Possible role of DNA hypomethylation in the induction of SLE: Relationship to the transcription of human endogenous retroviruses

H. Ogasawara, M. Okada, H. Kaneko, T. Hishikawa, I. Sekigawa[1], H. Hashimoto

Department of Internal Medicine and Rheumatology, Juntendo University School of Medicine, Tokyo; [1]Department of Medicine, Juntendo University Izu-Nagaoka Hospital, Shizuoka, Japan.

Abstract

Objective

We investigated the contribution of DNA methyltransferase activity to the transcription of human endogenous retroviruses (HERV), which have been reported to be a plausible causative agent for systemic lupus erythematosus (SLE).

Methods

The reverse transcriptase-polymerase chain reaction (RT-PCR) and real-time quantitative-PCR (RQ-PCR) were used.

Results

Our results indicated that treatment with 5-aza-deoxycytidine (5-aza C), a demethylating agent, increased the transcription of messenger RNA (mRNA) for HERV clone 4-1 and decreased mRNA for DNA methyltransferase-1 (DNMT-1; methylation-regulating enzyme) in peripheral blood mononuclear cells (PBMC) from normal individuals. Also, transcription of DNMT-1 mRNA in PBMC from patients with SLE was lower than in cells from normal controls.

Conclusion

DNA hypomethylation seems to play a significant role in the transcription of HERV clone 4-1 and may be related to the pathogenesis of SLE.

Key words

Systemic lupus erythematosus, human endogenous retroviruses, DNAmethyltransferase.

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Please address correspondence and reprint requests to: Iwao Sekigawa MD, Department of Medicine, Juntendo University Izu-Nagaoka Hospital, 1129 Nagaoka, Izu-Nagaoka-cho, Tagata-gun, Shizuoka 410-2295, Japan.
E-mail: isekigawa@mva.biglobe.ne.jp

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Introduction

HERV are widely found in human genomic DNA and generally lack the extracellular phase that is normally characteristic of retroviruses because of several interrupters, including termination codons, deletions, frame shift mutation, or methylation sites (1). Several reports have suggested that expression of HERV proteins induced by the activation of HERV sequences may play a significant role in the development of autoimmune diseases such as SLE (2-4). We recently reported that the transcription and translation of HERV clone 4-1-like sequences (clone 4-1 is a member of the HERV-E family) are markedly increased in patients with SLE when compared with normal controls (4-10). This raises the possibility that HERV clone 4-1 may contribute to the pathogenesis of SLE in certain patients.

Cytosine methylation of the regulatory sequences of some genes is associated with transcriptional inactivation, while hypomethylation of these sequences is associated with active transcription (11-13). Previous studies have detected hypomethylation of T cell nuclear proteins from SLE patients and have shown that inhibitors of DNA methylation such as 5-aza C can induce SLE-like autoreactivity and autoantibody production both *in vitro* and *in vivo* (13-18). DNA methyltransferase (DNMT) is the enzyme responsible for the methylation of DNA in mammalian cells and DNMT-1 is the first member of this family (19, 20). Accordingly, we investigated the relationship between HERV clone 4-1 and DNMT-1 mRNA transcription, and assessed the possible role of DNA hypomethylation in the development of SLE.

Patients and methods

Patients and cells

The patients (7 women and 2 men aged 21-48 years) investigated in this study were diagnosed as having SLE according to the 1982 revised criteria of the American College of Rheumatology (21). Control samples were obtained from age- and sex-matched healthy volunteers. The main clinical manifestation of SLE was nephropathy and our patients showed high serum levels of anti-DNA antibodies and low CH50 levels. Three patients had not received any steroid therapy, while the remaining 6 patients had been on prednisolone for between 1 and 48 months. No patient received any immunosuppressive agent before the collection of samples and none of the subjects was pregnant.

PBMC were separated from peripheral blood samples of the SLE patients and normal volunteers by centrifugation on a Ficoll-Paque cushion. The cells were cultured with 100 μM 5-aza C (Sigma Chemical Co., St Louis, MO) for 48 hours in a 5% CO_2 incubator at 37ºC using RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (Gibco Laboratories, Grand Island, NY).

RT-PCR analysis

Total mRNA was isolated from PBMC (1 x 10^7) using a QIAamp RNA Blood Mini Kit (QIAGEN Inc., Valencia, CA). Then cDNA was obtained from the purified RNA using a First Strand cDNASynthesis Kit (Amersham International plc, Buckinghamshire, England) with avian myeloblastosis virus (AMV) RT. Briefly, mRNA (2 μg) was incubated with 2.5 μg of oligo-dT, 10 μl of the first strand synthesis reaction buffer, 2.5 μl of sodium pyrophosphate solution, 5 μl of dNTP mixture, and 5 μl of human placental ribonuclease inhibitor in the presence of AMV RT (20 units) at 42ºC for 50 min.

RT-PCR of cDNA with primers for the DNMT-1 gene (20) was performed using Clontech Amplimer Sets (Clontech Laboratories, Inc., Palo Alto, CA) that contained the following primers: 5,-TTC CGG CTG AAC AAC CC-3, (DNMT primer 1, nt 3766-3782) and 5,-TGT CTC GCA CCG TGA TGG TCC G-3, (DNMT primer 2, nt 4324-4345) (Fig. 1). An aliquot (1 μg) of cDNA was used as the template for each PCR, which involved 35 cycles of denaturation at 94ºC for 1 min, annealing at 56ºC for 90 sec, and elongation at 72ºC for 2 min in the standard PCR mixture (Perkin-Elmer Cetus, Emeryville, CA, USA). To examine DNA contamination, samples containing

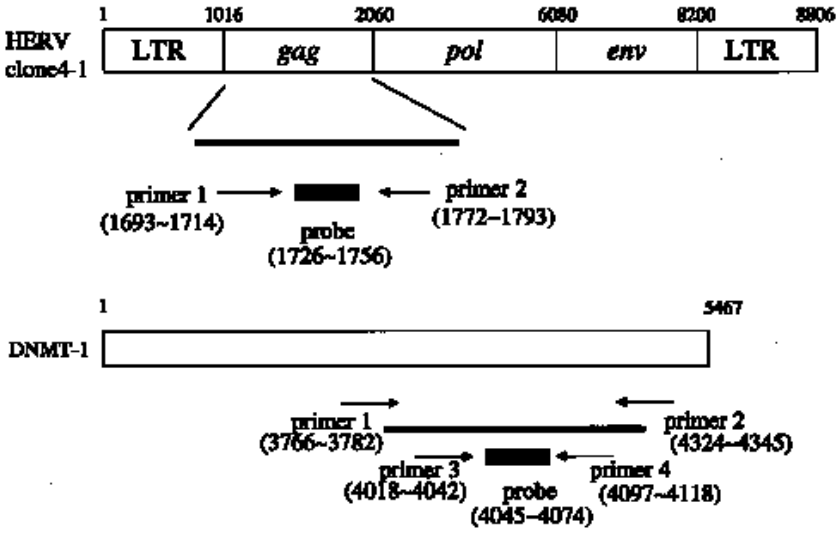

Fig. 1. Structure of HERVclone 4-1 and DNMT-1. Numbers indicate the nucleotide numbers.

mRNAwithout RT were also amplified under the same conditions. The PCR products were run on 1% agarose gel containing 0.5 μg/ml ethidium bromide.

RQ-PCR analysis

Quantitative analysis of HERV clone 4-1 (gag region) and DNMT-1 mRNA was performed by RQ-PCR using a TaqMan fluorogenic system (ABI Prism 7700 Sequence Detection System, Perkin Elmer Applied Biosytems, Forster City, CA), as described previously (9, 10). TaqMan primers and probes were designed with the Primer Express (Parkin Elmer Applied Biosystems), Oligo 4.0 (National Biosciences, Polymouth, MN) computer programs. The following primers were employed:

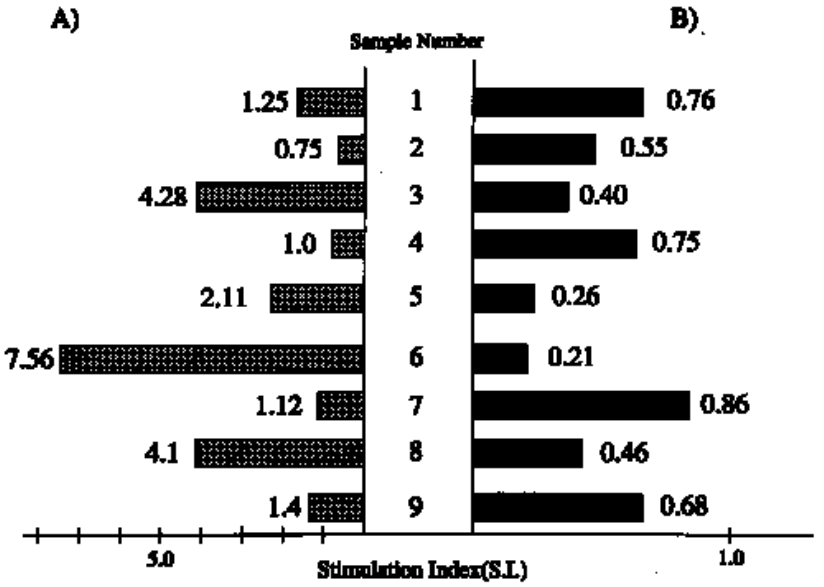

Fig. 2. Effects of 5-aza C on the expression of HERV clone 4-1 mRNA (**A**) and DNMT-1 mRNA (**B**) in normal individuals. The stimulation index (S.I.) is shown. Absolute amounts of clone 4-1 and DNMT-1 mRNAmeasured by RQ-PCR in patients 1-9 without 5-aza C treatment were as follows: normal 1; 58 (clone 4-1) and 4644 copies/μg (DNMT-1), normal 2; 124 and 858 copies/μg, normal 3; 156 and 858 copies/μg, normal 4; 17 and 764 copies/μg, normal 5, 46 and 384 copies/μg, normal 6; 110 and 2734 copies/μg, normal 7; 2 and 211 copies /μg, normal 8; 23.6 and 1654 copies/μg, normal 9; 442 and 1726 copies/μg, respectively.

HERV clone 4-1; 5,-CAC ATG GTG GAG AGT CGT GTT T-3, (gag primer 1; nt 1693-1714) and 5,-GCT TGC GGC TTT TCA GTA TGT G-3, (gag primer 2; nt 1772-1793), DNMT-1; 5,-CGG TTC TTC CTC CTG GAG AAT GTC A-3, (DNMT primer 3; nt 4018-4042) and 5,-CAC TGA TAG CCC ATG CGG ACC A-3, (DNMT primer 4; nt 4097-4118) (Fig. 1). The TaqMan probes for the HERV clone 4-1 gag region and DNMT-1 were labeled with FAM reporter dye (6-carboxytetrafluorescein) at the 5, -end and with TAMRA (6-carboxytetramethylrhodamine) at the 3, -end as the quencher dye (5,-TTC ACC TCT GCC GAC CTT CTC AAC TGG A-3, (gag probe; nt 1726-1756) and 5,-AAC TTT GTC TCC TTC AAG CGC TCC ATG GTC-3, (DNMT probe; nt 4045-4074), respectively) (Fig. 1). Reaction mixtures (total volume: 25 μl) contained 10 x TaqMan buffer (2.5 μl), 3.5 μM $MgCl_2$, 0.5 μl dATP, dCTP, dGTP, and dUTP, 200 nM of each primer, 100 nM of the probe, 0.25 units of AmpliTaq Gold DNA polymerase, 0.125 units of AmpErace uracil N-glucosidase (UNG) (TaqMan PCR core reagent kit: Perkin Elmer Applied Biosystems) and 100 ng of sample DNA. The amplification conditions comprised 2 min at 50ºC for UNG activation and 10 s at 95ºC for TaqGold activation and predenaturation, followed by 40 cycles of denaturation at 95ºC for 15 sec and annealing at 60ºC for 1 min. The standard was drawn with data for five points (10^2, 10^3, 10^4, 10^5, and 10^6 copies) using HERV clone 4-1 gag and pol cDNA(nt 1630-2893) and DNMT-1 cDNA (nt 3766-4345). Experiments were performed in duplicate for each data point. The stimulation index (S.I.) was calculated as the mean amount of mRNA obtained by three separate experiments (standard deviation < 15%) in normal PBMC treated with 5-aza C divided by the amount in untreated normal PBMC.

Statistical analysis

Statistical analysis was performed by calculating Pearson's correlation coefficients and by using Student's t-test. The analysis was performed with commercially available statistical software

(SPSS, Chicago, IL, USA) and P< 0.05 was considered significant.

Results

In order to investigate the role of methylation in the transcription of HERV clone 4-1 mRNA, we examined the effect of 5-aza C treatment on clone 4-1 mRNA expression by PBMC from normal individuals. As shown in Figure 2, this treatment enhanced the expression of clone 4-1 mRNA and inhibited that of DNMT-1 mRNAin many of the normal controls. There was a significant inverse correlation between the increase of clone 4-1 mRNA and the decrease of DNMT-1 mRNA in 5-aza C-treated normal PBMC ($p < 0.05$).

RT-PCR analysis showed that DNMT-1 mRNA expression was lower in the PBMC of SLE patients than in cells from normal controls (Fig. 3). Similar fragments were not detected in the absence of RT, so the possibility of contamination by DNMT-1 DNA was ruled out (data not shown). RQ-PCR also revealed that the quantity of DNMT-1 mRNA in PBMC from SLE patients was lower than in cells from normal controls (the average amount ± SD of DNMT-1 mRNA in cells from SLE patients and normal controls was respectively, 183.6 ± 89.8 copies/μg and 1264.8 ± 489.8 copies/μg, $p < 0.01$) (Fig. 4). In addition, representative data suggested that the expression of DNMT-1 mRNA by PBMC from SLE patients was increased after steroid treatments (Fig. 5).

Discussion

Retroviruses, especially endogenous retroviruses, have repeatedly been suggested as etiological factors for SLE in mice and humans (2-4, 22, 23), although their precise role is still unknown. Recently, we reported that HERV clone 4-1 sequences show increased transcription in SLE patients as compared with normal controls (the average amount of clone 4-1 mRNA in 15 SLE patients and 15 normal controls was 3173 ± 692 and 31 ± 12 copies/μg, respectively, in our previous study), and that antigens and serum antibodies to this HERV are detected in SLE patients at an incidence of approximately

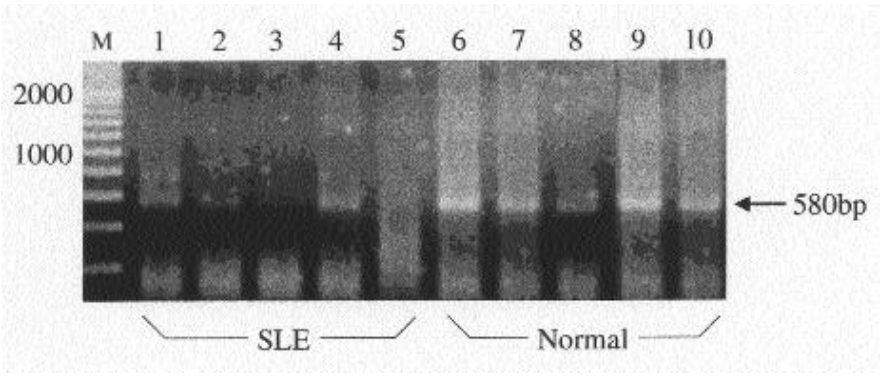

Fig. 3. Expression of DNMT-1 mRNA in SLE patients and normal individuals shown by RT-PCR. Lanes 1-5; SLE patients, lanes 6-9; normal controls. Arrow indicates the molecular weigh (M) of DNMT-1 mRNA(580 bp).

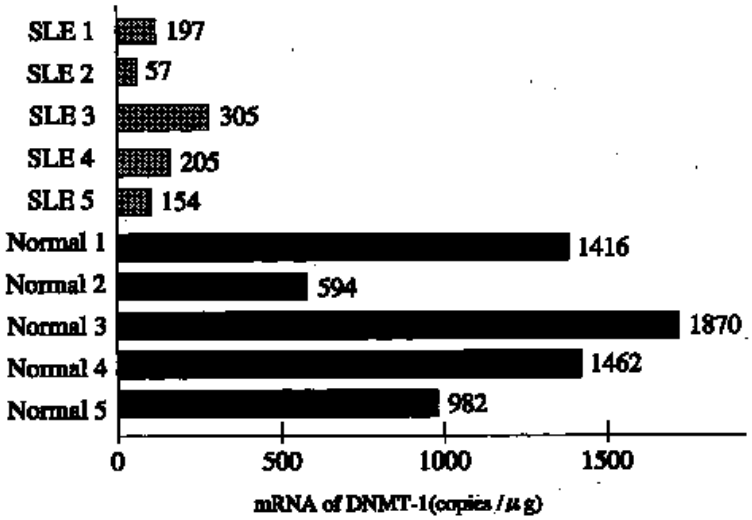

Fig. 4. Quantitative analysis of DNMT-1 mRNAin SLE patients and normal individuals.

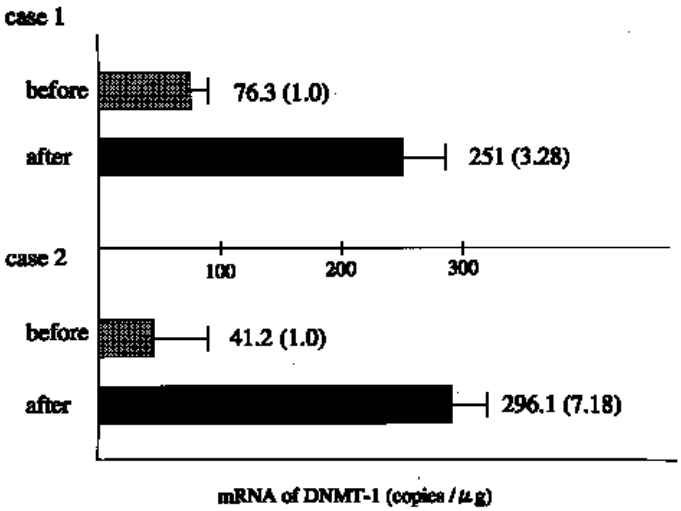

Fig. 5. Comparison of the expression of DNMT-1 mRNA in SLE patients before and after steroid treatment (40 mg/day of prednisolone) for two weeks. Absolute amounts of DNMT-1 mRNAand the S.I. in parentheses before and after treatment are shown. Bars indicate the standard deviation of three separate experiments. There were significant differences of the mRNAlevels between before and after steroid therapy in cases 1 and 2 ($p < 0.01$).

50%, but not in normal individuals (4, 5,9). The inactivation of stop codons observed in clone 4-1 from SLE patients (but not controls) may also be related to an increase of its translation in the patients (7).

Mitogens such as concanavalin A (Con A) do not enhance the transcription of clone 4-1 sequences in normal PBMC (10). This indicates that cellular activation *per se* does not result in increased transcription of these sequences, although SLE lymphocytes are generally activated *in vivo*. In the present study, we found that transcription of this HERV was partly regulated by DNMT-1 and that the amount of DNMT-1 mRNA was lower in SLE patients than in normal controls (Figs. 2 and 4). Unlike cells from normal individuals, 5-aza C treatment of PBMC from SLE patients did not have a perceptible influence on DNMT-1 mRNA expression (data not shown), or on HERV clone 4-1 mRNA expression, as we have previously reported (10). Thus, hypomethylation may play an important role in the transcription of HERV clone 4-1 sequences in SLE patients, although there has been no direct evidence to prove this issue. Furthermore, we cannot exclude the possible contribution of other factors, such as enhancer activity in the long terminal repeat (LTR) region, which also promotes clone 4-1 sequence transcription in SLE, because the amount of clone 4-1 mRNA detected in normal PBMC after 5-aza C treatment was lower than in the cells of SLE patients (9, 10).

Hypomethylation of T cell DNA, as suggested by low DNA (cytosine-5) methyltransferase activity, is observed in patients with SLE (15), and this is reported to be associated with reduced activity of DNMT-1 (24). Recent evidence has revealed that DNA hypomethylation in SLE patients may be regulated by a decrease in signaling through the ras-mitogen-activated protein kinase (ras-MAPK) pathway (24). When antigen-specific cloned CD4+ T cells are treated with 5-aza C, antigen restriction is lost and the cells respond to autologous class II major histocompatibility complex (MHC) determinants in the absence of the relevant antigen, thus becoming autoreactive (14). After 5-aza C treatment, CD4+ T cells show functional similarities to SLE T cells *in vitro*, and adoptive transfer of such cells into syngeneic mice can induce the development of several SLE-like manifestations *in vivo* (17). These experiments have suggested the important role of lymphocyte function-associated antigen 1 (LFA-1; CD11a/CD18) expression in the development of SLE-like autoimmunity (16, 17). In addition, it has been reported that the overexpression of perforin on SLE-T cells is induced by hypomethylation and this may account in part for the increase of T cell mediated-apoptotic cells in the patients (25).

Interestingly, drugs like procainamide and hydralazine that induce a lupus-like illness are known to act as demethylating agents and procainamide-treated cells show similar phenotypic and functional abnormalities to those caused by 5 aza-C treatment (18). In addition to these findings, our present results indicate that 5-aza C can suppress transcription of the DNMT-1 gene and can induce overexpression of HERV genes in normal PBMC. In contrast, steroids enhance DNMT-1 mRNA expression in SLE PBMC, although the expression level by steroids does not reach that of normal PBMC (Fig. 5). Previously, we also reported that steroid therapy could inhibit HERV clone 4-1 mRNA transcription in SLE PBMC (9). The effect on SLE manifestations of several drugs, such as procainamide and steroids, may be influenced by the level of DNA methylation, including that of DNMT-1.

The DNMT family includes DNMT-2 and 3a/3b in addition to DNMT-1. It has been suggested that DNMT-1 preferentially methylates hemimethylated DNA and is a maintenance methyltransferase, while DNMT-3a/3b mainly methylates unmethylated DNA and shows *de novo* activity, but the function of DNMT-2 still remains unclear (13, 19). We could only detect DNMT-1 (not DNMT-2 and -3) mRNAin PBMC from SLE patients and normal controls using our RT-PCR or RQ-PCR systems. 5-aza C is thought to be an inhibitor of DNA methyltransferase (13) and our data revealed that this agent can also suppress the transcription of DNMT-1 mRNA. Furthermore, our results supported previous findings that SLE is a disease associated with hypomethylation. Recent evidence has indicated that mutations of DNA methyltransferase can induce immunodeficiency, e.g., the immunodeficiency-centromeric instability-facial anomalies syndrome (ICF syndrome) (26). Furthermore, methyltransferase activity is decreased in the leukocyte membrane fraction from allergic patients and gene hypomethylation contributes to interleukin (IL)-4-associated IgE production in such patients (27, 28). Thus, abnormalities of methylation-regulating genes seem to be related to the pathogenesis of immune abnormalities such as autoimmunity, immunodeficiency, and allergy, in addition to cancer (13) though the transcription level of DNMT-1 mRNA in other autoimmune diseases besides SLE is unknown. Epigenetics means transcriptional regulation that occurs independently of the nucleotide sequence and includes mechanisms like DNAmethylation (11, 12). Further epigenetic investigations will be needed to obtain a deeper understanding of the pathogenesis of SLE and new therapeutic strategies for this disease.

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