested that the RAG recombinase evolved from an ancient transposase consisting of RAG genes flanked by RSS sequences. The RAG proteins can catalyze transpositional insertions *in vitro.* Thus, a one-ended transposition involving an RSS from an Ig or TCR locus could link it to a random site on another chromosome. Usually VDJ recombination occurs in immature lymphocytes, but reactivation of *rag* gene expression in germinal centers of the spleen has recently been reported.[37] This resolves the dilemma posed by the fact that many lymphomas have a mature phenotype and gives even mature B cells the opportunity to misuse the VDJ recombinase in the event of chromosomal translocations. Whether the process of VDJ recombination is indeed contributing to the development of lymphomas in humans and whether aberrant control of epigenesis can be at fault remains to be determined.

ROLE OF GENOMIC METHYLATION DURING LYMPHOID DEVELOPMENT

The importance of genomic methylation during murine development has been demonstrated in distinct mouse models. Targeted deletion of genes that are involved in genomic methylation results in lethality, as shown in *DNMT1*$^{-/-}$, *DNMT3b*$^{-/-}$, *Mbd3*$^{-/-}$, and *Lsh*$^{-/-}$ knockout mice.[38–42] In order to investigate a specific role for methylation during lymphoid development or the immune response, cell lineage–specific demethylation had to be achieved so as to avoid the detrimental effect of demethylation on the whole organism. Lsh (lymphoid-specific helicase) is a regulator of global genomic methylation.[39] Lsh belongs to the SNF2 family of chromatin remodelers based on its seven conserved ATPase/helicase motifs,[44] thus linking chromatin remodeling and DNA methylation. Although ubiquitously detectable during embryogenesis, Lsh is preferentially expressed in lymphoid precursor cells and activated lymphocytes in the adult animal.[43,44] *Lsh*–/– mice die shortly after birth, with a greatly hypomethylated genome.[39,45] In order to evaluate the effect of Lsh on lymphoid development, hematopoietic precursor cells from fetal liver were injected into *Rag*–/– mice that lack any lymphoid development on their own.[46] Defects in T and B cell development were observed, with reduced T and B cell numbers in thymus, spleen, and lymph nodes. T cells and B cells were able to undergo VDJ recombination and differentiated to mature cells: B cells released normal Ig serum levels and T cells were able to produce cytokines. However, T and B cells were reduced in their ability to proliferate *in vitro* in response to mitogens, and T cells underwent apoptosis after stimulation. Thus, hypomethylation in the lymphoid system impacts the normal rate of lymphocyte production and their *in vitro* response; however, differentiation and maturation are still possible.

DNA methyltransferase 1 activity shows *in vitro* preference for hemimethylated substrates, and its biologic activity may be primarily but not exclusively maintenance of methylation patterns after replication occurs.[47,48] Loss of DNMT1 is lethal around day 9 to 11 of gestation. To examine its effect in the lymphoid lineage, conditional knockouts were generated, deleting DNMT1 activity in T cell precursors.[49] As a result, T cell numbers in the thymus and in peripheral organs were dramatically reduced, with reduced survival of TCR-$\alpha\beta$ cells in the thymus. In addition, the abundant appearance of $\gamma\delta$T cells with unusual CD8 expression was reported. Deletion of

DNMT1 at later stages of T cell development (using a CD4 cre recombinase) resulted in normal T-cell development. The growth rate *in vitro* was reduced, but cytokine production was normal or even enhanced (e.g., interferon-γ, or IFN-γ). Another effect of DNMT1 expression on the immune response has been reported in heterozygotic mice with a targeted deletion of one allele of DNMT1.[50] These mice survive normally into adulthood and surprisingly show evidence of genomic hypermethylation and dysregulation of the methyl-DNA binding protein MeCP2. Signs of autoimmunity and senescence develop more slowly than in wild-type controls.

ROLE OF METHYLATION IN CYTOKINE REGULATION OF $CD4^+$ T CELL IMMUNE RESPONSES

The regulation of expression of many cytokine and chemokine genes and their receptors in lymphoid cells has been widely studied for many years; however, the epigenetic control of these genes has not been extensively defined. Most of the work on the epigenetic regulation of cytokine gene expression has focused on gene expression in T cells. In particular, the maturation of $CD4^+$ T helper cells into a T helper 1 (TH1) phenotype (defined as producing IFN-γ, tumor necrosis factor [TNF], and lymphotoxin) or a T helper 2 (TH2) phenotype (defined as producing, IL-4, IL-5, IL-9, and IL-13 but not IFN-γ) has been the subject of much of the past work within this field.[51–55] Within this context, epigenetic regulation of the IFN-γ and IL-4/IL-13 loci has been analyzed in some detail.

DNASE 1 HYPERSENSITIVITY ANALYSIS OF THE IFN-γ LOCUS

Epigenetic regulation of IFN-γ messenger RNA (mRNA) expression was originally investigated by two common techniques. The first method, DNAse 1 hypersensitivity, is based on the ability of DNAse 1 to cut DNA in intact nuclei. The sensitivity of a region of genomic DNA to cutting by DNAse 1 signifies that a gene locus has an "open" chromatin conformation. Hardy and coworkers found a good correlation between DNAse1 hypersensitivity and IFN-γ gene expression.[56,57] In particular, in human cell lines that were induced to express the IFN-γ mRNA, strong increases in DNAse 1 hypersensitivity were observed in the promoter as well as the first intron. The promoter site was located at a egion that was defined as being critical for IFN-γ promoter function and interacted with the CREB/ATF and AP-1 transcription factors.[58–60] The first intron region was later identified as containing an enhancer element that bound NF-κB[61,62] and was also very near to an intronic region containing numerous STAT-binding sites.[63] Similar DNAse 1 hypersensitive sites were observed in human $CD4^+$ TH1 cells but not in IL-4–primed naïve $CD4^+$ T cells.[64] A detailed analysis of IFN-γ DNAse hypersensitive sites has also been performed on murine TH1 and TH2 clones.[65,66] In TH2 clones, no sites were observed in either the resting, differentiated, or activated state. In contrast, resting TH1 cells contained a site in the first intron, differentiated TH1 cells had two new sites in the first and third introns, and activated cells lost the site that was present in the resting cells but retained the new sites generated upon differentiation. Furthermore, introduction of T-bet, a transcription factor found to be important in $CD4^+$ T-cell and NK-

cell expression of IFN-γ,[67] induced cellular differentiation, resulting in the appearance of a hypersensitive site in the first intron of the IFN-γ locus that appears to correspond to the site generated upon TH1 differentiation.[68] These results indicate that the IFN-γ locus does undergo a chromatin reconfiguration that correlates with the ability of the cells to transcribe the gene. Furthermore, the histone deacetylase inhibitor sodium butyrate or trichostatin A could enhance IFN-γ gene expression, even in the presence of antibodies to IL-12, a potent IFN-γ–inducing agent.[68]

DNA METHYLATION ANALYSIS OF THE IFN-γ LOCUS

A second measure of epigenetic regulation of gene expression is through the analysis of DNA methylation. The first clear definition of a role for methylation in IFN-γ gene regulation was reported by Pang and coworkers, who found that a site in the IFN-γ promoter (−73 to −48) recognized by the methylation-sensitive restriction enzyme SnaB1 (TACGTA) was hypomethylated in human B cell lines that expressed IFN-γ.[69] Young and colleagues further demonstrated a strong correlation between hypomethylation of this site in murine TH1-cell clones and IFN-γ expression.[70] TH2 clones that did not express IFN-γ were methylated at this site but could be reactivated to express IFN-γ by treatment with 5-aza-cytidine (5-aza-C). Furthermore, methylation of the site *in vitro* resulted in a loss of DNA-protein complexes. This result was confirmed and extended by Penix and coworkers, who demonstrated that CREB, ATF-2, and c-Jun bound much less efficiently to an oligonucleotide containing the SnaB 1 site when the CpG was methylated.[59] A strong correlation between hypomethylation of the promoter SnaB1 site and a Hpa II site in the first intron of the human gene was also observed in human $CD4^+$ and $CD8^+$ T cells.[71] In this report, neonatal T cells and thymocytes were completely methylated at these sites, while $CD8^+$ T cells and memory $CD4^+$ T cells showed substantial hypomethylation at both the *SnaB*I and *Hpa* II sites.

The development of a new technique, bisulfite genomic DNA sequencing, has permitted the analysis of the methylation state of all CpG dinucleotides within a target region of genomic DNA. Fitzpatrick and coworkers applied this technique to a clonal analysis of murine $CD8^+$ T cells.[72] These investigators first found that 5 aza-C treatment of primary murine $CD8^+$ T cells resulted in a substantial increase in the ability to express IFN-γ. Analysis of the IFN-γ promoter (–203 to +170) in $CD8^+$ clones revealed an association of widespread promoter demethylation with high levels of IFN-γ production. Furthermore, while there was clonal variation in the methylation patterns, demethylation of sites at –203, −191, and –53 (SnaB1 sites) was closely correlated with high IFN-γ mRNA expression. In addition, symmetrical or hemidemethylation at –45 and –34 was observed with a very high frequency. These authors extended their work to demonstrate that the demethylation patterns were stably inherited and maintained through 16 generations, even if the clones were no longer expressing IFN-γ mRNA.[73] Thus, the epigenetic changes became a part of the T cell memory and did not require continued active transcription at the IFN-γ locus. This conclusion was consistent with a report by Katamura and colleagues in which it was demonstrated that IL-4 and prostaglandin E2 treatment of $CD4^+$ T cells inhibited promoter and intronic hypomethylation of the IFN-γ gene and made these

cells unable to express IFN-γ. Furthermore, these authors found that the effects of IL-4 and prostaglandin could be overcome by 5-aza-C treatment of the cells.[74]

Additional support for an important role for site-specific methylation/demethylation of the IFN-γ promoter has come from White and coworkers.[75] These investigators analyzed the methylation status of the IFN-γ promoter in cord blood and adult $CD4^+/CD45RO^-$ T cells, $CD8^+/CD45RO^-$ T cells, and $CD56^+/CD16^+/CD3^-$ NK cells. They found that the promoter in $CD8^+$ T cells and NK cells from both cord blood and adults was largely hypomethylated. In contrast, in $CD4^+$ neonate T cells, 94–96% of CpG sites were methylated, while in adult T cells only 62–74% of the sites were methylated. In addition, in a most unusual result, these investigators reported that there was a fivefold higher number of methylated CpT and CpA sites in the IFN-γ promoter in neonate $CD4^+$ T cells than in adult $CD4^+$ T cells. CpT/CpA methylation was not observed in the IL-4, TNFα, or IFNGR1 (IFN-γ receptor α-chain) promoters. This increased methylation of CpG and non-CpG sites correlated well with the strongly reduced capacity of neonate $CD4^+$ T cells to express IFN-γ in response to PMA (phorbol 12-myristate 13-acetate)/ionomycin. This result suggests that methylation inhibited the binding of transcription factors (e.g., AP-1, ATF) required for activation of IFN-γ expression.

Lee and coworkers took a different approach towards understanding the role of DNA methylation in T cell development and cytokine gene expression, using a mouse model in which Dnmt1 was conditionally inactivated in the T cell lineage.[49] These authors found that the sites at −191 and −53 but not +17 of the IFN-γ gene were largely hypomethylated in the mutant mice, and this loss of methylation correlated well with the increased IFN-γ expression seen in both naïve $CD4^+$ cells and $CD8^+$ cells following activation. Thus, epigenetic changes at the IFN-γ gene locus appear to play a critical role in regulating the transcriptional control of this important immunoregulatory cytokine.

DNASE HYPERSENSITIVITY IN THE IL-4/IL-13 LOCI

Agarwal and coworkers and Takemoto and coworkers found five clusters of DNAse 1 hypersensitive sites spread over 19 KB of the IL-4 locus in differentiated murine TH2 cells, a murine mast cell line, and primary mast cells.[76,77] In contrast, a TH1 clone showed only a single hypersensitive site in the IL-4 locus. The multiple hypersensitive sites seen in the TH2 cells were the result of chromatin remodeling, as naïve $CD4^+$ cells showed a single hypersensitive site until stimulated with IL-4^+ antigen. Similar to the IL-4 gene, the IL-13 gene also demonstrated chromatin remodeling upon activation. Two DNAse 1 hypersensitive sites also appeared in the intergenic region between the IL-5 and IL-13 genes in TH2 but not TH1 cells. Other studies showed that the $CD4^+$ T cell chromatin remodeling was dependent on STAT6, as T cells from STAT6 KO (knockout) mice induced to differentiate under TH2 conditions did not show any evidence of the appearance of new DNAse 1 sites and did not produce significant IL-4. However, the deficiency of STAT6 could be overcome by treatment of cells with a combination of 5-aza-C + TSA.[78] Thus, STAT6 itself is not essential for expression of IL-4 upon T cell activation, and chromatin remodeling is a critical step. Introduction of the STAT6 inducible transcription

factor GATA-3 into a TH1 clone resulted in the appearance of the hypersensitive site present in intron 2 of the IL-4 gene.[79] Furthermore, ectopic expression of both STAT6 and GATA3 in TH1-polarized cells resulted in chromatin remodeling as detected by generation of DNAse 1 hypersensitivity at sites flanking both the IL-4 and IL-13 genes.[77] However, GATA-3 binding to target DNA sequences was not required, as a GATA-3 mutant deficient in DNA binding could still redirect a TH1 cell to TH2 cytokine expression. Thus, the chromatin changes observed at the IL-4/IL-13 locus may be dependent upon the expression and binding of specific transcription factors as well as the recruitment of chromatin remodeling complexes to accessible segments of the IL-4/IL-13 intergenic regulatory region.

DNA METHYLATION IN IL-4 GENE EXPRESSION

Bird and coworkers clearly demonstrated that treatment of naïve T cells with either sodium butyrate or 5-deoxyaza-C did increase the frequency of cells expressing IL-4.[78] Demethylation of the IL-4 and IL-5 genes between days 4 and 7 was seen during differentiation of T cells to the TH2 phenotype. Similar to the results obtained with IFN-γ, these changes were likely to be stable, as a resting differentiated TH2 clone could reexpress IL-4 within 4 hours of stimulation.

Agarwal reported demethylation at two SMA 1 sites, one each in the IL-4 and Il-13 loci, upon differentiation of naïve T cells to the TH2 phenotype.[65] Consistent with these observations, Lee and coworkers observed a three- to fourfold higher level of IL-4 gene expression in naïve T cells isolated from mice with a targeted T cell deficiency in the DNA methytransferase Dnmt1 than in comparable cells isolated from control mice.[49] Yet, this increase in IL-4 expression is relatively modest compared to that seen for IFN-γ (nearly eightfold) in the Dnmt1-deficient mice. Thus, in contrast to the IFN-γ gene, demethylation of specific sites does not appear to play as important a role in the activation of IL-4 gene expression. Instead, IL-4 expression may be regulated more by chromatin remodeling or by critical *trans*-acting factors. In summary, we are becoming increasingly aware of the importance of the role played by epigenetic regulation of the IFN-γ and IL-4/IL-13 loci in the control of gene transcription.

Additional evidence was provided by investigation of patients with the ICF (immunodeficiency, centromeric instability, facial anomalies) syndrome.[80] ICF is a recessive disease that is caused by mutations in the Dnmt3b gene.[42,47] Since a loss of Dnmt3b in mice is lethal at the embryonic stage, the genetic mutation in ICF patients is thought to leave residual Dnmt3b activity. Patients suffer from facial anomalies, genomic instability at centromeric regions, and immune deficiency. Mitogen activation of peripheral lymphocytes leads to typical chromosomal abnormalities, and patients suffer from low serum Ig levels. A recent analysis of gene expression using microarrays revealed dysregulation of a number of genes that play a role in the function of immune cells.[81] However, the molecular mechanisms leading to transcriptional changes remain uncertain. Not all of the affected genes are upregulated, and the methylation levels at the promoter regions were found to be unaltered, suggesting that there is not a direct link between hypomethylation and transcriptional upregulation.

ABERRANT METHYLATION DURING VIRAL INFECTIONS

The immune response to viral and bacterial infections is regulated by Th1 $CD4^+$ T cells, while the response to helminth infections is regulated by Th2 $CD4^+$ T cells. Since specific hypomethylation is responsible for the activation of cytokine genes that control $CD4^+$ T cell differentiation, it is possible that viral infection could result in dysregulation of methylation, allowing viral escape from immune functions. However, only recently has the link between viral infections and the methylation machinery been observed.

One could postulate several effects of viral infection on the methylation machinery. These could be indirect effects before or after viral integration or direct effects of the expression of viral genes. A study by Heller *et al.* supports indirect effects. These authors demonstrated an association between chromosomal insertion of adenovirus type 12 (Ad12), plasmid, or bacteriophage l DNA and enhanced methylation of cellular DNA segments.[82] Further, they detected no changes in cellular DNA methylation if cells were abortively infected, leading them to conclude that Ad 12 early gene products were not directly involved in increasing cellular DNA methylation; rather, the alterations were due, at least in part, to insertion of foreign DNA into the genome.[82] These data support the theory of Bestor and others that at least one function of DNA methylation is as a host defense to silence the transcription of transposons and retroviruses that have accumulated in the mammalian genome.[83,84] Indeed, a number of studies have shown that methylation of not only the genomes of the human pathogenic retroviruses, human T cell lymphotropic virus-I (HTLV-1), and human immunodeficiency virus (HIV), but also γ-herpesviruses such as EBV plays an important role in suppression of viral gene expression and latency.[85,86] As these viruses are not classic oncogenic viruses but persistent viruses in which infections result in tumorigenesis in only a small percentage of the infected population, several mechanisms, including indirect mechanisms resulting from either the host response to the infection, chronic immune stimulation, or inflammation, likely play an important role in oncogenesis. Acute infection of T cells with HIV-1 results in increased expression and activity of Dnmt1, an overall increase in methylated genomic DNA in infected cells, and the *de novo* methylation of the IFN-γ promoter and subsequent downregulation of IFN-γ production in infected cells.[87] Further, acute HIV infection also results in hypermethylation of CpG island–containing genes such as *p16*. In this study it was found that integration of the virus was not required for *de novo* methylation to occur. In agreement with the results of Heller *et al.*, the increased aberrant methylation and Dnmt1 expression were not caused by the mere presence of foreign DNA in the cell, as an RT (reverse transcriptase)-negative mutant had no effect.[88]

Adults with AIDS have an increased susceptibility to B cell lymphomas, Kaposi's sarcoma, and primary effusion lymphomas (PELs). Children with AIDS have an increased occurrence of leiomyosarcomas.[89,90] All of these tumors have been associated with the γ-herpesviruses, EBV, KSHV/HHV8, and HHV6, as well as with aberrant methylation of tumor suppressor genes.[91] EBV is associated with various malignancies, including Hodgkin's lymphoma,[92] nasopharyngeal carcinoma (NPC),[93,94] B cell lymphomas,[95] gastric carcionomas (GC),[96] and some types of breast cancer.[97] The fact that the EBV genome is present in nearly 100% of EBV-positive tumor cells but only a small subset of normal B cells, suggests that the virus

is involved in tumor development; however, this is controversial. Data are accumulating linking aberrant methylation in almost all of these EBV-associated tumors. Kang *et al.* showed a relationship between EBV-positive gastric carcinoma and aberrant methylation of multiple genes.[96] A tumor with an epithelial component associated with latent EBV infection is NPC. The tumor suppressor genes *p16* and *RASSF1A* have been shown to be extensively hypermethylated in NPC.[98] EBV latent infection was detected in 100% of these tumors, leading to speculation that inactivation of *p16* and *RASSF1A* together with EBV latent infection may be the critical events for NPC tumorigenesis.[98]

The tumor suppressor gene *p16* has recently been shown to be hypermethylated and silenced in KSHV/HH8-associated PEL.[99] In addition to aberrant methylation of CpG dinucleotides, a recent study demonstrated aberrant methylation of C^mC(A/T)GG sites of the B 29 promoter in KSHV-associated PEL.[100] Methylation of non-CpG cytosines is usually seen in early mammalian embryo and germ cells, plants, and fungi; but little is known concerning the gene specificity, frequency, and functional significance of this type of non-CpG methylation. This type of methylation is similar to that reported in epigenetic silencing by the *de novo* methyltransferases Dnmt 3a and 3b, leading these authors to the conclusion that B cell gene silencing in PEL may occur by a similar mechanism. A very rare tumor, PEL is almost always seen in individuals coinfected with HIV and HHV8. This tumor, which lacks expression of lineage-specific genes, is only seen in those patients with longstanding AIDS, tempting the speculation that HIV infection is involved in aberrant methylation of both CpG and non-CpG sites.

Aberrant methylation of both tumor suppressor and immune response genes has also been detected recently in hepatocellular carcinoma (HCC) and more recently linked to hepatitis B and C infections.[101] Hypermethylation of SOCS-1 was seen in 65% of the primary human HCC samples analyzed.[102] SOCS-1 turns off cytokine signal transduction through direct interaction with Janus kinase (JAK) proteins. The authors proposed that SOCS-1 functions normally to suppress growth of hepatocyes and demonstrated that inhibition of the JAK STAT pathway using the small molecule inhibitor AG490 led to growth suppression of the tumor cells.[102] In support of this conclusion, Sun *et al.* have reported increased levels of Dnmt1 mRNA in chronic hepatitis and cirrhosis.[103]

The association of virus infection with aberrant methylation also includes the classic oncogenic viruses such as SV40 and human papilloma virus (HPV). A relationship between SV40 and malignant mesothelioma (MM) has been observed by demonstrating that aberrant methylation of *RASSF1A* was significantly higher in SV40 T-antigen (Tag)–positive MM than in SV40-negative MM.[104] Similar studies demonstrated promoter methylation of multiple genes in cervical cancer. In this case, the aberrant hypermethylation profile existed irrespective of expression of the HPV E6 and E7 oncogenes.[105]

Does aberrant methylation of immune response genes such as IFN-γ and SOCS-1 depress the tumor suppressor role of the primary immune response? A recent study showed that mice lacking functional lymphocytes either alone or in combination with an IFN-γ signaling defect were significantly more prone to spontaneous tumor development.[106] Could aberrant methylation of IFN-γ in AIDS patients be a mechanism for generating a number of associated malignancies of tissues not necessarily infected with the virus? Are specific interactions with viral proteins and the three

known functional DNMTs involved in the mechanism of aberrant methylation? A recent publication described the mechanism by which the oncogenic fusion protein PML-RAR, which is seen in acute promyelocytic leukemias, silences transcription from the *RARβ*-2 promoter by tethering DNMT1 and 3a in the complex at the target gene promoter.[107] This suggests similar mechanisms by which some viral proteins such as HIV Tat and HTLV-1 tax, which have been associated with HATs and HDACs in complexes with CBp300 at the promoters of many cellular genes, could result in aberrant regulation of the methylation machinery.[108–110] These data suggest that specific interactions of viral proteins, which disrupt the normal complex formation of the methylation machinery, will be found. Whether or not specific interactions can be identified and provide new molecular targets for early detection and/or therapeutic intervention, the association of specific viruses with particular tumors could be of prognostic value and present new opportunities for treatment of tumors with inhibitors of methylation alone or in combination with antiviral agents.

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