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Alteration of DNA methylation levels in MRL lupus mice

M. MIZUGAKI, T. YAMAGUCHI, S. ISHIWATA, H. SHINDO, T. HISHINUMA, S. NOZAKI* & M. NOSE†
*Department of Pharmaceutical Sciences, Tohoku University Hospital, Miyagi, *Pharmacy Division, Ohta Nishinouchi Hospital, Fukushima and †Department of Pathology, Tohoku University School of Medicine, Miyagi, Japan*

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SUMMARY

Recent reports suggest that DNA methylation is involved in the cause of autoimmune disease. We investigated the alteration of DNA methylation levels in lupus strains of mice, MRL/lpr as a model, which develop an age-dependent lymphadenopathy and autoimmune disease. DNA methylation levels of thymus and axillary lymph nodes in 20-week-old MRL/lpr mice, which are in an autoimmune disease state, were lower than those of 4-week-old MRL/lpr mice with no symptoms as yet. No significant changes were observed in MRL/+ strain mice, which seemed normal at least 20 weeks, while DNA methylation levels in the spleen of both strains of mice increased significantly from the age of 4 to 20 weeks. However, no significant changes of DNA methylation levels in peripheral blood were observed with ageing in MRL strains. Moreover, we clarified that administration of 5-azacytidine had a strong effect on longer survival of MRL/lpr mice and reduced DNA methylation levels in the axillary lymph nodes and spleen. The possible relevance of DNA methylation levels to the progression of autoimmune disease is discussed.

Keywords DNA methylation 5-methyldeoxycytidine 5-azacytidine MRL/Mp-*lpr/lpr* mice autoimmune disease

INTRODUCTION

5-Methyldeoxycytidine (5mdC) methylated at the 5 position of deoxycytidine (dC) is the only modified nucleoside naturally found in mammalian DNA. DNA methylation is thought to play important roles in inactivating the X-chromosome [1], altering the formation of chromatin or DNA structures [2], modifying the DNA-protein interaction [3], genomic imprinting [4], etc. In particular, regulation of gene expression is thought to be a key function [5], and in general the increased methylation state correlates with decreased gene expression.

The ratio of 5mdC to total cytidine residues in mammalian DNA (DNA methylation level) is between 2% and 5%, and varies in a tissue- or cell-specific manner [6]. It has been reported that the DNA methylation levels change in ageing, development and carcinogenesis [7–9]. Besides these changes, Corvetta *et al.* [10] have reported that the DNA methylation level in the peripheral blood of autoimmune disease patients was significantly lower than that of healthy donors. Also, *in vitro* evidence that DNA methylation might participate in autoimmune disease was reported by Richardson *et al.* [11–13]. However, the relation of their cause and effect is still unknown.

Correspondence: M. Mizugaki, Department of Pharmaceutical Sciences, Tohoku University Hospital, 1-1 Seiryo-machi, Aobe-ku, Sendai, Miyagi 980-77, Japan.

Strains of MRL/Mp mice are known to develop autoimmune disease. MRL/Mp-*lpr/lpr* (MRL/lpr) and MRL/Mp-*gld/gld* (MRL/gld) mice develop an age-dependent lymphadenopathy and autoimmune disease that resembles systemic lupus erythematosus (SLE) in humans [14,15]. However, their parental wild-type controls, MRL/Mp-+/+ (MRL/+) mice, show no symptoms of autoimmune disease at the age when MRL/lpr and MRL/gld mice are attacked. In this report we investigated the alteration of DNA methylation levels in lymphoid organs during the development of autoimmune disease using MRL/Mp strains as a model. Previously, Yoshida *et al.* [16] have reported that a DNA demethylating agent, 5-azacytidine (5AzC), reduced lymphadenopathy and acceleration of lupus-like syndrome in MRL/lpr mice. We also validated the effect of 5AzC administration on autoimmunity and investigated the DNA methylation level in the 5AzC-treated mice.

MATERIALS AND METHODS

Chemicals and enzymes

5-Methyldeoxycytidine, deoxycytidine and 5-azacytidine were purchased from Sigma (St Louis, MO). *Penicillium citrinum* nuclease P1 and calf intestinal alkaline phosphatase were obtained from Boehringer-Mannheim (Mannheim, Germany). RNase A and

proteinase K were from Sigma. The other chemicals were from Wako Pure Chemical Industries Ltd (Tokyo, Japan).

Mice

MRL/Mp-*lpr/lpr* (MRL/lpr) and MRL/Mp-+/+ (MRL/+) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were bred and housed in the Animal Research Institute of Tohoku University School of Medicine. Mice were used in the experiment at 4 and 20 weeks after birth. Mice were killed by ether anaesthesia, and peripheral blood, thymus, axillary lymph nodes, spleen and kidneys were obtained and stored at − 80°C.

DNA extraction and digestion

Peripheral blood or tissues were lysed with 0·1% (final concentration) sodium dodecyl sulphate, treated with 20 μg/ml RNase A and 100 μg/ml proteinase K, and DNA was extracted by the phenol extraction method as described by Maniatis [17]. DNA digestion was performed by the method of Gehrke *et al.* [18]. Briefly, 50 μl of purified DNA were degraded to deoxyribonucleoside monophosphates by incubation with 100 μl of 30 mM sodium acetate (pH 5·3), 5 μl of 20 mM zinc sulphate, 7 μl of nuclease P1 (0·3 unit/μl) and 1·5 μl of alkaline phosphatase (1 unit/μl) at 37°C for 2 h. After addition of 10 μl of 1 M Tris, the sample was re-incubated for additional 2 h at 37°C for the phosphatase reaction.

Measurement of DNA methylation levels

The DNA methylation levels were measured by reverse-phase high-performance liquid chromatography (HPLC) as described by Gehrke *et al.* [18] with modification. The hydrolysed nucleosides were separated on a CAPCELL PAK C_{18} column (250×4·6 mm i.d., 5 μm spherical particles, 120 Å pore; SHISEIDO, Tokyo, Japan) with a mobile phase of 0·05 M KH_2PO_4 (pH 4·5) containing 1% methanol at a flow rate of 1 ml/min, and the absorbance was monitored at 277 nm. The percentage of methylated cytosine to total cytosine residues was referred to DNA methylation, which was calculated from the peak areas as [5mdC/(dC + 5mdC)] × 100 (%).

Administration of 5AzC to MRL/lpr mice

Four-week-old MRL/lpr mice were treated twice a week from 4 to 20 weeks with intraperitoneal injections of 50 μg of 5AzC diluted in 200 μl of PBS. PBS alone was injected in the same manner in the control group.

Pathological studies

MRL/lpr mice were sacrificed at 20 weeks of age. Whole body, thymus, axillary lymph nodes, spleen and kidneys were weighed. The average organ weights were expressed as divided value by each whole body weight. Tissue specimens from main organs were fixed with 10% formalin in 0·01 M phosphate buffer (pH 7·2) and embedded in paraffin. They were stained with haematoxylin and eosin or periodic acid-Schiff for histological examination by light microscopy. The severity of glomerulonephritis was graded from 0 to 3: 0, normal; 1, cell proliferation and/or infiltration; 2, ditto with mesangial proliferation and hyaline droplets; 3, ditto with crescent formation and/or hyalinosis, in more than 50% of renal glomeruli. Level 2 or higher was considered in crisis. The severity of arteritis was 0, normal or perivascular cell infiltration; 1, destruction of external elastic lamina; 2, ditto with myointimal thickening, in more than one lesion in main organs; and sialoadenitis was 0, normal or cell infiltration localized in periductal region; 1, cell infiltration extending to parenchyma; 2, ditto with fibrosis and/or granulomatous lesion, in more than one lesion in submaxillary gland. In these cases, level 1 or higher was considered in crisis.

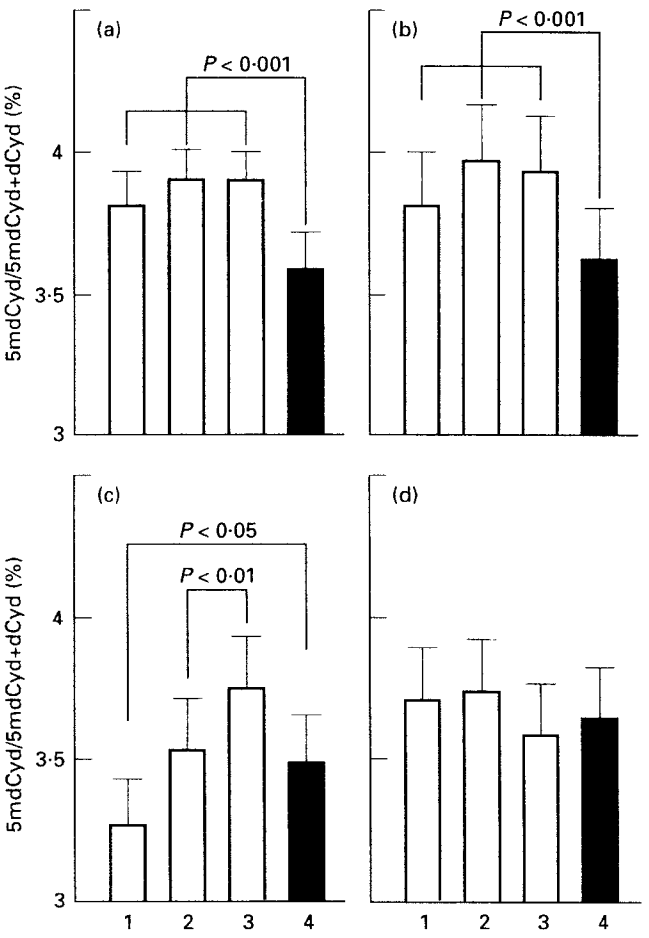

Fig. 1. DNA methylation level in various mice tissues. DNA methylation levels in thymus (a), lymph node (b), spleen (c) and peripheral blood (d) are expressed as the mean ± S.D. DNA methylation is presented as the percentage of methylated cytidine residues for methylated and non-methylated cytidine residues. 1, MRL/lpr − 4 weeks ($n = 9$); MRL/+ − 4 weeks ($n = 8$); 3, MRL/+ − 20 weeks ($n = 8$); 4, MRL/lpr − 20 weeks ($n = 8$).

Statistical analysis

The statistical analyses were performed by Student's *t*-test (unpaired *t*-test). The results were considered statistically significant if the *P* value was 0·05 or less.

RESULTS

Alteration of DNA methylation levels during the development of autoimmune disease in MRL/lpr mice (age-dependent model)

We investigated the alteration of the DNA methylation levels during the development of autoimmune disease in MRL/lpr mice by HPLC analysis. DNA methylation levels in each organ of 4- and 20-week-old MRL/lpr and MRL/+ mice are shown in Fig. 1. The mice exhibiting autoimmune disease (20-week-old MRL/lpr mice) revealed lower DNA methylation levels ($P < 0·001$) than those exhibiting no symptom of the disease (4- and 20-week-old MRL/+ mice). From 4 to 20 weeks DNA methylation levels of MRL/lpr mice reduced significantly in the thymus ($P < 0·01$) and axillary

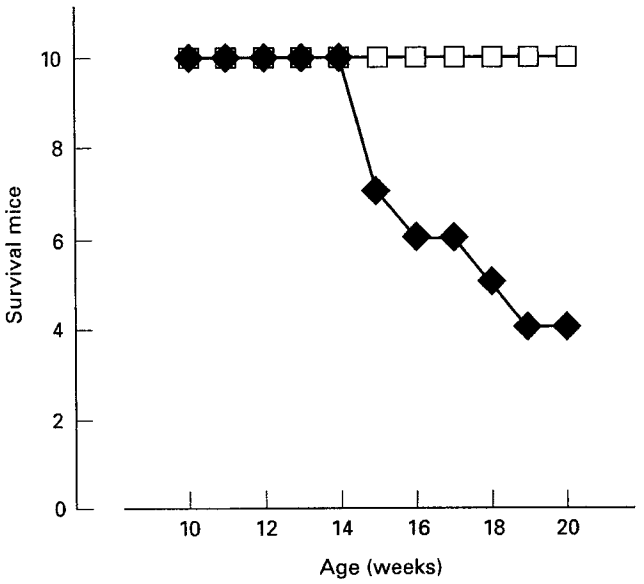

Fig. 2. Survival curve of MRL/lpr mice treated with 5-azacytidine. ◆, control mice ($n = 10$); □, 5-azacytidine-treated mice ($n = 10$).

lymph nodes ($P < 0.001$). However, no significant changes were observed in MRL/+ mice, while DNA methylation levels in the spleens of both MRL/lpr and MRL/+ mice increased significantly, as they grew older from 4 to 20 weeks (MRL/lpr mice: $P < 0.01$, MRL/+ mice: $P < 0.05$). No significant changes of the DNA methylation levels were observed in peripheral blood of any MRL strain. In both 4- and 20-week-old MRL/+ mice, higher levels of DNA methylation were observed in all tissues compared with those of MRL/lpr mice at the same ages.

Effect of 5AzC administration on survival and weights in MRL/lpr mice (5AzC-treatment model)

The effect of 5AzC administration on the survival of MRL/lpr mice was investigated from 4 weeks to 20 weeks of age (Fig. 2). The control mice began to die from 15 weeks after birth, and only 40% (four of 10) of the mice survived at 20 weeks, whereas all of the 5AzC-treated mice were alive at the end of the experiment.

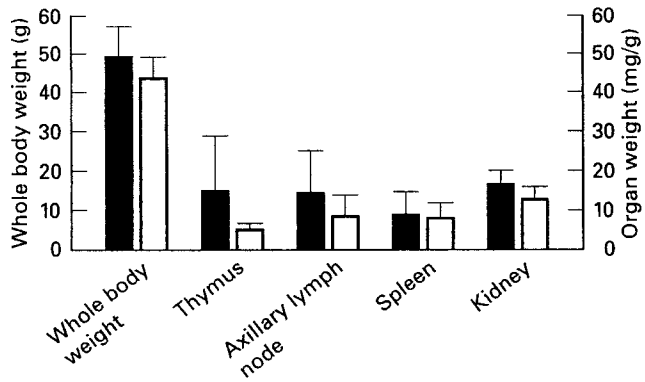

Fig. 3. Effects of 5-azacytidine on whole body weights and organ weights in MRL/lpr mice. The data are expressed as the mean ± S.D. Organ weights (mg/g) in this figure are calculated dividing measured organ weights (mg) by the whole body weight (g). ■, Control mice ($n = 4$); □, 5-azacytidine-treated mice ($n = 10$).

Table 1. Effect of 5-azacytidine treatment on histopathology in 20-week-old MRL/Mp-*lpr/lpr* mice

Group	Glomerulonephritis*	Arteritis†	Sialoadenitis†
Control	100% (4/4)	75% (3/4)	100% (4/4)
5-Azacytidine	40% (4/10)	20% (2/10)	20% (2/10)

* Glomerulonephritis was graded from 0 to 3, and a level higher than 2 was considered a crisis.

† Arteritis and sialoadenitis were graded from 0 to 2, and a level higher than 1 was considered a crisis.

The effects of 5AzC administration on the whole body weight and the organ weights in MRL/lpr mice are shown in Fig. 3. Administration of 5AzC caused a tendency for reduced weights of the thymus and axillary lymph nodes. However, no significant changes were observed in the whole body or any organs.

Effect of 5AzC administration on histopathology in MRL/lpr mice

The effects of 5AzC administration on glomerulonephritis, arteritis and sialoadenitis in MRL/lpr mice were investigated by histopathological studies (Table 1). The control group contracted glomerulonephritis (100%), arteritis (75%) and sialoadenitis (100%) with high risk. By contrast, the 5AzC treatment reduced the severity of all disease.

Effect of 5AzC administration on alteration of DNA methylation levels in MRL/lpr mice

The DNA methylation levels of each organ of the 5AzC-treated MRL/lpr mice are shown in Fig. 4. Treatment with 5AzC caused a

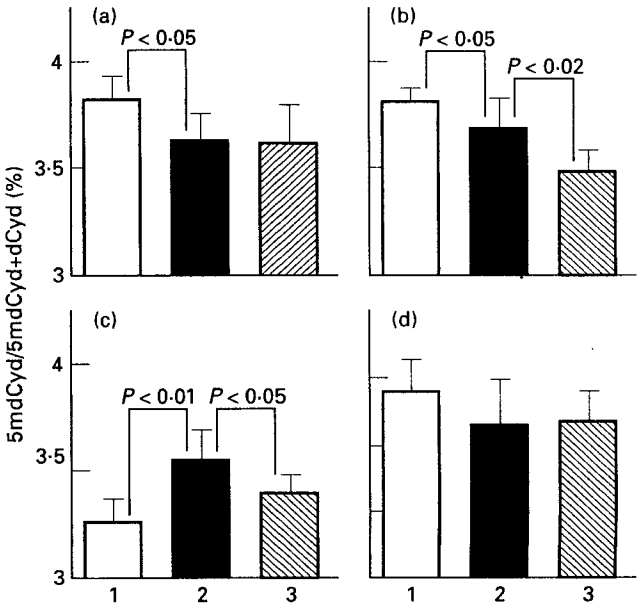

Fig. 4. Effects of 5-azacytidine treatment on DNA methylation level in 20-week-old MRL/lpr mice. DNA methylation levels in thymus (a), lymph node (b), spleen (c) and peripheral blood (d) are expressed as the mean ± S.D. DNA methylation is presented as the percentage of methylated cytidine residues for methylated and non-methylated cytidine residues. 1, MRL/lpr – 4 weeks ($n = 9$); 2 control – MRL/lpr – 20 weeks ($n = 4$); 3, 5-azacytidine-treated – MRL/lpr – 20 weeks ($n = 10$).

significant reduction of the DNA methylation levels in axillary lymph nodes ($P<0.02$) and spleen ($P<0.05$), but no significant change was observed in the thymus and peripheral blood.

DISCUSSION

Recent research revealed that the *lpr* gene of MRL/lpr mice was a deletion mutant of the *Fas* gene that mediates apoptosis [19]. In the *lpr* gene, an early transposable element is inserted into intron 2 of the *Fas* gene. As a result, premature termination and aberrant splicing of *Fas* transcripts occurred [20]. The *gld* gene is a replacement mutation of the Fas ligand (*Fas L*) gene, causing the production of non-functional *Fas L* [21]. As a Fas/Fas-mediated apoptosis is involved in clonal selection of T cells in thymus and periphery, mutation in the *Fas* or *Fas L* genes is regarded as the cause of lymphadenopathy and autoimmune disease in MRL/lpr and MRL/gld mice [22]. However, when the *lpr* or *gld* mutation is transferred into a number of other inbred strains with a different background, such as C3H/HeJ mice, lymphadenopathy and autoantibodies are induced but glomerulonephritis and arteritis are not [15,23,24]. These results indicate that autoimmune disease may comprise not only an abnormality of signal transduction in a Fas/Fas L system but also an unknown genetic background.

In this report, we have shown that DNA methylation levels in thymus and axillary lymph nodes decreased with the progress of autoimmune disease in MRL/lpr mice. Decreased methylation levels were related with increased gene expression [5]. Reduced methylation levels in bacterial DNA and synthetic oligodeoxynucleotides induced various immune effects including B cell activation and secretion of IgM and IL-6 [25,26]. It is possible that hypomethylated DNA originating from thymus and axillary lymph nodes facilitates the progress of autoimmune disease. Most, if not all, of the studies on DNA methylation levels have reported an age-dependent decline in various tissues [27]. However, this decline seems to have few effects in the short-term experiment. In fact, there were no changes in the DNA methylation levels of thymus and axillary lymph nodes in MRL/+ mice with ageing, only if they had no deficit in Fas-mediated apoptosis, followed by the development of autoimmune disease, while DNA methylation levels in the spleen increased significantly regardless of a deficit in Fas-mediated apoptosis and suffering from autoimmune disease, and those of peripheral blood were unchanged. Although the cause of these conflicting results is not clear at the moment, this is the first report that DNA methylation levels alter in an organ-specific manner. It is possible that the decreased methylation levels in thymus and axillary lymph node and/or increased methylation levels in spleen also play an important role in the genetic background for progressing autoimmune disease in MRL/lpr mice.

It is reported that cloned T cells treated with 5AzC lost the requirement for antigen and could be activated by autologous HLA-D molecules alone *in vitro* [11,12]. An *in vivo* experiment has also shown that treatment with 5AzC induced autoimmune disease in the chicken [28]. These reports regarded the reduction of the DNA methylation levels as one cause of autoimmune disease. By contrast to these observations, Yoshida *et al.* [16] have reported that 5AzC treatment prolonged survival and inhibited glomerulonephritis and lymphoproliferation in MRL/lpr mice. This paradox prompted us to examine the effects of 5AzC on the DNA methylation levels in various lymphoid organs and their relationship to autoimmune disease. As a result, we validated prolonged survival, and reduced glomerulonephritis, arteritis and sialoadenitis in 5AzC-treated mice, although significant inhibitions of lymphadenopathy and splenomegaly, as Yoshida *et al.* had observed [16], were not detected in our study. 5AzC treatment decreased DNA methylation levels in axillary lymph nodes and spleens, while those in thymus and peripheral blood remained unchanged. 5AzC is thought to decrease DNA methylation levels independent of methylated sites, inhibiting DNA methylase. However, the result which we obtained showed that the sensitivity of the methylation to 5AzC differed in lymphoid organs of these mice. This suggests that there are particular mechanisms for maintaining DNA methylation levels in each organ.

The results obtained from the age-dependent model seem inconsistent with the 5AzC-treatment model at the point in which the opposite direction of DNA methylation changes cause autoimmune disease. 5AzC is thought to decrease methylation levels in a non-specific and artificial manner. Therefore, gene product(s) being repressed by DNA methylation and harbouring activities to suppress autoimmunity might be expressed in axillary lymph nodes and/or spleen. We think that the age-dependent model reflects the actual autoimmune disease and the result accords with the many previous reports. However, the 5AzC-treatment model uncovered the organ-specific DNA methylation sensitivity to 5AzC and the possible existence of the gene product suppressing the disease. There is no report on variation of the DNA methylation levels in organs associated with autoimmune disease so far. Further understanding of the relationship of DNA methylation and autoimmune disease in MRL mice with a deficit in Fas-mediated apoptosis could possibly provide new insights into the biological basis of autoimmunity.

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