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DNA methylation and silencing of gene expression role in pathogenesis of systemic lupus erythematosus

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Systemic lupus erythematosus (SLE) is a prototypical autoimmune disease characterized by production of autoantibodies against a wide array of host nuclear antigens. Though the causes and etiology of the disease is not clearly understood, some genetic, epigenetic and environmental issues have been found responsible as risk factors. The impact of environment, which is majorly reflected by the epigenetic mechanisms, especially DNA methylation and epigenetic regulation of gene expressions are generally considered as key players in the due course of SLE pathogenesis. The repertoire of the evidences has indicated that DNA hypomethylation in T cells is an important characteristic of SLE. DNA hypomethylation mechanisms in the genes like CD11a (ITGL), perforin (PRF1), CD70 (TNFSF7), CD40LG (CD40), IFN-γ, IL-4, have been designated them as classic methylation-sensitive autoimmunity related genes. In addition to these, the genome wide analyses have reported the hypomethylation in IL10 and IL-1R2 genes in SLE. On the other hand, certain drugs such as procainamide, hydralazine, 5-azacytidine (5-azaC) which are competitive DNA methyltransferases inhibitors, have been proposed as potential agents causing DNA hypomethylation in lupus. In this review, we summerise the current understanding of aberrant DNA methylation in T cells and consequently altered gene expression in SLE. Based on DBA 2 and AKR mice models, we have summarized the alterations in ERK (Ras-MAPK) signaling pathway and overexpression of LFA-1 caused by certain drugs contributing to the development of manifestations of the disease.

Keywords: DNA methylation, gene silencing, autoimmunity, systemic lupus erythematosus (SLE), pathogeneisis

Introduction

Systemic lupus erythematosus (SLE) is a complex autoimmune disease that has multiple organ involvement and is characterized by production of autoantibodies against a large array of host nuclear antigens. It is predominantly observed in females[1]. Although the etiology of lupus is unknown, it has been implicated that environmental factors can alter genetic mechanisms especially epigenetic regulation of gene expression and may play an important role in SLE pathogenesis[2]. Epigenetics is defined as the heritable chromatin-based mechanisms like DNA methylation, histone modifications, microRNA interference which are involved in the regulation of gene expression without any alterations in the DNA sequence[3]. Of these, DNA methylation plays an important role in maintaining normal function of T-cells. Improper levels and patterns of this DNA methylation can result in T-cell autoreactivity *in vitro* and autoimmunity *in vivo*. Growing body of evidences exhibiting development of sporadic autoreactive T-cell mediated lupus-like autoimmunity on exposure to drugs like procainamide, hydralazine and ultra violet light indicates role of exogenous agents in SLE pathogenesis[4-7]. In this review, we discuss the role of epigenetic dysregulation, specifically DNA methylation and the associated silencing of gene expression in pathogenesis of lupus. The knowledge of epigenetic targets in SLE can be used not only for understanding SLE pathogenesis but also has diagnostic and therapeutic potential in thenear future.

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DNA Methylation and Silencing of Gene Expression

DNA methylation is associated with condensed nuclease-resistant heterochromatin and silencing of gene expression[8,9]. It plays a vital role in X chromosome inactivation[10], parental imprinting[11,12], repressing the expression of tumor suppressor genes in cancer[13] and is essential for development. In mammals, methylation of the 5'-position of cytosine residues is a reversible covalent modification of DNA in the palindromic sequence 5'-CpG-3' with the methyl groups projecting into the major groove of the DNA

structure[15,16]. DNA methylation is of two types namely maintenance methylation and *de novo* methylation. These methylation processes are catalyzed by three different enzymes i.e. DNA methyltransferases (DNMTs) encoded by different genes on three distinct chromosomes and are named DNMT1, DNMT3a, DNMT3b. DNMT1 is recruited to replicating DNA during cell division to reproduce the methylation pattern identical to the parental strands onto the newly synthesized daughter strands hence the name maintenance methylation[17,18]. DNMT3a and DNMT3b, involved in *de novo* methylation, are capable of methylating CpG dinucleotides of unnmethylated and hemimethylated DNA and are important in the establishment of DNA methylation patterns within the embryo and during fetal development.

It is observed that most CG pairs in the mammalian genome are methylated with exception of those CG pairs that are present in the promoters of actively transcribed genes. While hypomethylation of the promoter renders these genes transcriptionally active, methylation of other genes makes them inactive and silences their expression[19]. This association is the strongest for CpG islands, which are CG rich sequences located in promoters of ~50-60% of mammalian genes, but is also true for CG pairs in CpG island lacking genes depending on the number and location of CG dimers and strength of the promoter under study[20]. Hence DNA methylation is a remarkably stable epigenetic modification[16] capable of regulating transcription of multiple genes. Among the 70-80% of CG dinucleotides that are methylated in normal mammalian cells, most are the repressive marks associated with transcriptional silencing of parasitic DNA sequences, X chromosome inactivation, genomic imprinting, mammalian embryonic development and lineage specification[17,18]. DNA methylation can silence gene expression in several ways. Firstly, the projecting methyl group on the DNA can act as direct interference to the binding of specific transcription factors that have methylated CpGs within their response elements[21]. Secondly, the methylated DNA can act as binding site for methyl-CpG binding proteins like MBD1, MBD2, MBD4 and MECP2[18]. These proteins can themselves interfere with binding of transcription factors to the CpG-containing response element or can recruit co-repressors and histone deacetylases (HDACs) which lead to chromatin remodeling, increase in chromatin density and induces a tightly packed chromatin structure inaccessible for transcription[22].

Role of Methylation and Silencing in T-Cell Gene Expression and Function

DNA methylation plays an important role in maintaining normal function of T-cells. It has been observed that patients with autoimmune diseases like rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) show presence of T-cells with DNA-hypomethylation indicating a relation between DNA hypomethylation and autoimmunity[23,24,25]. Large amount of evidence has been gathered suggesting that DNA-hypomethylation may play an important role in pathogenesis of autoimmune diseases[26]. Various methods were employed to identify the genes affected by hypomethylation that may lead to disease. One approach among them is to inhibit maintenance DNA methyltransferase activity during mitosis by employing use of 5-azacytidine (5-azaC) which is a cytosine analog. 5-azaC is incorporated into the newly synthesized DNA where it covalently binds to DNA methyltransferases during synthesis (S)-phase cytosine transmethylation reaction, depleting intracellular pools of the enzymes[27], thereby causing genome-wide DNA hypomethylation and altered expresiion of variety of genes in the cell[28]. Cloned normal T-cells treated with 5-azaC, showed increased expression of a number of methylation sensitive genes like ITGAL (CD11a)[29], TNFSF7 (CD70)[30], CD40LG (CD40L)[31], IFN-ɣ[32], IL-4 and PRF1 (perforin)[33].

In order to understand the mechanism by which DNA methylation affects the expression CD11a, CD70, CD40L, IFN-ɣ, IL-4 and perforin, bisulphate DNA sequencing was carried out to precisely quantitate methylated and unmethylated dC bases in the regulatory regions of the above genes. Bisulphite sequencing of the CD11a promoter and 5'-flanking region showed that the region is partially methylated except for hypermethylated series of alu repeats located ~1 kb upstream to the transcription start site. The 5-azaC demethylates this region and hence leads to CD11a overexpression[34]. Similarly, bisulphate sequencing of the perforin promoter and upstream enhancer demonstrates that perforin expression correlates with methylation status of the region between the enhancer and the promoter, where methylation of this region suppresses promoter function. Hypomethylation of this region alters chromatin structure around the enhancer facilitating perforin overexpression[33]. The methylation status of the IFN- ɣ, IL-4 and CD40L promoters has been similarly studied and hypomethylation of specific dC

bases was found to correspond with IFN- ɣ[32], IL-4[35,36] and CD40L[37] overexpression, respectively.

The DNA methylation inhibitors show profound effects on various T-cell functions like T-cell antigen recognition, B-cell activation, T-cell-macrophage interactions, etc which lead to autoimmunity. Cloned and polyclonal human and murine CD4+ T cells become responsive to normally sub-threshold stimuli, including self-class II MHC determinants without specific antigens[38-41], a form of autoreactivity resembling alloreactive responses. This autoreactivity is in part due to the LFA-1 (CD11a) overexpression which overstabilizes the normal low-affinity interaction between the TCR and self class II MHC molecule without the presence of an appropriate antigenic peptide, allowing the T-cell receptor (TCR) signaling apparatus to assemble[42]. More conclusive evidence comes from studies in which human and murine antigen specific CD4+ T cells transfected with CD18 also overexpressed LFA-1 and responded to sub-threshold stimuli, including self-class II MHC molecule without antigen, identical to 5-azaC treated T cells[6,43]. However, the autoreactivity due to 5-azaC treatment or transfection can be inhibited by adding small amounts of anti-CD11a[6,43]. When 5-azaC-treated human CD4+ T cells are cocultured with autologous B-cells, the 5-azaC-treated human CD4+ T cells shows increased expression of B-cell co-stimulatory molecules including IFN-ɣ, IL-4 and CD-70 which directly stimulates B-cell IgG secretion[30,32,41]. Similar results were also obtained on co-culture of murine Th1 clones with syngeneic B-cells. Polyclonal CD4+ T cells treated 5-azaC overexpress CD70 which further stimulates the B-cell and increases IgG secretion related to untreated controls. This increase is inhibited by employing small amounts of anti-CD70, indicating the important role of CD-70 as a B-cell co-stimulatory molecule.

During the course of normal immune response, after activating the CD4+ T-cells, antigen-presenting macrophages die by apoptosis due to apoptotic signals delivered by T-cell FasL, TWEAK and TRAIL[45-47]. However, 5-azaC treated CD4+ T cells respond to autologous or syngeneic macrophages without specific antigen, promiscuously killing the stimulating macrophages using the same apoptotic signals[38]. Recent evidences indicate that 5-azaC-treated CD4+ T cells also overexpress perforin which plays an important role in the killing of the macrophages by the hypomethylated, autoreactive T cells. This autoreactive killing is inhibited by a selective perforin antagonist namely concanamycin A[33]. The pathological significance of the T-cell changes caused by DNA hypomethylation has been tested in animal models. Female mice receiving 5-azaC-treated polyclonal CD4+ T-cells from syngeneic DBA/2 mice developed lupus-like disease with an immune complex glomerulonephritis, anti-DNA antibodies and a lupus band test. The female mice receiving untreated polyclonal CD4+ T-cells from the syngeneic DBA/2 mice did not show any such pathological consequences[38].

The mechanisms by which hypomethylated CD4+ T cells induce autoimmunity are incompletely understood. However, the presently available knowledge, allows us to construct a rough scheme regarding how DNA hypomethylation can cause autoimmunity. Firstly, DNA hypomethylation leads to overexpression of LFA-1 and possibly other adhesion molecules, causing T-cell responsiveness to normally sub-threshold stimuli including self-class II MHC molecules presenting inappropriate or no antigen. These T-cells are capable of promiscuously killing the syngeneic antigen-presenting macrophage with the help of perforins and stimulating the autologous B-cells by overexpressing B-cell co-stimulatory molecules including IFN-ɣ, IL-4 and CD-70. This macrophage killing and secretion of cytokines further promotes B-cell differentiation and contributes to the development of anti-DNA antibodies and other disease manifestations. Recent reports suggest that apoptotic rates exceeding clearance capacity can lead to the development of anti-DNA antibodies: injecting large numbers of apoptotic thymocytes induces anti-cardiolipin, anti-ssDNA, anti-dsDNA and ANA in normal mice[48]. The killing of macrophages would both increase the amount of apoptotic material and decrease its clearance. The rate of apoptosis correlates with the amount of nucleosomal material released, which suggests that abnormal apoptosis might play a vital role in providing nuclear antigens that drive the immune reponse in lupus[49]. However, only apoptotic DNA and not necrotic or normal DNA, is capable of inducing auto-immunity via TLR-9 stimulation, which is activated by hypomethylated CpG DNA[50,51]. The accumulation of this apoptotic material may lead to its presentation by other APCs like dendritic cells to nucleosome-reactive T cells, inducing an autoimmune response. Together with cytokines promoting IgG synthesis, an anti-DNA response may develop.

Drug Induced Lupus

By now it is clear that exposing T-cells to DNA methylation-inhibitors is sufficient to cause lupus-like disease. There are more than 100 known lupus-inducing drugs and it is possible that most or all of them are DNA-methylation inhibitors. These lupus-inducing drugs can be classified as low risk, moderate risk and high risk drugs for inducing drug induced lupus (DIL). Although some of these drugs belong to certain chemical natures, for e.g., aromatic amines (procainamide, sulfapyridine), and aromatic hydrazines (hydralazine, isoniazide), there is no general property predisposing a drug to be at high risk for inducing DIL. However the ability of the individuals to metabolize and biotransform these xenobiotic drugs in the liver may be a decisive factor in determining if they end up suffering from DIL or not.

Among the many lupus inducing drugs procainamide and hydralazine are the most strongly associated to cause DIL[53]. Procainamide is a competitive DNA methyltransferase inhibitor of some but not all DNA methyltransferase activity[54] while hydralazine selectively inhibits signaling through the ERK (Ras-MAPK) signaling pathway, preventing upregulation of DNMT1 and DNMT3a in stimulated T-cells exposed to hydralazine, eventually resulting into DNA hypomethylation[4]. Human CD4+ T-cells that have been treated with procainamide, hydralazine or 5-azaC show decreased T-cell genomic dmC content with 5-azaC found to have the most potent hypomethylation activity[5]. In the polyclonal T-cell/DBA2 mice model, procainamide and 5-azaC induce LFA-1 overexpression and autoreactivity *in vitro*, and the treated cells induce similar titres of anti-DNA-antibodies *in vivo* by demethylating the same sequences 5' to the CD11a promoter[38,55]. Both procainamide and hydralazine induce LFA-1 overexpression and autoreactivity in D10 cells, a cloned Th2 line isolated from AKR mice, which cause anti-dsDNA antibodies in D10/AKR mice model[7]. The antibody titres are comparable to those induced with 5-azaC in similar models[39]. Since hydralazine inhibits DNA methylation by inhibiting ERK signaling pathway, the effects of hydralazine and other ERK pathway inhibitors like U0126 have been compared by treating D10 cells with either hydralazine or U0126. The D10 cells treated by either of the above drugs overexpressed LFA-1 and became autoreactive *in vitro*, inducing anti-dsDNA antibody production *in vivo*[4]. On summarization it can be said that some drugs may cause lupus-like disease by inhibiting T-cell DNA methylation by at least two mechanisms. One of these mechanisms involve both 5-azaC and procainamide which directly inhibit DNA methyltransferases[27,54], while the other mechanism involves hydralazine and other ERK signalling pathway inhibitors like U0126 which downregulate DNA methyltransferase levels and eventually cause DNA-hypomethylation[4].

To complicate matters further, many immune-genetic factors have been suggested to be predisposing agents towards occurrence of DIL keeping in mind the low attack rate of DIL in drug-treated patients. Various MHC molecules of human leukocyte antigens (HLA) system like HLA-DR2, HLA-DR4, HLA-B44, and HLA-DQw7 have been implicated in generating a T-cell dependent anti-chromatin IgG autoantibody response to lupus-inducing drugs[56-63]. Furthermore, the complement system genes, which form a part of the MHC class III genetic loci are also thought to be involved as predisposing factors in DIL. Null alleles of C4 genes (encoded separately from MHC Class III loci), namely C4A and C4B, were found to be more prevalent in patients with procainamide induced lupus[63]. Females[64], Caucasians[65] and patients previously diagnosed with SLE[66] were also found to be more prone to suffer DIL. Unlike in SLE, the ANA in DIL are predominantly IgG autoantibodies against chromatin which is the histone-DNA complex within eukaryotic cell nucleus[67]. While procainamide has been highly implicated in induction of anti-chromatin IgG antibodies in majority (~90%) of patients, there have been reports suggesting production of anti-chromatin IgGs in patients suffering from DIL induced by other drugs[68]. Other laboratory features exhibited by patients with DIL may vary from mild anemia, elevated erythrocyte sedimentation rates, leukopenia, thrombocytopenia, hypergammaglobulinemia, positive Coomb's tests, elevated levels of rheumatoid factor, circulating immune complexes, ANCA, complement activation, and reduced complement levels[68].

DNA Hypomethylation in Idiopathic Lupus

The role of DNA hypomethylation in causing idiopathic lupus has been studied by many research groups. T-cells of patients with active lupus show reduced level of DNA methyltransferases activity alongwith decreased levels of DNMT1 mRNA and genomic dmC content. Furthermore, T-cells from patients with active lupus show that the same sequences in CD11a promoter are demethylated as in

the 5-azaC or procainamide-induced lupus[55]. CD4+ T-cells of lupus patients showed increased CD11a mRNA expression and hence CD11a molecule overexpression which shows negative correlation with DNA methylation[69]. Similarly, T cells form lupus patients show selective overexpression of LFA-1 similar to T-cells treated with DNA methylation inhibitors, such that LFA-1 overexpression correlates with the lupus disease activity[29]. CD70 is another methylation sensitive gene which codes for a B-cell co-stimulatory molecule that is overexpressed in CD4+ T-cells from lupus patients in amounts comparable to procainamide and hydralazine treated T-cells. It contributes to excessive B-cell stimulation *in vitro*[70]. The same genetic element that suppresses CD70 expression if methylated was found to be demethylated in T-cells from lupus patients and T-cells treated with procainamide and hydrazlazine leading to CD70 overexpression and overstimulated B-cell immune–globulin G (IgG) production[70,71]. Another molecule found to be overexpressed in CD4+ T-cells treated with DNA methylation inhibitors and CD4+ T-cells from active lupus patients is perforin. Demethylation of same region in the perforin gene that is demethylated in T-cells treated with DNA methylation inhibitors is responsible for perforin overexpression in patients with active but not inactive lupus[33,72]. A more recent genome-wide study reported that the IL-10 and IL-1R2 genes was significantly hypomethylated in SLE patient samples compared to healthy control samples. It was also noted that the SLE patients with hypomethylated IL-10 and IL-1R2 genes appeared to have higher disease activity[73].

T-cells from patients with active lupus show decreased levels of ERK pathway signaling and the levels of DNMT1 mRNA are also found to be reduced to the same degree as in T-cells treated with ERK signaling pathways inhibitors like hydralazine or U0126[74]. The mechanism causing decreased ERK pathway signaling was unknown, however impaired T-cell ERK pathway signaling is implicated clearly in pathogenesis of lupus[2]. The reason for the ERK signaling defect in lupus CD4+ T-cells and hydralazine treated T-cells was localized to protein kinase C (PKC) delta on analysis of the ERK signaling pathway phosphorylation cascade[75]. Many research efforts have been made to obtain the explanation for the predilection of lupus to affect women. Women have 2 X-chromosomes, one of which is inactivated by mechanisms that include DNA methylation to form Barr body. Global demethylation in the genome leads to demethylation of sequences on the inactivated X chromosome which may lead to certain genes being overexpressed uniquely in women, which predisposes them to lupus[76]. CD40L is a methylation sensitive gene located on X-chromosome which on demethylation shows doubled expression in CD4+ T-cells from women as compared to its expression in CD4+ T-cells from men. This description explains the tendency of lupus to affect women more frequently and would help explain the gene-dose effect described from the number X-chromosome in lupus patients[76,77].

Summary

DNA methylation is a heritable chromatin-based epigenetic mechanism which is actively involved in the regulation of gene expression without any alterations in the DNA sequence. DNA methylation levels and patterns in the genome are controlled by a group of enzymes called DNA methyltransferases, whose expression to some extent is regulated by the signals through ERK signaling pathway. Down regulation of DNA methyltransferases activity leads to hypo–methylation of regulatory sequences of various genes that are necessary for the normal cellular functioning. This in turn makes these genes available for expression. Under normal circumstances genes with hypomethylated regulatory sequences are actively transcribed while those genes which possess hypermethylated regulatory sequences are transcriptionally inactive i.e. they are silenced. Aberrant DNA methylation hence disrupts normal cellular functioning and has pathological consequences. Increasing mass of evidences suggest that abnormal hypomethylation of several genes in the CD4+ T-cells is associated with T-cell autoreactivity *in vitro* and autoimmunity *in vivo*. Although our knowledge regarding etiology of lupus which is a polygenic disease is quite primitive, DNA hypomethylation in CD4+ T-cells and its associated altered gene expression profiles have improved our understanding of lupus pathogenesis. Pharmacological modifications of DNA methylation pattern has also provided us with mechanistic explanation as of how drug-induced lupus is caused and how environmental factors can play an important role in triggering a lupus-like autoimmune disease in genetically predisposed individuals. As such, a common explanation for the interaction between environmental factors and genetic elements that may produce lupus and perhaps other autoimmune diseases may be

abnormal DNA methylation in itself. A number of hypomethylated genes have been discovered and elucidated in lupus, most notable of which are CD11a, CD70, perforin, CD40L and various cytokines some of which are IFN- ɣ, IL-4 and IL-10. Such studies may aid in identifying potential biomarkers and steps in lupus pathogenesis suitable for therapeutic intervention. These biomarkers that correlate with the pathogenic process and/or disease activity of SLE, may be used to diagnose, monitor, stratify, and predict individual response to therapy. Keeping in mind the role of DNA hypomethylation in SLE pathogenesis a few obvious therapeutic approaches may involve: a) targeted inactivation of the upregulated gene products using monoclonal antibodies like anti-CD11a (efalizumab), b) remethylating the hypomethylated regulatory sequences of genes by transfection of lentiviral vectors encoding methylases (perhaps DNMT1) in CD4+ T-cells to raise the cellular global methylation levels, c) site-directed remethylation by introduction of fusion proteins with methylase activity, and d) inhibition of HDAC activity to alter global methylation levels. While these are some of the epigenetic therapeutic approaches for lupus which are presently under study, the possibility of introducing a global hypermethylated state in bystander cells is something to be considered during development of effective therapy or drug. Furthermore, advances in understanding the pathogenesis of of SLE and DIL might suggest consideration of immunogenetic factors like HLA genotype, complement and C4 allelic status, gender, race and previous history for SLE of a patient population before developing and/or prescribing any drug with possible risk for inducing DIL or exacerbating existing SLE. However, despite all the advances in lupus research, further work is needed to understand what actually triggers the abnormal hypomethylation of these genes in lupus patients. A complete understanding of the role of this epigenetic aberration in development and progression of this complex multifactorial disease is still lacking. Nevertheless, the possibility of targeting the epigenome of lupus patients to provide a long-term therapeutic effect is still alluring.

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