DNA Methylation in the Pathogenesis of Systemic Lupus Erythematosus

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Abstract: Some forms of drug-induced lupus may be due to inhibition of T cell DNA methylation. DNA methylation modifies gene expression. In general, methylation of regulatory elements suppresses gene expression, while hypomethylation promotes gene expression. Methylation patterns are replicated during mitosis by a family of DNA methyltransferases, whose expression is regulated in part by signals transmitted through the extracellular signal-regulated kinase (ERK) pathway. Inhibition of DNA methylation during mitosis results in aberrant gene expression in the daughter cells, sometimes with pathologic consequences. A recent series of reports demonstrates that treating T lymphocytes with DNA methyltransferase inhibitors such as 5-azacytidine and procainamide, or ERK pathway inhibitors including hydralazine and the MEK inhibitor U0126, inhibits DNA methylation, alters gene expression, and induces autoreactivity. The autoreactive cells cause a lupus-like disease in animal models. Importantly, the same DNA sequences are demethylated in T cells from patients with active lupus, with identical effects on gene expression. These observations suggest that certain drugs and chemicals, and possibly as yet unidentified environmental toxins, can modify DNA methylation patterns through effects on DNA methyltransferase activity or expression, resulting in disordered gene expression and the subsequent development of a lupus-like disease.

INTRODUCTION

Systemic lupus erythematosus is a complex autoimmune disease characterized by the production of autoantibodies directed against a host of cellular components. While the etiology of lupus is unknown, a number of genetic and environmental factors have been implicated. Evidence for a genetic contribution comes from familial aggregation of autoimmunity in ~20% of lupus cases [Priori 2003], a higher concordance rate for lupus among monozygotic twins (~25%) compared to dizygotic twins (2%) [Deapen 1992], a number of known lupus-associated genetic polymorphisms, and the evidence for linkage at multiple loci across the human genome as shown by several genome wide scans of subjects with familial lupus [Kelly 2002]. The observations that the majority of lupus is sporadic, that drugs such as procainamide, hydralazine and others, as well as UV light, trigger lupus-like autoimmunity, the association with Epstein-Barr virus infection, and the lack of complete concordance in identical twins similarly indicate a prominent role for exogenous agents [Yung 2003, James 2001]. How the environmental agents interact with the different genetic loci in the production of autoantibodies, the hallmark of lupus, is poorly understood. A growing body of evidence indicates a possible role for autoreactive T cells in activating B cells, resulting in autoantibody production. Work from our group has demonstrated that inhibiting DNA methylation in mature CD4+ T cells induces autoreactivity *in vitro* and a lupus-like disease *in vivo*. DNA methylation, a post-synthetic modification of deoxycytosine (dC) residues in CG pairs, plays an important role in maintaining T cell function. Importantly, some lupus-inducing drugs are DNA methylation inhibitors.

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In this manuscript we review the role of DNA methylation in the regulation of gene expression, T cell function, and the evidence for abnormal DNA methylation in drug induced and idiopathic lupus.

DNA METHYLATION AND REGULATION OF GENE EXPRESSION

DNA methylation plays an important role in the regulation of gene expression. In mammals, methylation occurs at position 5 of the cytosine residues in the CG pairs. Most CG pairs are methylated with the exception of CG pairs in the promoter sequences of transcriptionally active genes. Promoters of active genes are typically hypomethylated, while methylation of promoter sequences renders the genes transcriptionally inactive [Busslinger 1983]. This association is strongest for CpG islands, which are GC-rich sequences located in the promoters of ~50% of mammalian genes, but is also true for CG pairs in genes lacking CpG islands, depending on the number and location of the CG dimers and the relative strength of the promoter [Antequera 2003]. Thus, DNA methylation can play an important role in the transcription of multiple genes. DNA methylation suppresses gene expression by inhibiting the binding of transcriptional factors to the promoter sequences [Comb 1990, Clark 1997], and by promoting chromatin condensation into an inactive configuration [Jones 1998]. The pattern of DNA methylation varies according to the cell type. Promoter sequences of genes that are important for the function of a particular cell type are typically methylation free, while the promoters of genes that are not needed are heavily methylated. The pattern and level of DNA methylation in various cells is determined during development then replicated during cell division.

A group of enzymes, the DNA methyltransferases, are responsible for establishing and maintaining DNA

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methylation patterns in the different cell types. DNA methyltransferase 3a (DNMT3a) and DNA methyltransferase 3b (DNMT3b) are responsible for de novo DNA methylation during development, while DNA methyltransferase 1 (DNMT1) maintains the levels and patterns of methylated DNA during mitosis. Any change in the level or pattern of DNA methylation in the promoters of mature cells may have pathologic consequences. For instance, aberrant methylation of CpG islands associated with tumor suppressor genes has been proposed to contribute to carcinogenesis [Baylin 2002].

5-Azacytidine is a cytosine analogue that binds covalently with the DNA methyltransferases when incorporated into DNA [Santi 1984]. Cells that are treated with 5-azacytidine during the S-phase will incorporate the drug into the newly synthesized DNA in place of cytosine. This results in depletion of the DNA methyltransferases with subsequent genome wide hypomethylation of the newly synthesized DNA. In studies designed to characterize the role of DNA methylation in regulating T cell function, T cells were treated with 5-azacytidine. 5-Azacytidine was found to inhibit T cell DNA methylation and cause overexpression of multiple genes, as well as inducing T cell autoreactivity [Richardson 1986]. This was confirmed in both human and murine T cells. Genes that are potentially relevant to autoimmunity and that are overexpressed in 5-azacytidine treated T-cells include CD11a, CD70, perforin, INF- , and IL-4 [Richardson 2003]. The effects of 5-azacytidine on CD11a, perforin, CD70, and INF- have been studied in some detail.

CD11a or LFA-1 is a subunit of the 2-integrin LFA-1 (CD11a/CD18, L 2). LFA-1 is expressed on the cell surface of leukocytes and is important in immune responses, where it mediates binding to intracellular adhesion molecules on other cells. For example, LFA-1 is involved in the interaction between T cells and other immune cells including B cells, macrophages, and dendritic cells, as well as to endothelial cells [Hynes 2002, Hynes 2003]. Initial studies demonstrated that T cells treated with 5-azacytidine increase LFA-1 expression, through effects on CD11a [Richardson 1992]. This suggested that DNA methylation might regulate CD11a expression. To demonstrate that DNA methylation plays an important role in the CD11a expression, bisulfite sequencing was used to compare methylation patterns in the promoter and the 5' flanking region of *ITGAL* gene, encoding for CD11a, in human T cells that normally express CD11a and fibroblasts which do not. In fibroblasts, the entire region was found to be heavily methylated, while in T cells the promoter and 5' flanking region was hypomethylated except for a series of *alu* repeats located ~ 1 kb 5' to the start site. This region was demethylated in 5-azacytidine treated T cells that overexpressed LFA-1. Finally, selective methylation of this region in reporter constructs suppressed promoter function, indicating that methylation of the flanking region modifies promoter function [Lu 2002].

The second molecule examined was perforin. Perforin is a cytotoxic molecule expressed by natural killer (NK) cells, and by a subset of cytotoxic T lymphocytes. 5-azacytidine was found to increase perforin expression in CD4+ and CD8+ T cells. Again using bisulfite sequencing, the perforin promoter and upstream enhancer were found to be nearly completely unmethylated in a transformed NK cell line, but heavily methylated in fibroblasts, which do not express perforin. In primary CD4+ and CD8+ cells, perforin expression is associated with hypomethylation of an area residing between the upstream enhancer at -1 kb and the distal promoter at -0.3 kb. Treating T cells with the DNA methyltransferase inhibitor 5-azacytidine selectively demethylates this area and increases perforin expression. Similar to CD11a, methylation of this region also suppresses promoter function [Lu 2003].

5-Azacytidine was also found to increase CD70 expression on CD4+ T cells. CD70 is a costimulatory molecule, interacting with CD27 on B cells and promoting IgG synthesis [Oelke 2004]. Again, using bisulfite sequencing and regional methylation of reporter constructs, CD70 overexpression was found to be due demethylation of a region flanking the promoter (unpublished data). Together, these results indicate an important role for DNA methylation in the regulation of these and likely other T cell genes.

DNA methylation plays an important role in regulating INF- expression. Fukunaga *et al* demonstrated that the extent of DNA methylation at CCGG sequences within and around the INF- gene was inversely correlated with the level of INF- produced in several T cell lines [Fukunaga 1986]. Furthermore, in murine Th1 cells, which normally express INF- , a CpG dinucleotide located within a TATA proximal regulatory element of the INF- promoter is hypomethylated [Young 1994]. In contrast, murine Th2 cells which do not express INF- are heavily methylated at this regulatory element [Young 1994]. Treatment of murine Th2 cells with 5- azacytidine, a DNA methylation inhibitor, will convert them to INF- producing cells [Young 1994]. Human naive CD4+ cells produce low levels of INF- . However, when stimulated via the T-cell receptor, the CpG dinucleotide within the TATA proximal regulatory element of the INF- gene will become hypomethylated, coinciding with increased INF- expression [Katamura 1998]. Both INF- expression and CpG hypomethylation were inhibited by IL-4 and prostaglandin E. However, the ability to produce INF- was restored after treatment with 5-azacytidine [Katamura 1998].

DNA METHYLATION AND T-CELL FUNCTION

The changes in T cell gene expression caused by DNA demethylation have important effects on T cell function, including antigen recognition, interactions with macrophages and interactions with B cells, due to increases in LFA-1, perforin, and CD70, respectively.

CD4+ T cells, from both mouse and human, that are treated with DNA methyltransferase inhibitors like 5-azacytidine become autoreactive, largely due to overexpression of CD11a. These cells become responsive to self-class II MHC without the presence of the appropriate antigen in the antigen binding cleft of the class II molecule. This autoreactive response is reversed by adding anti-CD11a monoclonal antibodies to the 5-azacytidine treated T cells, while antigen reactivity is retained, suggesting that the autoreactive immune response results from CD11a

overexpression and that inhibiting the additional molecules reverses this [Richardson 1992]. This hypothesis was confirmed when antigen specific CD4+ cells, from both mice and humans, were transfected with CD18. This resulted in LFA-1 overexpression and MHC-specific autoreactivity that was again reversed by anti-CD11a monoclonal antibodies, identical to 5-azacytidine treated cells. It is possible that the autoreactivity observed is due to stabilization of the normally weak interaction between the T cell receptor and the class II MHC in the absence of a specific antigen [Kaplan 2000].

The autoreactive CD4+ T cells also promiscuously kill autologous or syngeneic antigen presenting cells. In normal immune responses, the antigen presenting macrophage dies by apoptosis after stimulating the T cell [Richardson 1993]. However, once T cells are made autoreactive by treatment with DNA methylation inhibitors, they will respond to and kill autologous or syngeneic macrophages without the appropriate antigen, reflecting the autoreactivity, and this killing correlates with the induction of perforin expression in the autoreactive T cells. Concanamycin, a selective perforin inhibitor, inhibits this killing [Lu 2003], suggesting that the perforin is involved.

The CD70 overexpression also has functional significance. Others have reported that cells overexpressing CD70 by transfection provide signals increasing B cell IgG production [Kobata 1995]. CD4+ T cells overexpressing CD70 from 5-azacytidine treatment, then cultured with autologous B cells, similarly overstimulate immunoglobulin production relative to untreated controls. Further, anti-CD70 inhibits this response, implicating CD70 overexpression in the augmented IgG production. However, the 5-azacytidine treated T cells also overexpress IL-4 and IFN- [Quddus 1993], cytokines known to be involved in T cell mediated B cell stimulation [Golbus 1988, Singh 2003], and these may contribute as well.

DNA METHYLATION IN DRUG INDUCED LUPUS

Several drugs are capable of inducing a lupus-like illness in man, most notably procainamide and hydralazine. Similar to what was described with 5-azacytidine, procainamide and hydralazine also inhibit DNA methylation in T cells. Indeed, when antigen specific CD4+ T cells were treated with either procainamide or hydralazine, these cells became autoreactive, similar to what was seen with 5-azacytidine [Cornacchia 1988]. Procainamide and hydralazine induced autoreactivity at a low concentration (10^{-7} M), and the autoreactivity increased in a dose-dependent fashion. Interestingly, the concentrations of procainamide and hydralazine inducing autoreactivity *in vitro* fall in the therapeutic concentrations of these drug *in vivo* and are similar to the concentrations that induce lupus. Moreover, both procainamide and hydralazine were shown to inhibit DNA methylation. Diminished methylation of newly synthesized DNA in T cells treated with procainamide or hydralazine, was demonstrated using a biosynthetic labeling technique. This was confirmed by a high pressure liquid chromatography (HPLC) analysis [Cornacchia 1988]. Finally, procainamide and hydralazine also increase LFA-1 expression, similar to 5-azacytidine [Yung 1997].

UV light exposure can also trigger lupus flares [Sanders 2003, Caricchio 2003]. Others have reported that UV light can demethylate DNA, affecting expression of genes such as metallothionein-I [Lieberman 1983]. Brief exposures of T cells to UV light, less than what would cause sunburn in fair skinned people, induces DNA hypomethylation, LFA-1 overexpression and T cell autoreactivity similar to that observed with procainamide and hydralazine [Richardson 1994], further supporting a possible association between T cell DNA hypomethylation and autoimmunity.

Subsequently, it was shown that procainamide is a competitive inhibitor of T cell DNA methyltransferase [Scheinbart 1991]. In contrast, hydralazine does not inhibit DNA methyltransferase enzyme activity, but instead inhibits the extracellular signal-regulated kinase (ERK) pathway, thereby decreasing DNA methyltransferase expression [Deng 2003]. Murine T cells treated with the MEK inhibitor U0126 also have lower expression of DNMT1 and DNMT3a and have hypomethylated DNA, similar to human T cells treated with hydralazine [Deng 2003]. To determine if inhibiting the ERK pathway in T cells can cause autoimmunity in mice, D10 cells were treated with the MEK inhibitor U1026 or hydralazine. U1026 or hydralazine treated cells overexpressed LFA-1, and responded to syngeneic antigen-presenting cells in the absence of the antigen, thereby resembling T cells treated with 5-azacytidine.

To test whether T cells made autoreactive with DNA methylation inhibitors can cause autoimmunity *in vivo*, polyclonal CD4+ T cells from DBA/2 mice were treated with 5-azacytidine or procainamide, then injected into unirradiated syngeneic female mice. These mice developed an immune complex glomerulonephritis, a positive lupus band test, and anti-DNA and anti-histone antibodies [Quddus 1993]. This experiment was repeated using conalbumin-reactive, cloned Th2 cells treated with 5-azacytidine. The treated cells overexpressed LFA-1, became autoreactive, and produced large amounts of IL-6 [Yung 1995]. Adoptive transfer of the treated cells into female AKR mice induced immune complex glomerulonephritis, pulmonary alveolitis, and central nervous system pathologies including fibrinoid necrosis, karyorrhexis, and meningitis. In addition, these mice developed bile duct proliferation with periportal inflammation similar to primary biliary cirrhosis. Serologically, these mice responded by producing autoantibodies directed against single stranded and double stranded DNA as well as anti-histone antibodies [Yung 1995]. The same model system was used to demonstrate that procainamide was more potent than N-acetylprocainamide in inducing LFA-1 overexpression, autoreactivity *in vitro* and autoimmunity *in vivo*, and that hydralazine was more potent than phthalazine, the parent compound, in the same assays [Yung 1997]. Since hydralazine is an ERK pathway inhibitor [Deng 2003], D10 cells were made autoreactive by treatment with the ERK pathway inhibitor U0126 or hydralazine. Adoptive transfer of the drug-treated D10 cells into nonirradiated syngeneic female mice induced the production of anti-double stranded DNA antibodies, confirming that ERK pathway inhibition could induce autoimmunity [Deng 2003].

Together, these studies indicate that T cells hypomethylated by treatment with DNA methyltransferase inhibitors or ERK pathway inhibitors, are sufficient to induce a lupus-like disease *in vivo*. This suggests a mechanism which might contribute to the development of some forms of drug-induced lupus, and possibly idiopathic lupus. We therefore asked if similar changes in DNA methylation, gene expression and T cell function occurred in idiopathic lupus.

DNA METHYLATION IN THE PATHOGENESIS OF IDIOPATHIC LUPUS

In initial studies, a subset of T-cells from patients with active lupus was found to overexpress CD11a, similar to antigen-specific normal CD4+ T cells treated with DNA methylation inhibitors. The increase in the CD11a overexpression in the lupus T cells correlates with the disease activity [Richardson 1992]. Furthermore, using bisulfite sequencing, the same sequences of the *ITGAL* flanking region were demethylated as seen in T cells treated with 5-azacytidine or procainamide. As noted above, methylation of this region suppressed *ITGAL* promoter function, suggesting a mechanism for LFA-1 overexpression on lupus T cells [Lu 2002].

Subsequent studies demonstrated that CD4+ T cells from patients with active lupus abnormally express perforin and promiscuously kill autologous macrophages, and this macrophage killing is inhibited by Concanamycin, identical to CD4+ T cells treated with DNA methylation inhibitors [Kaplan 2004]. Further, the same sequences in the perforin promoter region demethylate in lupus as in the experimentally hypomethylated cells, indicating further parallels between the drug-induced model and idiopathic lupus [Kaplan 2004].

Further, CD4+ T cells from patients with active lupus also overexpress CD70 and overstimulate IgG production by autologous B cells, and this effect is abrogated by antibodies to CD70 [Oelke 2004]. This is also similar to the experimentally hypomethylated T cells. Again, the same sequences flanking the CD70 promoter region are demethylated in lupus as in the drug treated cells.

The reason for DNA hypomethylation in lupus T cells may relate to abnormal signaling. T cells from patients with active lupus have global decreases in genomic deoxymethylcytosine ($d^{m}C$) levels, decreased levels of DNA methyltransferase activity, and decreased levels of DNA methyltransferase 1 mRNA [Richardson 1990, Deng 2001]. Similar to hydralazine treated T cells, T cells from active lupus patients have decreased signaling through the ERK pathway resulting in reduction in the DNMT1 mRNA levels. Indeed, the DNMT1 mRNA levels are decreased to the same degree in T cells from active lupus compared to T cells treated with ERK pathway inhibitors [Deng 2001]. This suggests that decreased ERK pathway signaling contributes to both hydralazine induced and idiopathic lupus.

Abnormal DNA methylation may be also involved in the development of autoimmunity in some lupus-prone mice strains, for example MRL/*lpr* mice that spontaneiously develop age-dependent lymphadenopathy and severe autoimmune disease resembling lupus. 20-weeks old MRL/*lpr* mice with autoimmune disease have lower levels of DNA methylation in the thymus and axillary lymph nodes compared to 4-weeks old MRL/*lpr* mice with no autominnune disease yet [Mizugaki 1997]. In addition, MRL/*lpr* mice with autoimmune disease have lower levels of DNA methylation compared to age-matched MRL/+ mice. Peculiarly however, in both strains the level of DNA methylation increased in the spleen and did not change in the peripheral blood as they aged from 4 to 20 weeks [Mizugaki 1997].

Of interest, lymph nodes form *lpr* mice express high levels of *c-myb* proto-oncogene mRNA [Mountz 1984]. The increased c-myb protein, is primarily produced by an abnormal T cell population that does not express the mature T cell markers L3T4 and Lyt-2 (double-negative cells) [Evans 1987]. Evans *et al* demonstrated that the *c-myb* gene in double-negative cells from the *lpr* mice is hypomethylated compared to normal mice, suggesting a role for DNA methylation in regulating *c-myb* expression in this lupus mouse model [Evans 1987].

Overall, these observations strongly support the hypothesis that DNA hypomethylation may contribute to the pathogenesis of some forms of drug induced lupus as well as idiopathic lupus. The DNA hypomethylation may be induced by DNA methyltransferase inhibitors such as 5-azacytidine or procainamide, or by ERK pathway inhibitors such as U0126 and hydralazine, and as occurs in idiopathic lupus. Further, the parallel effects on DNA methylation and gene expression seen in the drug-induced model and in idiopathic lupus suggest that the *in vitro* model may be used to predict genes abnormally expressed in lupus T cells, and which may contribute to disease pathogenesis. These genes may be targets for therapeutic intervention.

CONCLUSION

DNA methylation plays an important role in regulating gene expression. Methylation of regulatory gene sequences is carried out by a group of enzymes, the DNA methyltransferases, whose expression is regulated at least in part by signals through the ERK pathway. DNA methyltransferases are responsible for both determining and maintaining the patterns of DNA methylation across the genome. Hypomethylation of the regulatory sequences of genes that are necessary for the function of a particular cell type make them available for expression, while the regulatory sequences of genes that are not expressed are typically heavily methylated. Aberrant DNA methylation has pathologic consequences. In lupus, there is strong evidence that abnormal hypomethylation of several genes in the CD4+ T cells is associated with T cell autoreactivity *in vitro* and autoimmunity *in vivo*. While we believe that lupus is most likely a polygenic disease and that our understanding of lupus genetics is poor, pharmacologic modification of DNA methylation has certainly improved our understanding of drug induced lupus. Furthermore, alteration of DNA methylation patterns might be the mechanistic explanation of how certain environmental factors trigger an autoimmune

response in genetically predisposed individuals. Indeed, abnormal DNA methylation provides a possible common pathway to explain the interaction between the environment and the genetic elements to produce lupus and perhaps other autoimmune diseases. A number of candidate genes have been shown to be hypomethylated in lupus, most notably CD11a, perforin, and CD70. However, further work is needed to understand what triggers the abnormal hypomethylation of these genes in lupus patients.

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