Epigenetics signifies stable and heritable changes in gene expression without changes in the genetic code. There is a wealth of emerging evidence for such processes in promoting autoimmunity. The first clue is that inhibition of DNA methyl transferases (DNMTs) induces systemic lupus erythematosus (SLE) in animals. Similar immune-mediated disorders have been generated by injecting normal T cells incubated with DNMT inhibitors into healthy mice. Further, monozygotic twins display differences in DNA methylation that parallel discordances in SLE. Moreover, defects in DNA methylation characterize lymphocytes from SLE, synoviocytes from rheumatoid arthritis, and neural cells from multiple sclerosis patients. It has also been shown that DNA hypomethylation of T and B cells correlates with reduced DNMT efficacy and histone acetylation in SLE. Once a gene promoter has been demethylated, the gene recovers its capacity to be transcribed, e.g., genes for cytokines, activation receptors on cells, and endogenous retroviruses. This outcome has been associated with a blockage of the Erk pathway and/or a growth arrest at the G0/G1 interface of the cell cycle. Of importance is the fact that these changes can be reversed. For example, blockade of the interleukin-6 autocrine loop in SLE B cells restores DNA methylation status, thus opening new perspectives for therapy. (Keio J Med 60 (1) : 10–16, March 2011)

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Introduction

The sequence of events that leads to autoimmunity remains unknown. Although variations in a number of genes have been claimed to be associated with susceptibility to anti-self responses, there is growing evidence to indicate that, besides genetics, environmental factors participate in the development of autoimmunity (e.g., drugs, ultraviolet light, infection and diet). The involvement of such factors is reflected by the facts that the frequency of the consequential abnormalities differs from country to country and that geographic segregation of patients suffering from a given autoimmune disease has been identified. The perplexing observation that concordance with respect to systemic lupus erythematosus (SLE) is never 100% in monozygotic twins (MTs) raises the question as to what happens to the transcription and translation machineries between the genotype upstream and the phenotype downstream. The discordance results, at least in part, from epigenetics, which has been cited as a mechanism by which cells with as few as 30,000 genes differentiate into so many different cell types and vary so extensively at different developmental and functional stages.

In essence, gene mutations are permanent and affect all cells when passed through the germline. In contrast, epigenetics consists of stable (but reversible), and cell type-specific (but heritable) changes in gene expression.
which are unrelated to DNA alterations. Thus, several mechanisms govern gene expression over the cell cycle, regulate lineage development throughout its ontogenesis, and produce the response to environmental stimulations and to biological modifications.

This series of events supports the notion that the immune system is tightly regulated at the epigenetic level. Furthermore, ensuing alterations antedate the emergence of autoimmune traits and render genetically predisposed individuals at risk of developing overt autoimmune disease. Of critical importance, these disturbances are not restricted to idiopathic disorders, but have been implicated in the pathogenesis of autoimmune diseases induced by chemicals or drugs.

Epigenetic modifications of the kind described above are associated not only with autoimmune disorders but also with various pathological conditions, including cancer, heart insufficiency and skin afflictions. This recognition is the reason why the main purpose of the Epigenetics Session on the occasion of the 10th International Symposium on Sjögren’s Syndrome, in Brest, France, was to focus on academic works to allow a more comprehensive view of the current state of the related research. Further insights into these mechanisms may lead to the ability to restore epigenetic mechanisms, thus offering an exciting way to control the inflammatory process.

The Scope of Epigenetic Mechanisms

Main processes

DNA methylation

Three main epigenetic checkpoints exist in a normally regulated genome, DNA methylation, histone adjustments and micro-RNA. For transcription factors (TFs) to promote gene expression, they need to attach to their binding sites on DNA promoters, thereby activating them, and their target DNA must be accessible. Post-translational modifications of TFs contribute to nuclear translocation, oligomerisation, and binding to their target DNA. This bulk of reactions accounts for the earliest epigenetics processes. The most efficient way to silence gene transcription is to prevent the binding of TFs to DNA. To achieve this, DNA methyl transferases (DNMTs) convey a methyl group to the 5' carbon position of cytosines of cytosine-P-guanosine (CpG) dinucleotides (Fig. 1).

Most CpG is gathered together within specific regions, collectively designated CpG islands, that exist in regulatory areas of the gene. The concentration of CpG motifs reveals the propensity of 5-methylcytosine to mutate to thymidine when CpG motifs are present outside active genomic areas. A number of distinct DNMTs can be mobilized, viz. DNMT1, DNMT2, DNMT3a, DNMT3b, and DNMT3L. Among them, DNMT3a and DNMT3b induce new methylations, whereas DNMT1 requires a methylated cytosine on either of the strands and contributes to the maintenance of the DNA methylation pattern.

DNA demethylation can be passive or active. Cellular replication encourages passive DNA demethylation of the newly synthesized strand, as confirmed by inhibition of DNMTs at the G0/G1 interface of the cell cycle. In addition, DNA can be actively demethylated by enzymes independently from DNA replication.

The reverse phenomena may also occur, and, instead of being methylated by DNMTs, DNA is demethylated by methyl-CpG-binding domain (MBD) proteins. Methyl-CpG-binding domain protein-4 (MBD4) is a DNA glycosylase that acts preferentially on hemi-methylated CpG, and thereby completes demethylation by replacing 5-methylcytosine with unmethylated cytosine. However, some controversy exist over the efficacy of this process in mammalian cells.

Histone adjustments

The nucleosome is the basic subunit of chromatin. It comprises 146 base pairs (bp) of DNA wrapped around an octamer of two copies each of H2A, H2B, H3 and H4 classes of histones. Nucleosomes, present at an estimated 10 million per cell, are organized into regular arrays (Fig. 2). These structures present as small glomerular proteins with a flexible N-terminal tail protruding from the nucleosome that is accessible to modifications that
impart functional capacities to the histones. The modifications include acetylation, methylation, ubiquitination, phosphorylation, sumoylation, deimination/citrullination, ADP-ribosylation and proline isomerization. Each modification serves a specific purpose. Good examples of opposite effects are enhancement of transcription by histone H3K9 acetylation and repression of transcription by histone H3K9 methylation.

DNA methylation and histone adjustments are intertwined by multiple mechanisms. For example, it has been shown that the MBD proteins selectively bind methylated CpG and recruit histone deacetylase or histone methyltransferase. In contrast H3K4m3, a transcriptionally active variant, blocks the binding of DNMT3L to DNA, which is essential for the action of DNMT3a, and abrogates DNA methylation within transcriptionally active regions.

**Micro-RNA**

Micro-RNAs (miRNAs) consist of 21-23 bps of RNA and function as post-translational regulators of gene expression. One-third of the human transcriptome is regulated by 1,000 miRNAs. Some of them interact with transcripts of genes that modulate DNA and histone methylation. Conversely, miRNA expression can be affected by DNA methylation and histone accommodation.

**Factors of epigenetics**

**Age**

Given the absence of genetic differences between MTs, they are ideal to evaluate epigenetic modifications that are caused by environmental factors. Using peripheral blood lymphocytes from elderly and young MTs, Fraga et al. have established that the pattern of DNA
methylation of the two members of a pair of MTs, as well as their H3 and H4 acetylation profile, diversifies increasingly as they age. One reason for this is that total genomic 5-methyl cytosine content decreases with age, but at different rates in one twin than the other. Similarly, in tissue culture, genomic DNA is demethylated in the long term, particularly DNA of those genes involved in cell differentiation. A supplementary reason for increased diversity of acetylation with age is that environmental exposure is cumulative, so that there is every likelihood that small initial differences will become substantial with time. Interestingly, the environmental theory was further supported by the observation that MTs with the greatest differences in epigenetic modifications were in those who had spent less of their life together.

Gender
Females produce equivalent transcripts from their X-linked genes, as compared with males, even though they have two X chromosomes. Equivalency results from the inactivation of one of the two X chromosome in each cell by a process termed dosage compensation. The inactive X chromosome is referred to as Xi, and is characterized by high levels of DNA methylation, histone modifications, and recruitment of the silencing histone variant macro H2A. Of note, 35% of Xi-p genes and 5% of Xi-q genes are partially active. Whether the maternally derived or paternally derived X chromosome is inactivated is randomly determined at the embryonic stage and is maintained throughout life.

Drugs
A number of drugs have been suspected of causing SLE, most notably, procainamide, hydralazine and 5-azacytidine. Evidence has been provided by Richardson’s group16, 17 that these drugs inhibit DNA methylation. The same investigators have reported data supporting an increase in lymphocyte function-associated antigen expression and the ensuing proliferation of autoreactive T cells in SLE patients.18

Human endogenous retroviruses
Eight per cent of the human genome derives from the integration of retroviral sequences, together with their long terminal repeats, that were incorporated into our DNA more than 25 millions years ago.19 These human endoretroviruses (HERVs) generally lack an extracellular phase, and the genetic material is neutralized by methylation.

Hypomethylated T Lymphocytes in SLE
Accumulation of autoreactive lymphocytes and production of antibodies (Abs) against a wide range of self antigens (Ags) are the hallmarks of autoimmune diseases that damage kidneys, skin, lung and other organs. The role of genetics has been suggested by the observation that the incidence of SLE is 5-fold higher in MTs than in dizygotic twins. However, the concordance rate between MTs can range from 5% to 75%, and these variations suggest the involvement of additional triggers from the environment.

CD4+ T cells are hypomethylated in SLE
The CD4+ lymphocytes of SLE patients, but not their CD8+ T lymphocytes, manifest a defective capacity to methylate their DNA; the degree of this inhibition correlates with disease activity.22 The consequence of this fault is that several methylation-sensitive autoreactivity-promoting genes are overexpressed in CD4+ T cells, including23-28 those for perforin, immunoglobulin (Ig)-like receptor, interleukin (IL)-4, IL-6, and the B cell co-stimulatory molecules CD70, CD6 and CD154.

However, the results of studies of methylating and demethylating enzymes in SLE patients are conflicting. For example, DNMT1 and DNMT3a are down-regulated in CD4+ T cells from SLE patients with active disease in some,29, 30 but not in all studies.31, 32 According to some investigators, two DNMT1-targeting miRNAs are up-regulated in a number of patients,23 whereas, according to other investigators, the MBD4 protein is up-regulated in CD4+ and CD8+ T cells, but not in B cells, from patients with SLE.31, 34, 35

Variations have also been described in histones of CD4+ T cells from patients with SLE.36 In particular, H3 and H4 acetylation levels have been shown to correlate negatively with disease activity. Nevertheless, the degree of methylation of global histone H3K9, but not that of methylation of H3K4, is reduced in CD4+ T cells from SLE patients, irrespective of disease activity.

Regulatory T cells and hypomethylation
Regulatory T cells (Treg) are characterized phenotypically by the expression of CD25 on their surface and TF Foxp3 in their cytoplasm. Functionally, they are defined by their suppressive effects on effector T lymphocytes, B lymphocytes, and Ag-presenting cells. Foxp3 is the master TF of Treg cell development. Its expression is indispensable for Treg cells to exert their function. However, it is believed that not all Foxp3-positive T cells possess suppressive function. Supporting this suspicion is the fact that some individuals develop SLE despite having a normal number of Foxp3+ T cells.37 One possible explanation is that such Foxp3+ T cells are not genuine Treg cells, but result from hypomethylation-induced overexpression of Foxp3 in T cells that are not likely to serve as Treg cells.38
**X chromosome and CD40 ligand**

The greater prevalence of SLE in men with Klinefelter’s syndrome suggests that, despite the presence of one Y chromosome, the coexistence of two X chromosomes may lead to the development of SLE.39 Similarly, the recent observation that demethylation of the inactivated X chromosome in CD4+ T cells from female SLE patients is associated with overexpression of the B cell-stimulating CD40 ligand points to a potential reason for the female sex predominance in SLE.28

**B Cell Abnormalities in SLE**

**CD5+ B cells are hypomethylated**

The co-receptor CD5 is expressed in all T lymphocytes and in a minor subpopulation of B lymphocytes at various developmental and activation stages. Thus, CD5-expressing B lymphocytes are referred to as B1 cells, whereas conventional CD5-nonexpressing B lymphocytes are designated B2 cells. The finding that B1 cells produce polyreactive Abs with low-affinity binding to multiple Ags, including self Ags, suggests that they are the main source of natural Abs, and leaves the possibility that, subsequently, they can also secrete pathogenic autoAbs.40

Several facts support the view that DNA in B1 cells is hypomethylated, and thereby the gene for CD5 is likely to be transcribed into messenger RNA (mRNA). First, the activity of DNMT1 is reduced in CD5+ B cells.35 Second, CD5+ B cells express a fusion transcript between an HERV and the CD5 gene.41 This HERV was integrated into the genome at a time between the divergence of New World monkeys from Old World monkeys and the divergence of humans from Old World monkeys.42 When we reported this phenomenon, the new exon 1 was designated exon 1B, and the known exon 1 was renamed exon 1A. The transcription of exon 1B is normally regulated by methylation, and, contrary to all expectations, its expression is restricted to B1 cells, i.e., it depends on the methylation status. The third supporting fact is that incubation of B2 cells with DNA methylation inhibitors induces the expression of CD5-E1B similar to that of B1 cells.35

Interestingly, selection of HERV-exon 1B downregulates the membrane level of the protein product in B lymphocytes of patients with SLE.43 Exon 1B-containing mRNA codes for a truncated variant of CD5. Owing to the lack of leader peptide, the protein cannot be translocated to the membrane and be used to facilitate anergy of autoreactive B cells. This raises the possibility that HERV demethylation participates in the pathogenesis of SLE.44,45

**CD5-negative B cells are hypomethylated in SLE**

Given that B cell receptor-stimulated B2 cells from SLE patients, but not from controls, express high amounts of CD5-E1B, we have explored the DNA methylation status of B2 cells from patients with SLE.35 Analysis of the CpG sites in the U3 promoter region present in the 5’ long-terminal repeats of HERV-CD5 using methylation-sensitive endonuclease assays followed by polymerase chain reaction and bisulfite sequencing revealed that CpG motifs are hypomethylated in B2 cells from patients with SLE, as compared with those from healthy controls (Fig. 3).

In addition to these observations, our studies have indicated that cytokines may be involved in influencing epigenetics. For example, IL-6 enables B cell differentiation, maturation and Ig secretion. This multifunctional cytokine is synthesized predominantly by monocytes, fibroblasts and endothelial cells, but can also be produced by T and B cells. In humans, IL-6 gene polymorphism is associated with SLE, and elevated IL-6 levels parallel high disease activity and raised titres of anti-double-stranded DNA Abs. Further, it has been shown that autoreactive B cells from SLE patients release high amounts of IL-6 and that IL-6 experimental antagonism abolishes spontaneous Ig production by restoring DNA methylation in SLE B cells.

**Effects on the genesis of SLE**

The direct implication of hypomethylated DNA in B cells at the outset of SLE has recently been confirmed.48 B cells were purified from mice, treated ex vivo with DNMT inhibitors, and subsequently reintroduced in syngeneic mice by adoptive transfer. The treatment promoted antinuclear autoAb production in the recipient mice.

**Conclusions**

One of the most intriguing characteristics of epigenetics is the reversibility of its modifications. Although there remains a need for new animal models and specific cell-lines to test for suitable agents, some drugs have already been used to treat murine malignancies. Because controlling DNA methylation remains a challenge, several approaches have been proposed. These include blocking the autocrine IL-6 loop in SLE B cells,35 preventing adenosylmethionine degradation by decarboxylase, or regulating miRNAs. While our knowledge of epigenetics in SLE is limited, the epigenome revolution has started, and new tools for diagnosis, prognosis, and therapy should emerge in the near future.49,50
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