Ultraviolet B exposure of peripheral blood mononuclear cells of patients with systemic lupus erythematosus inhibits DNA methylation

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Systemic lupus erythematosus (SLE) is an autoimmune inflammatory disease, in which sunlight (especially ultraviolet B (UVB) 290–320 nm) is known to induce exacerbation of disease. DNA methylation regulates gene expression, and hypomethylation is associated with abnormal cell function in SLE. The purpose of this study was to investigate the effect of UVB on DNA methylation in SLE and its significance in the pathogenesis of SLE. Forty-five patients with SLE and 20 healthy controls were enrolled in the study, which involved the investigation of DNA methylation and DNA methyltransferase 1 (DNMT1) of peripheral blood mononuclear cells with UVB irradiation. Our results demonstrate the following: The level of DNA methylation in patients with SLE was lower than that in the control group. DNA methylation was decreased after UVB irradiation at different dosages especially in patients with marlar rashes and leucopenia, but no significant difference was observed in the DNMT1 mRNA expression. DNA methylation levels in patients with active SLE were more sensitive to UVB. In conclusion, UVB exposure is able to inhibit DNA methylation, which subsequently takes part in the pathogenesis of SLE. Lupus (2009) 18, 1037–1044.

Key words: DNA methylation; DNA methyltransferase 1; lupus erythematosus; systemic; ultraviolet

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterised by the production of autoantibodies and the involvement of multiple organ systems and has a higher prevalence in females than in males. The molecular mechanisms initiating the autoimmune process in lupus remain unknown. Its multifactorial aetiology includes genetic, hormonal, environmental and immunologic factors. Environmental effects on lupus could play a role in mediating epigenetic changes in immunity. Epigenetic modifications including DNA methylation and histone modifications have a significant impact on gene expression that occur without a change in DNA sequence and are meiotically or mitotically heritable. DNA methylation is a fundamental determinant of chromatin structure with potent suppressive effects on gene expression. DNA methylation in eukaryotes occurs at the 5th position of the cytosine ring in the context of CpG dinucleotides and is catalysed by DNA methyltransferases.1 Maintenance of DNA methylation is mediated by DNA methyltransferase 1 (DNMT1), whereas de novo methylation is carried out by DNMT3a and DNMT3b.2 In general, inactive genes are silenced by methylation, whereas the promoters of transcriptionally active genes are typically hypomethylated. The first evidence of the role of aberrant changes in the DNA methylation patterns in the development of SLE was that T cells from patients with active lupus were shown to exhibit globally hypomethylated DNA.3 Additional evidence of the role of methylation changes in the development of SLE comes from studies with DNA demethylating drugs. One of the most common demethylating drugs used to induce SLE in mice is 5-azacytidine. Other demethylating drugs used to induce SLE are procainamide,4 a competitive DNMT inhibitor, and hydralazine, whose demethylating...
activity has been explained as an indirect result of the inhibition of the ERK pathway signalling, decreasing DNMT1 and DNMT3a levels during mitosis.5

In addition to these drugs, ultraviolet (UV) light can also potentially induce or aggravate lupus. Up to 73% of patients with SLE report photosensitivity.6 Most cutaneous lupus lesions can be triggered by sunlight exposure. Sunlight exposure, especially ultraviolet B light (UVB, 290–320 nm), can even induce systemic disease activity. A link between UV irradiation, defective post-replication repair and altered methylation has been reported: UV irradiation was shown to lead to specific demethylation events during subsequent rounds of replication.7 As a consequence of aberrant repair or repair methylation, UV irradiation has also been used to activate the transcription of a quiescent metallothionine gene, which, based on 5-azacytidine-reactivation experiments, is thought to be under methylation control.8 However, whether UVB can influence SLE through altering DNA methylation has remained uncertain.

In this study, we investigated the level of DNA methylation and DNMT1 gene expression in patients suffering from SLE following different dosages of UVB irradiation. Our data suggested that a decrease of DNA methylation in patients with SLE after irradiation may play a role in the pathogenesis of SLE.

Materials and methods

Study participants

The study was performed in 45 patients with SLE, 40 females and 5 males (mean age: 36 years, range: 18–61 years). Twenty healthy volunteers, sex- and age-matched, served as a control group (2 men, 18 women; mean age 31 years, range: 22–46 years). Patients with SLE met at least four of revised criteria of the American College of Rheumatology.9 The study was performed on patients with SLE available during the period of collecting samples, actually diagnosed or under observation, with inactive or active disease, untreated or treated with steroids. SLE activity was assessed by the SLE disease activity index (SLEDAI),10 and those with an SLEDAI of ≥10 were considered to have active disease. In 45 patients, 21 had active disease (10 cases with newly diagnosed lupus and 11 cases with lupus flare-ups), the other 24 patients were stable. The 10 patients with newly diagnosed lupus had not received any treatments. The 11 patients with lupus flare-ups were given prednisone at a dosage no greater than 10 mg/day before the time the blood was drawn. All the stable patients received prednisone at a dosage no greater than 10 mg/day.

Isolation of peripheral blood mononuclear cells

A total of 20 ml of ethylenediaminetetraacetic acid (EDTA)-K2-preserved venous peripheral blood was drawn from both patients and controls. Peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll gradient centrifugation. PBMCs were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated foetal bovine serum (FBS), 2 mM sodium pyruvate, 100 IU/ml penicillin and 100 μg/ml streptomycin.

Irradiation protocol

UVB irradiation was performed using Waldman UV109B lights with TL-12 lamps (Waldman Lighting Ltd., Germany), which emit most of their energy within the UVB range (290–320 nm) with an emission peak at 311 nm. PBMC were irradiated in PBS with different doses of UVB (0, 50 and 100 mJ/cm²). PBS was removed after irradiation, and then RPMI 1640 containing 10% FBS was added and the cells were cultured for 24 h. Following culture, DNA and RNA were extracted. Doses of UVB were chosen based on the World Health Organisation guidelines for sun exposure11 and on the standard erythemal dose (SED), a cumulative measure of erythemal or sunburning solar UV irradiation.12

DNA extraction and high-performance capillary electrophoresis

Five deoxynucleoside standards, 2′-deoxyadenosine (dA), 2′-deoxythymidine (dT), 2′-deoxyguanosine (dG), 2′-deoxycytidine (dC) and 5-methyl-2′-deoxy- cytidine (mdC), were purchased from the International Laboratory (CA, USA). All the nucleosides were dissolved in ddH2O water. DNA extraction was carried out with a genomic DNA extraction kit (QIAamp, DNA mini kit; Qiagen, Hilden, Germany). RNA digestion was performed by adding 20 μg/μl RNAase A (Sigma-Aldrich, St. Louis, MO). For DNA hydrolysis, 18 μl of DNA samples were heated for 2 min in a boiling water bath and cooled rapidly in ice; 4.5 μl of 10 mM ZnSO4 and 7.5 μl of nuclease P1 (Sigma-Aldrich, St. Louis, MO) were added and the mixtures were incubated for 16 h at 37 °C. Then 7.5 μl of Tris (0.5 M, pH 8.3), 4.5 μl of alkali phosphate (Sigma-Aldrich, St. Louis, MO) and 50 units/ml in 2.5 M (NH4)2SO4 were added and the mixtures were incubated for an additional 2 h at 37 °C. Samples were stored at 4 °C

An uncoated fused-silica capillary (60 cm × 75 μm, effective length 57 cm) was used in a CE system (BECKMAN P/ACETM MDQ) connected to a
Millenium data-processing station. The buffer was 48 mM NaHCO₃ (pH 9.6) containing 60 mM SDS. Constant voltage (20 kV) and temperature (25 °C) were used. All samples were pressure injected for 5 s. Hydrolysed DNA or 0.1 mM of free deoxynucleoside standard was injected into a high-performance capillary electrophoresis (HPCE) analysis system equipped with a photodiode array (PDA) detection. Absorbance was monitored at 256 nm. Before each run, the capillary was conditioned by washing first with 1 M NaOH for 2 min and then with 1 mM NaOH for 1 min. Finally, it was filled with the running buffer for 3 min. Buffers and washing solutions were filtered through 0.45 μm pore size filters. Hydrolysed samples were injected hydrostatically for 30 s from a height of 9.8 cm above the cathode. By comparing the migration time and peak area of each sample electropherogram with the standards, DNA methylation levels can be calculated. Quantification of the relative methylation of each DNA sample was determined as the percentage of mdC of total cytosines: mdC/(dC + mdC) × 100%.

RNA isolation and real-time PCR

The total RNA was isolated from PBMCs using Trizol reagent according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). Reverse transcription was carried out using the Reverse Transcription System (Promega, Madison, WI, USA). For cDNA synthesis, 1.0 μg total RNA was used in a single-round reverse transcriptase reaction (total volume 20 μl), containing 0.5 μg oligo (dT) 15 primer, 2.0 μl dNTPs, 2.0 μl reverse transcriptase 10× buffer, 15 μl AMV reverse transcriptase and 0.5 μl recombinant RNasin ribonuclease inhibitor. Real-time PCR was subsequently performed in the ABI Prism 7500 Sequence Detection System using the SYBR Premix Ex Taq Kit (TaKaRa Bio Inc.). Each reaction contained 10 μl of 2× SYBR green Master Mix, 0.2 μM primers, 5 μl of 1:5 dilution of the cDNA prepared above and 20 μl of water. The reactions were then followed by 40 cycles for 30 s at 94 °C, 40 s at 60 °C and 60 s at 72 °C. The primers used in this study were as follows: β-actin, forward 5'-TGGCCACGACATAATGAA-3' and reverse 5'-CTAA GTCATAGTCCGCTTAGAAGCA-3'; DNM1, forward 5'-CTTCGGCAACCATCTGGGACA-3' and reverse 5'-CTTGGGCAACATACAAAGC TTGA-3'. All primers were purchased from TaKaRa. The standard curve was constructed with serial dilutions of reverse transcription products corresponding to different concentrations of purified PCR production. Expression was compared with the standard curve and reported in equivalent quantity of purified PCR production from the reference cell line. Normalisation of RNA amounts was performed using β-actin expression analysed with the same procedure. Finally, expression ratios between the gene of interest and β-actin were calculated.

Statistical analysis

Either the Mann–Whitney U-test or one-way ANOVA for equality of means (along with the Levene’s test for equality of variances) was used to compare values. Spearman’s rank correlation was used to examine the relationship between two continuous variables. P-values of <0.05 were considered significant. All analyses were performed using SPSS, version 10.0 (SPSS Inc., Chicago, IL, USA).

Results

Electropherogram

Under the analytical condition selected, five deoxynucleoside standards could be well separated. After samples were treated, the corresponding peak appeared under the conditions of a running buffer of 48 mM NaHCO₃, at pH 9.6, containing 60 mM SDS, with a separation voltage of 20 kV, injection pressure of 0.7 psi, at 25 °C and hydrodynamic injection of 5 s (Figure 1).

Calibration curves

Calibration curves were established with the peak area as the ordinate (y) versus the concentration of analyte as the abscissa (x). Different quantities standards of dC and mdC were added to a mixture of dA, dT and dG (0.1 mM), to obtain the following dC and mdC concentration: dC (0.01, 0.02, 0.04, 0.06, 0.08 and 0.09 mM); mdC (0.001, 0.002, 0.0025, 0.005, 0.01 and 0.02 mM). Each mixture was prepared three times and three runs of each were performed. According to the peak area station, the corresponding concentration was achieved (Figure 2).

The comparison of the level of genomic DNA methylation between the SLE group and the control group

To determine whether global methylation was decreased in patients with SLE, DNA was extracted from 45 patients and 20 normal controls. The level of DNA methylation in patients with SLE was significantly lower than that in the control group (P = 0.042). DNA methylation in the active and stable...
SLE groups significantly decreased when compared with the control group, \( (P = 0.032, P = 0.032) \), while no apparent differences were found between the two SLE groups \( (P = 0.931) \) (Figure 3).

Comparison of DNA methylation after different dosages of irradiation

DNA methylation level after different dosages of irradiation is summarised in Table 1. DNA methylation level decreased after UVB irradiation in the SLE group and the control group. DNA methylation decreased significantly after 50 mJ/cm\(^2\) irradiation in active SLE group, while decreased significantly after 100 mJ/cm\(^2\) irradiation in the stable SLE group and the control group. Overall, patients with active SLE after irradiation in particular had very low levels of DNA methylation (Figure 4).

Comparison of the expression of DNMT1 mRNA in PBMC between the SLE group and the control group

Once the methylation status was found to be decreased in patients with SLE, quantitative real-time PCR assays were carried out to evaluate the
mRNA levels of DNA (cytosine-5) methyltransferases 1. β-actin was chosen as a control to normalise mRNA levels because its expression does not change in SLE. Patients and controls showed similar mRNA levels of DNMT1, no apparent differences were found among the active SLE group, the stable SLE group and the control group.

When the UVB radiation was taken into account, no significant differences could be found in the expression of DNMT1 after UVB radiation (50 mJ/cm² and 100 mJ/cm², respectively) in patients with SLE and the control group (Figure 5).

Table 1  The level of DNA methylation after different dosages of irradiation in SLE and control groups (% ± s)

<table>
<thead>
<tr>
<th>Group</th>
<th>Cases</th>
<th>0 (mJ/cm²)</th>
<th>50 (mJ/cm²)</th>
<th>100 (mJ/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active SLE group</td>
<td>21</td>
<td>13.3 ± 5.3</td>
<td>8.9 ± 3.5*</td>
<td>6.8 ± 4.6*</td>
</tr>
<tr>
<td>Stable SLE group</td>
<td>24</td>
<td>13.5 ± 4.0</td>
<td>11.7 ± 4.4</td>
<td>8.3 ± 3.2*</td>
</tr>
<tr>
<td>Control group</td>
<td>20</td>
<td>17.6 ± 7.4</td>
<td>14.1 ± 5.6</td>
<td>11.5 ± 4.6*</td>
</tr>
</tbody>
</table>

Values shown represent the mean ± standard deviation.
*Statistically significant differences were found with DNA methylation between different dosages of irradiation versus with 0 mJ/cm² (P < 0.01).

We then wanted to ascertain whether a relationship between DNMT1 mRNA expression level and DNA methylation existed and if such behaviour was identical both in controls and in patients with SLE. No significant correlation was found before or after different doses of UV radiation.

Correlation between DNA methylation and SLE manifestations, immunological parameters and glucocorticoid treatment

Among all 45 patients with SLE, 10 were newly diagnosed lupus with no underlying treatment, and 35 were patients receiving prednisone at a dosage no greater than 10 mg daily before blood was drawn. The DNA methylation levels in the newly diagnosed patients were significantly lower than that in patients using glucocorticoid (P = 0.01).

Among the 45 patients with SLE, 13 had malar rashes and 14 had haematological lesions (of which 10 had leucocytopenia and 8 had thrombocytopenia). Table 2 indicates that methylation levels for all patients decreased after UVB irradiation irrespective of the presence or absence of malar rashes and haematological lesions. Furthermore, methylation levels in patients with malar rashes were lower than that seen in patients without rashes, irrespective of UVB irradiation at any dosage. After UVB irradiation, methylation level in patients with leucopenia was significantly lower than that in patients with normal leucocyte numbers. No obvious differences were observed.
between patients with or without thrombocytopenia, serositis, arthritis, lupus nephritis and SLEDAI.

To determine whether any of the methylation variables we had studied were correlated with the laboratory parameters, we performed Spearman’s correlation tests. No significant correlation was found between the level of DNA methylation and some indexes, including ANA titre, anti-dsDNA antibody, anti-nucleosome antibody, anti-C1q antibody, immunoglobin IgG, IgM, IgA and complement C3, C4.

Discussion

DNA methylation, a ubiquitous epigenetic modification in eukaryotes, plays an important role in the pathogenesis of SLE. After hydrazine and procainamide treatment, the expressions of LFA-1, CD70 and perforin in T-cell increased, which was in accordance with the hypomethylation of coding genes for promoter or enhancer. Similarly, the increased expression of LFA-1, CD70 and perforin and changes of DNA methylation were observed in patients with SLE.\textsuperscript{13–15} UVB exposure is an environmental factor and may play a role in the pathogenesis of SLE.\textsuperscript{13–15} UVB exposure can promote the secretion of IL-1, TNF-\(\alpha\) in keratinocyte cells, mast cells and Langhans’ cells, and can recruit and activate dendritic cells, T cells and plasma cell-like dendritic cells (pDC) to release IFN-\(\alpha\), further releasing chemotatic factors.\textsuperscript{16} Meanwhile, UVB exposure can induce proinflammatory cytokines in murine lupus PBMC.\textsuperscript{17} Kastan, \textit{et al.}\textsuperscript{7} found that the specific DNA demethylation may occur after UV radiation, which had been confirmed by subsequent study that the level of DNA methylation catalysed by DNMT was inhibited by UVB radiation in a phage DNA model.\textsuperscript{18} However, there are currently no published studies concerning the effect of UVB exposure on DNA methylation in the patients with SLE.

At present, there are many ways to detect genomic DNA methylation, such as HPLC, SSsI methyltransferase, immunochemistry, chloroacetaldehyde and HPCE. Compared with the traditional HPLC, HPCE is a relatively novel method, which is more simple, rapid and economic. In 2002, Fraga, \textit{et al.}\textsuperscript{19} used HPCE to analyse DNA hydrolysates and then compared them with standard preparations, followed by the detection of absorption peaks and volumes using UV light and obtained overall level of genomic methylation by calculating the ratio of 5 mdC/(5 mdC + 5 dC). In this study, HPCE was used to detect the level of genomic DNA methylation before and after UVB exposure.

We first analysed DNA methylation levels in patients with SLE and in control groups without UVB irradiation. The results showed that the level of DNA methylation in patients with SLE without UVB irradiation was significantly lower than that of the control group. These findings suggested that the down-regulation of DNA methylation might play an important role in the pathogenesis of SLE. To our great interest, DNA methylation decreased after different dosages of irradiation, suggesting that UVB exposure may play a role in the pathogenesis of SLE by decreasing DNA methylation. Moreover, DNA methylation decreased significantly after low dose of UVB (50 mJ/cm\(^2\)) irradiation in active SLE group, while decreased significantly after the high dose of UVB (100 mJ/cm\(^2\)) irradiation in the stable SLE group and the control group; active patients with SLE seem to have higher sensitivity to UVB exposure that results in significant decrease of DNA methylation level. This finding is consistent with clinical phenomenon that active patients are more sensitive to sun light especially in patients with anti-SSA(Ro) antibodies, which was highly expressed in patients with active SLE.

Among the DNMTs that have been studied,\textsuperscript{20} DNMT1 plays the most important role in methylation by maintaining the methylation for new subchains. The mechanisms of DNMT1 are as follows: 1) amino terminal of DNMT1 can have an effect on catalytic activity of carboxyl terminal by reaction to partial conformational change of DNA double helix, or interactions with other regulatory proteins;
2) amino terminal of DNMT1 contains a cysteine rich domain (similar to a zinc finger), which may bind with DNA duplexes in the major groove via specific base pairing. High expression of DNMT1 in tumours is always accompanied by hypomethylation of genomic DNA and cancer genes or hypermethylation of tumour suppressor genes. Balada, et al. found that there were no significant differences in DNMT1 gene expression in CD4+ T cells between the SLE group and the control group. Consistent with this result, our study demonstrated that no significant differences in DNMT1 mRNA expression were found in the SLE group and the control group after different doses of UVB radiation. DNA methylation decreased after irradiation, indicating that there may be some feedback mechanisms between DNA methylation and DNMT1 or DNA methylation level of DNMT1 coding gene itself may be affected by UVB exposure. The DNMT1 protein expression and its activity before and after UV radiation should be further studied.

Moreover, a correlation analysis was made between the level of DNA methylation and specific indices, including SLEDAI, ANA titre, anti-dsDNA antibody, anti-nucleosome antibody, anti-C1q antibody, immunoglobulin IgG, IgM, IgA and complement C3, C4. However, no correlation was found, which may be related to the following reasons: 1) DNA methylation is a kind of epigenetic modification and regulates gene expression. However, gene expression is affected by many factors like classical genetic alterations, so DNA methylation is not the only factor that determines the production of antibodies and immunoglobulins. 2) Current studies on DNA methylation in patients with SLE are more concentrated on T cells, rather than B cells and genomes; however, the antibody is mainly produced by plasma cells that are differentiated from B-cell. Therefore, the relationship between DNA methylation and antibodies needs to be further clarified. 3) The combination of acceleration of apoptosis and defec tion in clearance of apoptotic cells can cause abnormal methylation of nucleosome, release more GC-rich DNA, trigger self-stimulation and induce loss of self-tolerance in the process of autoimmune diseases, which has been proved by Huck, et al. In this experiment, no correlation between DNA methylation and anti-nuclear antibody was observed, a finding which merits further study.

In this study, we also find that the alterations of DNA methylation correlate with some clinical characteristics. Methylation level of patients with malar rashes decreases significantly. This is consistent with clinical observations. In clinical practice, patients with SLE with rash, especially those with photosensitivity, exhibit aggravated disease following UV irradiation. Following UVB irradiation, methylation levels in patients with leucopenia was significantly lower than that in patients without leucopenia. These results indicate that leucopenia is closely related to the methylation level.

Do DNA methylation levels change with glucocorticoid use? We find that DNA methylation levels in newly diagnosed patients with lupus who did not receive any therapy were significantly decreased. Until now, the relationship between glucocorticoid treatment and DNA methylation level had received little attention. Our results indicate that methylation levels increased after treatment with glucocorticoid. However, most patients receiving glucocorticoid had stable SLE, so it is possible that the change could be either as a result of glucocorticoid treatment or as a natural result of disease control.

In conclusion, DNA methylation decreases after UVB exposure especially in active lupus and patients with malar rashes and leucopenia, indicating that UVB may play a role in the pathogenesis of SLE, which provided the relevant experimental basis for our further studies on the mechanism of SLE. But the specific mechanism of how UVB changes DNA methylation in patients with SLE needs further investigation to prove. This study will advance the theory of epigenetic modification in SLE genesis and provide a rational basis for new preventions and treatments of SLE via sun protection.

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