A Methyl-Deficient Diet Modifies Early B Cell Development

Toshiaki Kurogi^a Hiroko Inoue^a Yun Guo^a Asako Nobukiyo^b Keiko Nohara^c
Masamoto Kanno^a

^aDepartment of Immunology, Graduate School of Biomedical Sciences and ^bNatural Science Center for Basic Research and Development, Hiroshima University, Hiroshima, and ^cEnvironmental Health Sciences Division, National Institute for Environmental Studies, Tsukuba, Japan

Key Words

Methyl deficiency · B cell development · Epigenetic alterations

Abstract

A functional methyl group donor is essential for the epigenetic regulation of all biological events due to the importance of DNA methylation and histone methylation as an epigenetic marker. However, the epigenetic alterations in the immune system due to methyl donor deficiency are not well known. In this study, we tried to address this question by studying the lymphocyte development and DNA methylation changes caused by a methyl-deficient diet (MDD). We fed one group of C57BL/6J mice with a methyl-sufficient diet (MSD) and the other group with an MDD for 5 months. Flow cytometry analyses of their immune systems showed a decrease in B220+ IgM+ (immature B) cells and an increase in B220+ IgM– (pro/pre-B) cells in the bone marrow of mice fed an MDD. By means of an in vitro OP9 coculture system, we recognized that this B220+ IgM– cell fraction from the MDD has an intrinsic developmental defect. When we quantitatively measured the mRNA expression levels of transcription factors and recombination machinery related to B cell development in the B220+ IgM– cell fraction of their bone marrow, we found that *ADA*, *EBF1*, *DNTT* and *Pax5* mRNA expression levels were significantly downregulated in mice fed

with an MDD. In addition, there was a drastic decrease in histone methylation profile H3K4me3 in the *Pax5* and *EBF1* promoters in these B220+ IgM– B cells. However, CpG-DNA methylation profiles had not changed and this revealed that these two promoters are demethylated even under an MSD condition. We also found changed expression levels of the *Polycomb* group genes (*mel18*, *bmi1*, *Pc1*, *Pc2*, *Ring1A*, *Ring1B*, *Ph1*) on semi-quantitative RT-PCR. These results indicate that under an MDD condition, early B cell development in bone marrow is easily affected by epigenetic alterations.

Copyright © 2012 S. Karger AG, Basel

Introduction

Recent studies have revealed that epigenetic modifications play pivotal roles in regulating gene expression. Epigenetics have also been implicated in changes in gene expression and adverse health effects caused by environment and diet [1]. Among the epigenetic mechanisms, DNA methylation and histone modifications (including histone methylation) are important for many biological events. These epigenetic modifications require a methyl donor e.g. methionine and S-adenosyl-methionine. However, the importance of methyl donors in the immune system is not well known. In this study, we tried to address this question by studying the lym-

phocyte development alteration caused by methyl donor deficiency.

Epigenetics regulate gene expression with DNA methylation, histone modification, and noncoding RNAs without changing the nucleotide sequence of DNA, and are required to activate the stable expression or repression of genes [2]. DNA methylation is an epigenetic modification that is critical for normal development, differentiation, gene regulation and the control of chromatin structure in mammalian cells [3]. It occurs on cytosine residues at symmetric CpG dinucleotides in genomic DNA and induces gene repression. This occurs through the methylation of CpG-enriched areas, which have been described as CpG islands [4]. Global patterns of DNA methylation are established by the action of the DNA methyl transferases (DNMTs): Dnmt1, Dnmt3a, and Dnmt3b [5]. Dnmt1 maintains DNA methylation during replication, while Dnmt3a and Dnmt3b play predominant roles in de novo DNA methylation [6, 7].

Polycomb group (PcG) proteins are related to 3 epigenetic mechanisms and silence gene expression. They reside in 2 main complexes termed Polycomb repressive complex 1 and 2 (PRC1 and PRC2) [8, 9]. PRC2, consisting of Eed, Suz12, and Ezh2, is thought to be required at the initiation stage of silencing, whereas PRC1, containing PcG proteins such as Bmi1, Mel18, M33/Pc1, Pc2, Rae-28/Mph1, Mph2, and Ring1A/B, is continuously required for the stable maintenance of the initiated PcG repression on specific target loci. Although PRC1 and PRC2 complexes have different functions, Ezh2/Eed-mediated nucleosome methylation on the trimethylation of histone H3 lysine 27 (H3K27) increases in vitro binding of the chromodomain protein Polycomb within PRC1 to the chromatin [10–12]. EZH2 serves as a recruitment platform for DNA methyltransferases [13], and both PRC1 and PRC2 complexes can interact with one another transiently at least [14].

B cell development occurs from hematopoietic stem cells in bone marrow [15]. Hematopoietic stem cells develop via various B-lineage-restricted precursors into immature B cells. An early step in hematopoiesis is the commitment of multipotent progenitors to either the lymphoid or the erythromyeloid lineages [16, 17]. The multipotent progenitors subsequently differentiate into the early lymphocyte progenitors (ELP) [18].

Immunoglobulin heavy (IgH) chain genes rearrange into pro-B cells, which require the expression of the lymphocyte-specific recombination enzymes RAG1, RAG2 and TdT [19]. These events result in the expression of the IgH proteins as part of the pre-B cell receptor, which acts

as an important checkpoint to control the transition of the pre-B cell stage [20–22]. Immunoglobulin subsequently rearranges the light chain into pre-B cells. Immature B cells express variable amounts of surface IgM that emigrate from the bone marrow to peripheral lymphoid organs [23].

In this study, we investigated the effect of a methyl-deficient diet (MDD) on the immune system. We showed abnormal early B cell development from B220+ IgM– (pro/pre-B) cell to B220+ IgM+ (immature B) cell in the MDD. Using an in vitro OP9 coculture system, we detected that this B220+ IgM– cell fraction from MDD has an intrinsic development defect. Therefore, we investigated the expression profiles of B cell development-related genes in pro/pre-B cells of the MDD. We found that the expression of ADA, EBF1, DNTT and Pax5 were downregulated and histone methylation of H3K4me3 levels was reduced in both *Pax5* and *EBF1* promoters. However, the CpG methylation profile of *Pax5* and *EBF1* was already demethylated in MSD and not changed in MDD. Concomitantly, the expressions of PcG genes were changed under an MDD. The results of the study reveal that methyl deficiency in the diet affects early B cell development in the bone marrow with alteration of *ADA*, *EBF1*, *DNTT* and *Pax5* expressions, histone methylation of H3K4me3 profiles and epigenetic regulator PcG genes expression.

Materials and Methods

Mice and Diet

Seven-week-old male and female C57BL/6 J mice were purchased from CLEA Japan (Tokyo) and were acclimatized to the environment for about 2 weeks prior to use. Throughout the experiment, the animals were maintained in a controlled environment at a temperature of $24 \pm 1^\circ\text{C}$, humidity of $50 \pm 10\%$ and a 12/12 h light/dark cycle. The mice were handled in a humane manner in accordance with the National Institute for Environmental Studies guidelines for animal experiments.

The methyl-sufficient diet (MSD) and the MDD – with a lower methionine, folic acid and choline content (online suppl. table 1; for all online supplementary material, see www.karger.com/doi/10.1159/000337290) – were prepared by CLEA Japan (Tokyo) [24]. Two groups each of males were fed the MSD and MDD ad libitum for 5 months. The LC/ESI-MS measurements revealed that the proportion of 5-methylcytosine tended to decrease in the order of the MSD group and MDD group among the male groups [24].

Flow Cytometry Analysis of Hematopoietic/Immune System for Developmental Abnormalities

The following fluorescein-conjugated mAbs were used: anti-IgM-FITC (II/41), anti-B220-APC (RA3–6B2), anti-CD4-FITC

(RM4-5), anti-CD8-PE (53-6.7), anti-CD25-FITC (7D4), anti-CD44-PE (IM7), anti-CD4-APC (RM4-5), anti-CD8-APC (53-6.7), anti-CD3-APC (145-2C11), anti-CD19-APC (1D3), anti-Mac1-APC (M1/70), anti-Gr1-APC (RB6-8C5), anti-Ter119-APC (Ly76) and anti-NK1.1-APC (PK136). The labeled cells were separated with an analytical FACS Vantage SE (Beckman Coulter, Miami, Fla., USA) and the data were analyzed with Flow Jo software (Tree Star, Inc., Ashland, Oreg., USA).

OP9 Cell Coculture System in vitro

B220+ IgM⁻ cells were isolated from the bone marrow cells of the C57BL/6J mice on MSD and MDD. The bone marrow cells were sorted for expression of c-kit and B220 and negative expression of IgM. Sorted c-kit+B220+IgM⁻ cells were seeded at 4×10^3 cells/well into 24-well tissue culture plates containing a confluent monolayer of OP9-GFP cells. All liquid cultures were performed in RPMI1640 (Invitrogen, Carlsbad, Calif., USA) plus 10% FBS, 50 mM 2-ME, 1 mM sodium pyruvate (Invitrogen) and $1 \times$ nonessential amino acid solution (Invitrogen). All cocultures were performed in the presence of 5 ng/ml IL-7 and 5 ng/ml SCF. After day 7 or day 12, the cultures were subjects for the flow cytometry analysis.

Quantitative RT-PCR Analysis of Master Regulators of Lymphocyte Development

The B220+ IgM⁻ cell fractions were sorted by negative selection with biotinylated anti-IgM and positive selection with biotinylated anti-B220 and streptavidin magnetic beads (Imag; BD Biosciences). Semi-quantitative RT-PCR was performed on cDNAs obtained from 4×10^7 sorted cells of B220+ IgM⁻ cells. Total RNA was isolated from the indicated cells using TRIzol reagent (Invitrogen Life Technologies, USA) and was reverse-transcribed using the Superscript III RT First-Strand synthesis system (Invitrogen) with random hexamer primers.

For quantitative PCR, we used a total volume of 20 μ l containing 2 μ l cDNA template (1/20 dilution), KAPA SYBR FAST qPCR master mix (Kapa Biosystems, USA), ROX Low and 0.2 μ M or 0.4 μ M of each primer. Each sample was amplified in triplicate or duplicate using the primers. A 2-step cycling protocol with combined primer annealing and elongation at 60°C was used. Gene expression was analyzed with an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, USA). TaqMan UBC and YWHAZ RNA control reagents were used as the endogenous normalization standard. The primers used for the PCR were *PU.1*, *Ikaros*, *ADA*, *Rag1*, *Rag2*, *TdT*, *EBF1*, *E2A* and *Pax5* (online suppl. table 2).

Expression Analysis of Master Epigenetic Regulator Families

B220+ IgM⁻ cells from bone marrow were isolated to the analysis expression level of DNA methylation-related genes, *Dnmt1*, *Dnmt3a*, *Dnmt3b* and Polycomb-related genes, *mel18* and *bmi1*, *Pc1*, *Pc2*, *Ph1*, *Ring1A* and *Ring1B* by quantitative PCR. For RT-PCR, we used a total volume of 25 μ l containing 4 μ l cDNA template (1/50 dilution), 0.2 μ M of each primer, AmpliTaq DNA polymerase (Applied Biosystems, USA), PCR buffer, 0.2 M of dNTP mixture. The cDNAs were amplified using various oligonucleotide primers chosen according to Oligo 4.0 software (National Biosciences, UK). The primers used for the PCR were *Dnmt1*, *Dnmt3a*, *Dnmt3b*, *mel18*, *bmi1*, *Pc1*, *Pc2*, *Ph1*, *Ring1A*, *Ring1B*, *UBC*, *YWHAZ* and *Rpl3a* (online suppl. table 2).

CpG Methylation Profile Analysis

Total DNA was isolated from the indicated cells using TRIzol reagent (Invitrogen). CpG methylation profile analysis was basically performed using Custom Methyl-Profiler PCR Array (SABiosciences, USA). DNA was treated with a Methyl-Profiler Methylation Enzyme kit (SABiosciences) at 37°C overnight. Quantitative RT-PCR was performed with RT2 SYBR Green qPCR master mix and Methyl-Profiler qPCR primers of *Pax5* and *EBF1* with an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Percentages of heavily methylated DNA were analyzed by the Δ Ct data analysis method of Methyl-Profiler DNA Methylation qPCR assays manual (SABiosciences).

For the bisulfite method, bisulfite-treated DNA isolated B220+IgM⁻ cells from mice fed an MSD or an MDD were sequencing at a site in the *Ebf1a* and *Ebf1b*, *Pax5a* and *Pax5b* regions. These sites were amplified by PCR with Go Taq Green master mix (Promega, Madison, Wisc., USA) and cloned into the pGEM-T easy vector (Promega) and transformed into *Escherichia coli* cell DH5 α (TOYOBO). Individual clones were sequenced using a dideoxy fluorescence terminator system (ABI 310). Primers are described in online supplementary table 2.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed essentially according to the Low Cell ChIP kit (Diagenode). ChIP assay with antitrimethylated H3K4 (Upstate Biotechnology) and antitrimethylated H3K27 (Upstate Biotechnology and SABioscience/QIAGEN), rabbit IgG control antibodies. We also used antibodies for dimethylated H3K4 and H3K27; however, all presented data were for trimethylated forms of H3K4 and H3K27. The results for dimethylated H3K4/H3K27 were not shown. The data from the dimethylated forms were essentially same as from trimethylated forms. Briefly, 2.0 μ g of primary antibody was bound to protein-A-coated paramagnetic beads. Isolated B220+ IgM⁻ cells from mice fed with an MSD or MDD were fixed with formaldehyde for 8 min at room temperature and then soluble chromatin was immunoprecipitated with antibodies overnight. PCR was performed with KAPA SYBR Fast qPCR master mix (Kapa Biosystems) for real-time PCR.

Results

MDD Inhibits B Lymphopoiesis in the Bone Marrow B220+ IgM⁻ Cell Stage

To determine the effects of an MDD on the immune system in vivo, male C57BL/6J mice were fed with a synthetic MSD or an MDD (online suppl. table 1) for 5 months. After this period, the mice that were fed an MDD had lost approximately 15% of their body weight (fig. 1).

To understand its effects on the development of the immune system, we performed flow cytometric analysis of the lymphocytes in bone marrow, thymus, spleen and lymph node. In bone marrow, we observed that an MDD leads to an increase in the percentage of B220+ IgM⁻ cells

Fig. 1. Mean body weight of mice fed an MSD or an MDD. Throughout the study, mice were weighed weekly and MSD/MDD food intake was determined once a week. Intake was measured by placing a specific quantity of food in the cage or liquid in the drinking bottle and measuring the remaining amount 24 h later. An experimental group consisted of 5–8 mice. These 5-month feeding experiments were repeated at least 4 times.

from 11 to 15% and a decrease of B220+ IgM+ cells from 26 to 17% (these percentages are mean values among 5 mice per group, see fig. 2a). The total number of cells in the bone marrow shows that under the MDD condition (4.5×10^7) there are more cells than the 3.3×10^7 cells MSD. On the other hand, we did not find a major difference in the T cell and B cell compartments in the thymus, spleen and lymph node (fig. 2b, c and data not shown). The results indicated that the MDD interferes with B lymphopoiesis at the B220+ IgM– cell stage in bone marrow.

This defect of early B cell development when on the MDD might be caused by both intrinsic factors within the lymphocyte and extrinsic factors from other parts of the body. In order to establish the possibility cell intrinsic defects in B220+ IgM– cells, we performed an in vitro B cell differentiation assay with OP9 cells [25]. The sorted 4×10^3 B220+ IgM– cells from both MSD and MDD bone marrow were cultured for 12 days with OP9, then analyzed by flow cytometry to be able to see the B220+ IgM+ cells. As shown in figure 2d, B220+ IgM– cells from the MDD condition lost their ability to develop sIgM+ B cells, and this in vitro data shows more severe effects than the in vivo data that we observed. At the least, this clearly indicates that MDD treatment has some intrinsic defects on B220+ IgM– B cells for their development.

MDD Decreases Transcription Factor and Recombination Machinery Expression in Bone Marrow B220+ IgM– Cells

Pro/pre-B cell development is regulated by several transcription factors and its stage precedes V(D)J recombination of IgH chain genes. To understand the mechanisms by which bone marrow B cell development was interfered with at the B220+ IgM– cell stage on the MDD, the B220+ IgM– cell fractions in bone marrow were isolated from 2 groups of mice fed an MSD or an MDD. We then analyzed the expression levels of the transcription factors related to B cell development (E2A, EBF1, Ikaros, Pax5 and PU.1) and V(D)J recombination genes (Rag1, Rag2, ADA and DNTT) in the B220+ IgM– cell fractions of their bone marrow, by quantitative RT-PCR (fig. 3). We found that *ADA*, *Pax5*, *EBF1*, *DNTT* and *Ikaros* mRNA expression levels were significantly downregulated on the MDD. These results showed that the MDD condition interferes with B lymphopoiesis at the B220+ IgM– cell stage by downregulating the expression of B cell lineage commitment genes.

The CpG Methylation Profile of the Pax5 Promoter and Epigenetic Factor Expression in MDD

The dietary manipulation of methyl donors can alter DNA methylation [26–28]. To examine the methylation status at the CpG islands of the *EBF1*, *DNTT* and *Pax5* that have large a difference between MSD and MDD in expression levels, we extracted the genomic DNA of the B220+ IgM– cells in bone marrow from 2 groups of mice fed an MSD or an MDD, and then applied the Custom Methyl-Profiler PCR Array for a rough analysis. We omitted the analysis of the DNTT promoter because there were no CpG islands. The *Pax5* and *EBF1* promoters each had two CpG-islands, Pax5a and Pax5b, and EBF1a and EBF1b. Previous reports showed that the Pax5b CpG island has no effect on *Pax5* gene expression because the HS-2 and HS-3 sites (Pax5b) of mouse embryo fibroblasts, embryonic stem cells and multipotent progenitor cells which had a low Pax5 expression had a low CpG methylation level [29]. By Custom Methyl-Profiler PCR Array analysis, we saw a dramatic difference in CpG methylation level on Pax5a, Pax5b and EBF1b regions, i.e. Pax5a was heavily methylated under the MSD condition (22%) and lower (0.2%) under the MDD condition (fig. 4b). However, for more precise nucleotide level analysis, we performed a bisulfite-sequencing method for analyzing CpG methylation status. Surprisingly, as shown in figure 4c, both Pax5a+b and EBF1a+b regions have a demethylated status even under the MSD condition.

Therefore, by analysis of the single nucleotide level, we detected no big differences between the MSD and MDD conditions in CpG methylation status on the Pax5 and EBF1 promoters. There is a discrepancy between the results of the 2 different methods; however, we prefer to take the result from the bisulfite-sequencing method, as this comprises single nucleotide level resolution.

Regarding the levels of DNA methylation, there are at least 2 variable factors: (1) the methyl donor substrate concentration which might be directly correlated with CpG methylation status or (2) the expression level of DNA methyltransferase. Therefore, we also analyzed the expression levels of *Dnmt1*, *Dnmt3a* and *Dnmt3b* in the B220+ IgM- cell fractions of the bone marrow by semi-quantitative RT-PCR (fig. 4d). Again, we could not detect much difference between MSD and MDD in *Dnmt1*, *Dnmt3a* and *Dnmt3b* mRNA expression levels. Collectively, these data suggest that CpG-DNA methylation levels undergo no drastic change and that the CpG-DNA methylation status of the Pax5 and EBF1 promoter may not be a cause of the B cell developmental defects that we observed in the MDD condition.

Employing the ChIP method, we also looked at the histone methylation status, i.e. H3K4me3 and H3K27me3 as representative markers of active and silent status, respectively, in the Pax5 and EBF1 promoter regions. These genes already have a transcriptionally active status (as mentioned above, interpreted by CpG-DNA methylation). In agreement, we observed a high level of the active marker H3K4me3 and a low level of the silencing marker H3K27me3 under the MSD condition. Under the MDD condition, the H3K4me3 level is significantly decreased (fig. 4e) and so is the H3K27me3 level. It is conceivable that histone methylation status at the promoter regions of Pax5 and EBF1 may be one cause of developmental defects on B cells due to the significant decrease of H3K4me3 level under the MDD condition, which may result in a 50% reduction in the transcription of Pax5 and EBF1.

The last possibility is that the MDD condition may influence the epigenetic regulators, so we looked at the expression level of epigenetic silencing-regulator PcG complexes, which were reported to be associated with global chromatin status including histone modification and DNA methylation. Both PRC1 and PRC2 complexes might be responsible for this status, and previous studies have indicated that they might be important in translating repressive chromatin modifications to gene repression [10, 13, 30, 31]. We analyzed the expression levels of PcG genes such as *mel18*, *bmi1*, *pc1*, *pc2*, *Ring1A*, *Ring1B*

Fig. 2. B lymphocyte differentiation in mice fed an MDD is blocked at the B220+ IgM- cell stage in bone marrow. Bone marrow cells (a) and thymocytes (b, c), isolated from mice fed an MDD or an MSD, were indicated and processed for flow cytometric analyses of B220 and IgM (a), CD4 and CD8 expression (b) or CD25 and CD44 expression in double negative thymocytes (c). The representative data were shown in 3 independent results. d Differentiation and proliferation of B220+ IgM- cells cultured on OP9 cells. The B220+ IgM- cells from MSD or MDD mice were cultured on OP9-GFP cells for 7 or 12 days, and the resultant ratio of B220+ IgM+ cell media were shown. The representative data were shown and experiments were repeated at least 3 times.

and *phl1* in the B220+ IgM- cell fractions of their bone marrow by semi-quantitative RT-PCR (fig. 5). We found that PcG gene mRNA expression levels were downregulated under the MDD condition. This result showed that the decreased expression levels of PcG associated with

Fig. 3. Gene expression of regulators on B cell development. B220+ IgM⁻ cells were isolated from bone marrow from mice fed an MSD or an MDD. Total RNA was extracted and real-time RT-PCR was performed using primers for Rag1, Rag2, ADA, E2A, EBF1, DNTT, Ikaros, Pax5 and PU.1. The total UBC and YWHAZ mRNA level was used for normalization. The gene expression level was set to 1.0 in mice fed an MSD. * $p < 0.05$, paired Student t test. The representative data were shown and experiments were repeated at least 3 times.

MDD might correlate with the chromatin status change on the *Pax5* and *EBF1* promoters.

These results also showed that the abnormality of B lymphopoiesis with downregulation of *Pax5* and *EBF1* expression under the MDD condition might be due to the change of epigenetic level.

Discussion

DNA methylation status change in the liver on an MDD has been well studied [24]. In this study, we focused on the immune system, detecting the developmental defect on B lymphopoiesis under the MDD condition, but only a little or no change in DNA methylation status on *Pax5* and *EBF1* promoters which play a pivotal role on B lymphopoiesis.

We observed abnormalities and partial developmental arrest in B cell development in vivo in a condition of MDD. Such observed defects might be caused by both intrinsic factors within the lymphocyte and/or extrinsic factors from other parts of body. For example, from a mouse model, it is known that an MDD can cause liver

injury [32]. A defect of early B cell development in MDD may be affected by changes in hormone balance from liver injury. We detected the presence of intrinsic defects on B lymphopoiesis, proved by the in vitro OP9 culture system. We therefore tried to focus on the analysis of intrinsic factors on B lymphopoiesis under the MDD condition. The defect occurs at the B220+ IgM⁻ cell stage in bone marrow in MDD mice, accompanied by a reduction in *ADA*, *EBF1*, *DNTT* and *Pax5* expression that are crucial for B cell development. As mentioned above, we could not detect the change of the CpG-DNA methylation status on the promoters of *Pax5* and *EBF1* which were already demethylated in MSD; however, we detected the difference in histone methylation status between MDD and MSD on B220+ IgM⁻ cells, i.e. a decrease of H3K4me3 under the MDD condition which might be a cause of the reduction of *Pax5* and *EBF1* transcripts.

We also detected the altered expression level in the epigenetic regulator genes, the *Polycomb* group genes, in the pro/pre-B cells. The supply of methyl donor may serve as an important factor for early B cell development through a change in transcription factor, recombination machinery gene expression and epigenetic factors, *PcG*

Fig. 4. Profiles of DNA methylation and histone methylation of Pax5 and EBF1 promoter regions. **a** CpG islands of Pax5 (Pax5a and Pax5b) and EBF1 (EBF1a and EBF1b). **b** CpG-DNA methylation level of Pax5 promoter (Pax5a and Pax5b) and EBF1 (EBF1a and EBF1b) in mice fed an MSD or an MDD were analyzed by the Custom Methyl-Profiler PCR Array system (open bar = MSD, filled black bar = MDD). Percentages of heavily methylated DNA were analyzed by the Δ Ct data analysis method of Methyl-Profiler DNA Methylation qPCR assays manual of SABiosciences. **c** Bisulfite-treated DNA from B220+IgM⁺ cells of mice fed with an MSD or an MDD were sequenced at a site in Ebf1a and Ebf1b, Pax5a and Pax5b. Methylated CpGs (closed circles) and unmethylated CpGs (open circles) are shown. **d** Dnmt1, Dnmt3a and Dnmt3b mRNA levels were analyzed using real-time RT-PCR. Total UBC and YWHAZ mRNA levels were used for normalization. The gene expression level was set to 1.0 in mice fed an MSD. **e** ChIP assays for trimethylated histone H3K4 and trimethylated histone H3K27 in regions of Ebf1 and Pax5. B220+IgM⁺ cells from bone marrow of mice fed an MSD or an MDD were used. Antibodies against trimethylated histone H3K4 and trimethylated histone H3K27 were used in the ChIP assays. Real-time PCRs were carried out with primers for each amplicon. The representative data were shown and experiments were repeated at least 3 times (all with essentially the same result).

Fig. 5. Expression of PcG genes. PcG genes (*mel18*, *bmi1*, *Pc1*, *Pc2*, *Ph1*, *Ring1A*, *Ring1B*) mRNA levels were analyzed using semi-quantitative RT-PCR. The total UBC mRNA level was used for normalization. The gene expression level was set to 1.0 in mice fed an MSD. The representative data were shown and experiments were repeated at least 3 times.

genes. However, the decrease in the Pax5 expression level that we observed cannot be explained by the CpG-DNA methylation status of the Pax5 promoter and expression of DNMTs. Transcription factor PAX5 is known to be crucial for B cell development at the pro/pre-B cell stage.

Pax5-deficient pro-B cells were reported to have the potential to differentiate into a variety of non-B cell lineages such as natural killer cells, macrophage/myeloid cells, granulocytes, osteoclasts and T cells.

Other studies have revealed that continuous *Pax5* expression was required to maintain the B cell identity [33–36]. In pro-B cells, PAX5 also regulates the accessibility of the IgH gene loci to the RAG1/2 recombinase, thereby controlling the V(D)J rearrangements necessary for the production of μ -heavy chain polypeptides [37–39]. It also controls the conformational changes in IgH gene loci in the nucleus.

However, *Pax5*^{+/-} mice, having 50% of gene dosage, do not have a B cell phenotype [40]. If both alleles of Pax5 genes are transcribed on pre-B cell stage, we cannot explain the observed B cell defects simply by a 50% reduction in the expression of Pax5 transcripts. However, several reports demonstrate that Pax5 gene is subject to allele-specific regulation during B cell development. Pax5 is predominantly transcribed from only one of its two alleles in early B-lymphoid progenitors and mature B cells,

while it transiently switches to a biallelic mode of transcription in pre-B and immature B cells [41, 42].

Therefore, the defect of B cell development could be explainable by a 50% reduction in the expression of Pax5 in MDD. The concerted downregulation of Pax5, ADA and DNTT might interfere with the process of the V(D)J rearrangement in the IgH gene in the MDD.

When it comes to DNA methylation status, the bisulfate method reveals that both Pax5 and EBF1 promoters are already demethylated even under the MSD condition. It is conceivable that the methylation status of the CpG islands of the Pax5 promoter and EBF1 are less likely to be a cause of the defects of B cell development.

Recent reports have suggested two links of PcG silencing with DNA methylation and histone methylation. Firstly, in the PRC2 complex, EZH2 interacts with DNMT1, DNMT3a and DNMT3b. This interaction is capable of recruiting DNA methylation machinery to certain genomic regions. In the PRC1 complex, BMI1 and HPC2 subunits interact with the DNMT1 and DNMT3a, respectively. Secondly, in the PRC2 complex, EZH2 has histone methyltransferase activity solely for H3K27me2/3 as a ‘writer’ for lysine residue 27 of H3 histone for silencing.

Any defect in B cell development can cause an immunodeficiency syndrome. An abnormal B cell repertoire might also cause an allergic or autoimmune disease.

PAX5 was also known to be the main target of somatic mutations in acute lymphoblastic leukemia [43]. Mulholland et al. [43] performed a genome-wide analysis of leukemic cells from 242 pediatric acute lymphoblastic leukemia patients using high-resolution single-nucleotide polymorphism arrays and genomic DNA sequencing. Their analyses revealed deletion, amplification, point mutation and structural rearrangement in genes encoding principal regulators of B lymphocyte development and differentiation in 40% of B-progenitor acute lymphoblastic leukemia cases. The *Pax5* gene was the most frequent target of somatic mutation. It was altered in 31.7% of the cases. The identified PAX5 mutations resulted in reduced levels of PAX5 protein or the generation of hypomorphic alleles. Therefore, the optimum Pax5 regulation is critically important for proper B cell development. The MDD-induced downregulation of Pax5 might contribute to causing B cell developmental defects observed under the MDD condition.

In conclusion, we reported here the B cell developmental defects in bone marrow under the MDD condition; there were some intrinsic developmental defects in B lymphocytes. It is conceivable that B cell development is regulated by transcription factors and epigenetic regulators. Changes in their expression levels might affect B cell development when on an MDD. However, the biological connections between Pax5, EBF1 and epigenetic regulators in B cell development are still unknown. Further studies are needed to decipher their relationships and mechanisms.

Acknowledgements

We thank Dr. Rieko Kanno for assistance with the FACS analysis and critical discussions. This work was supported by Environment Research and Technology Development Fund of the Ministry of Environment, Japan.

References

- Golka K, Hengstler JG, Marchan R, Bolt HM: Severe arsenic poisoning: one of the largest man-made catastrophies. *Arch Toxicol* 2010; 84:583–584.
- Dunn BK, Verma M, Umar A: Epigenetics in cancer prevention: early detection and risk assessment: introduction. *Ann NY Acad Sci* 2003;983:1–4.
- Li E: Chromatin modification and epigenetic reprogramming in mammalian development. *Nat Rev Genet* 2002;3:662–673.
- Gronbaek K, Hother C, Jones PA: Epigenetic changes in cancer. *APMIS* 2007;115:1039–1059.
- Goll MG, Bestor TH: Eukaryotic cytosine methyltransferases. *Annu Rev Biochem* 2005; 74:481–514.
- Li E, Bestor TH, Jaenisch R: Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* 1992;69: 915–926.
- Okano M, Bell DW, Haber DA, Li E: DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* 1999;99:247–257.
- Lund AH, van Lohuizen M: Polycomb complexes and silencing mechanisms. *Curr Opin Cell Biol* 2004;16:239–246.
- Ringrose L, Paro R: Epigenetic regulation of cellular memory by the Polycomb and Trithorax group proteins. *Annu Rev Genet* 2004;38:413–443.
- Cao R, Wang L, Wang H, Xia L, Erdjument-Bromage H, Tempst P, Jones RS, Zhang Y: Role of histone H3 lysine 27 methylation in Polycomb-group silencing. *Science* 2002; 298:1039–1043.
- Czermin B, Melfi R, McCabe D, Seitz V, Imhof A, Pirrotta V: *Drosophila* enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. *Cell* 2002;111:185–196.
- Kuzmichev A, Nishioka K, Erdjument-Bromage H, Tempst P, Reinberg D: Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein. *Genes Dev* 2002; 16:2893–2905.
- Viré E, Brenner C, Deplus R, Blanchon L, Fraga M, Didelot C, Morey L, Van Eynde A, Bernard D, Vanderwinden JM, Bollen M, Esteller M, Di Croce L, de Launoit Y, Fuks F: The Polycomb group protein EZH2 directly controls DNA methylation. *Nature* 2006; 439:871–874.
- Poux S, Melfi R, Pirrotta V: Establishment of Polycomb silencing requires a transient interaction between PC and ESC. *Genes Dev* 2001;15:2509–2514.
- Galy A, Travis M, Cen D, Chen B: Human T, B, natural killer, and dendritic cells arise from a common bone marrow progenitor cell subset. *Immunity* 1995;3:459–473.
- Akashi K, Traver D, Miyamoto T, Weissman IL: A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature* 2000;404:193–197.
- Kondo M, Weissman IL, Akashi K: Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell* 1997; 91:661–672.
- Igarashi H, Gregory SC, Yokota T, Sakaguchi N, Kincade PW: Transcription from the RAG1 locus marks the earliest lymphocyte progenitors in bone marrow. *Immunity* 2002;17:117–130.
- Jung D, Giallourakis C, Mostoslavsky R, Alt FW: Mechanism and control of V(D)J recombination at the immunoglobulin heavy chain locus. *Annu Rev Immunol* 2006;24: 541–570.
- Hardy RR, Carmack CE, Shinton SA, Kemp JD, Hayakawa K: Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. *J Exp Med* 1991;173:1213–1225.
- Li Y S, Hayakawa K, Hardy RR: The regulated expression of B lineage-associated genes during B cell differentiation in bone marrow and fetal liver. *J Exp Med* 1993;178: 951–960.
- Li YS, Wasserman R, Hayakawa K, Hardy RR: Identification of the earliest B lineage stage in mouse bone marrow. *Immunity* 1996;5:527–535.
- Meffre E, Casellas R, Nussenzweig MC: Antibody regulation of B cell development. *Nat Immunol* 2000;1:379–385.
- Nohara K, Baba T, Murai H, Kobayashi Y, Suzuki T, Tateishi Y, Matsumoto M, Nishimura N, Sano T: Global DNA methylation in the mouse liver is affected by methyl deficiency and arsenic in a sex-dependent manner. *Arch Toxicol* 2010;85:653–661.

- 25 Vieira P, Cumano A: Differentiation of B lymphocytes from hematopoietic stem cells. *Methods Mol Biol* 2004;271:67–76.
- 26 Locker J, Reddy TV, Lombardi B: DNA methylation and hepatocarcinogenesis in rats fed a choline-devoid diet. *Carcinogenesis* 1986;7:1309–1312.
- 27 Tsujiuchi T, Tsutsumi M, Sasaki Y, Takahama M, Konishi Y: Hypomethylation of CpG sites and c-myc gene overexpression in hepatocellular carcinomas, but not hyperplastic nodules, induced by a choline-deficient L-amino acid-defined diet in rats. *Jpn J Cancer Res* 1999;90:909–913.
- 28 Wainfan E, Dizik M, Stender M, Christman JK: Rapid appearance of hypomethylated DNA in livers of rats fed cancer-promoting, methyl-deficient diets. *Cancer Res* 1989;49:4094–4097.
- 29 Decker T, Pasca di Magliano M, McManus S, Sun Q, Bonifer C, Tagoh H, Busslinger M: Stepwise activation of enhancer and promoter regions of the B cell commitment gene Pax5 in early lymphopoiesis. *Immunity* 2009;30:508–520.
- 30 Negishi M, Saraya A, Miyagi S, Nagao K, Inagaki Y, Nishikawa M, Tajima S, Koseki H, Tsuda H, Takasaki Y, Nakauchi H, Iwama A: Bmi1 cooperates with Dnmt1-associated protein 1 in gene silencing. *Biochem Biophys Res Commun* 2007;353:992–998.
- 31 Paro R, Hogness DS: The Polycomb protein shares a homologous domain with a heterochromatin-associated protein of *Drosophila*. *Proc Natl Acad Sci USA* 1991;88:263–267.
- 32 Tryndyak VP, Han T, Muskhelishvili L, Fuscoe JC, Ross SA, Beland FA, Pogribny IP: Coupling global methylation and gene expression profiles reveal key pathophysiological events in liver injury induced by a methyl-deficient diet. *Mol Nutr Food Res* 2011;55:411–418.
- 33 Cobaleda C, Jochum W, Busslinger M: Conversion of mature B cells into T cells by dedifferentiation to uncommitted progenitors. *Nature* 2007;449:473–477.
- 34 Horcher M, Souabni A, Busslinger M: Pax5/BSAP maintains the identity of B cells in late B lymphopoiesis. *Immunity* 2001;14:779–790.
- 35 Lukin K, Fields S, Hartley J, Hagman J: Early B cell factor: Regulator of B lineage specification and commitment. *Semin Immunol* 2008;20:221–227.
- 36 Mikkola I, Heavey B, Horcher M, Busslinger M: Reversion of B cell commitment upon loss of Pax5 expression. *Science* 2002;297:110–113.
- 37 Bertolino E, Reddy K, Medina K, Parganas E, Ihle J, Singh H: Regulation of interleukin 7-dependent immunoglobulin heavy-chain variable gene rearrangements by transcription factor STAT5. *Nat Immunol* 2005;6:836–843.
- 38 Cain D, Kondo M, Chen H, Kelsoe G: Effects of acute and chronic inflammation on B-cell development and differentiation. *J Invest Dermatol* 2009;129:266–277.
- 39 Fuxa M, Skok J, Souabni A, Salvagiotto G, Roldan E, Busslinger M: Pax5 induces V-to-DJ rearrangements and locus contraction of the immunoglobulin heavy-chain gene. *Genes Dev* 2004;18:411–422.
- 40 Nutt SL, Vambrie S, Steinlein P, Kozmik Z, Rolink A, Weith A, Busslinger M: Independent regulation of the two Pax5 alleles during B-cell development. *Nat Genet* 1999;21:390–395.
- 41 Nutt SL, Busslinger M: Monoallelic expression of Pax5: a paradigm for the haploinsufficiency of mammalian Pax genes? *Biol Chem* 1999;380:601–611.
- 42 Rhoades KL, Singh N, Simon I, Glidden B, Cedar H, Chess A: Allele-specific expression patterns of interleukin-2 and Pax-5 revealed by a sensitive single-cell RT-PCR analysis. *Curr Biol* 2000;10:789–792.
- 43 Mullighan C, Goorha S, Radtke I, Miller C, Coustan-Smith E, Dalton J, Girtman K, Mathew S, Ma J, Pounds S, Su X, Pui C, Relling M, Evans W, Shurtleff S, Downing J: Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. *Nature* 2007;446:758–764.