Aging in Heterozygous Dnmt1-Deficient Mice: Effects on Survival, the DNA Methylation Genes, and the Development of Amyloidosis

Donna Ray,1 Ailing Wu,1 J. Erby Wilkinson,2 Hedwig S. Murphy,2,3 Qianjin Lu,1,5 Barbara Klueve-Beckerman,6 Juris J. Liepnies,6 Merrill Benson,6 Raymond Yung,1,4 and Bruce Richardson1,4

Departments of 1Medicine and 2Pathology, University of Michigan, Ann Arbor. Departments of 3Pathology and Laboratory Medicine and 4Medicine, Ann Arbor Veterans Administration Health Service, Michigan. 5Second Xiangya Hospital, Central South University, Changsha, China. 6Department of Pathology and Laboratory Medicine at the Indiana University School of Medicine, Indianapolis.

We previously reported that heterozygous DNA methyltransferase 1-deficient (Dnmt1+/−) mice maintain T-cell immune function and DNA methylation levels with aging, whereas controls develop autoimmunity, immune senescence, and DNA hypomethylation. We therefore compared survival, cause of death, and T-cell DNA methylation gene expression during aging in Dnmt1+/− mice and controls. No difference in longevity was observed, but greater numbers of Dnmt1+/− mice developed jejunal apolipoprotein AII amyloidosis. Both groups showed decreased Dnmt1 expression with aging. However, expression of the de novo methyltransferases Dnmt3a and Dnmt3b increased with aging in stimulated T cells from control mice. MeCP2, a methylcytosine binding protein that participates in maintenance DNA methylation, increased with age in Dnmt1+/− mice, suggesting a mechanism for the sustained DNA methylation levels. This model thus provides potential mechanisms for DNA methylation changes of aging, and suggests that changes in DNA methylation may contribute to some forms of amyloidosis that develop with aging.
Intestine Sections
Formalin-Fixed Paraffin-Embedded Mouse

Isolation and Identification of Amyloid Protein From
Intestine Sections

Amyloid protein was isolated from unstained, 4 μm-thick sections of intestine cut from a formalin-fixed, paraffin-embedded mouse tissue block and placed on glass slides (10). As previously described (11), tissue sections were deparaffinized and rehydrated, and intestine sections were scraped off and treated with 6M guanidine hydrochloride containing dithiothreitol. After alkylation with iodoacetate, the sample was centrifuged, filtered, exhaustively dialyzed against water, and lyophilized. The extract was digested with trypsin and fractionated on a SynChropak RP-8 column (100 × 4.6 mm; Synchrom, Lafayette, IN) eluted with an acetonitrile gradient. Samples were subjected to Edman degradation analysis on an Applied Biosystems (Foster City, CA) model 491cLC protein sequencer using the manufacturer’s standard cycles.

Immunohistochemical Characterization of Amyloid
Apolipoprotein AII (ApoAII) was detected immunohistochemically in paraffin-embedded tissues using the VECTASTAIN ABC method (Vector Laboratories, Burlingame, CA). Antiserum against mouse ApoAII was provided by Dr. K. Higuchi (Shinsh University Graduate School of Medicine) and used at a dilution of 1:1000.

T-Cell Isolation and Culture
Spleens were removed from the mice, and splenocytes were recovered as described (7). T cells were isolated with Miltenyi beads using protocols provided by the manufacturer, then stimulated with immobilized anti-CD3 and soluble anti-CD28 and cultured as previously described by our group (7).

Quantitative Reverse Transcription–Polymerase Chain Reaction
Unstimulated and stimulated T cells were suspended in Trizol (GIBCO-BRL, Gaithersburg, MD) and homogenized, and total RNA was purified according to the manufacturer’s protocol. Real-time reverse transcription–polymerase chain reaction was performed with a LightCycler thermocycler (Roche, Indianapolis, IN), using previously published protocols and primers for murine Dnmt1, Dnmt3a, Dnmt3b, and MeCP2 (7). At the completion of amplification, melting characteristics of the product were determined, and water was included as a negative control to rule out primer dimer formation. A series of five dilutions of one RNA sample was included to generate a standard curve; this curve was used to obtain relative concentrations of the transcript of interest in each of the RNA samples, using LightCycler software. Results are presented relative to β-actin or histone H4 transcripts, similarly amplified using the following primers, listed as forward; reverse: H4: 5'AGACCTTCAGCACACCGC and 5'CATACCCAAGAGGAAGGCTGG.

Statistical Analysis
Differences in survival between groups were tested using the Mantel–Haenszel test calculated with Systat 10 software (Evanston, IL). Differences between means were tested using Student’s t test, and between groups by chi-square analysis. Effects of T-cell stimulation on Dnmt expression...
over time were tested using the residual maximum likelihood (REML) method.

RESULTS

Effect of Dnmt1 Deficiency on Longevity and Cause of Death

The effect of the heterozygous Dnmt1 null mutation on longevity and cause of death was determined using 54 male mice heterozygous for Dnmt1 deficiency and 56 wild-type male littermates. Of these, 9 of the Dnmt1-deficient mice and 56 wild-type controls. The y-axis represents survival fraction, and the x-axis represents time in years.

Figure 1. Comparative survival rates of heterozygous Dnmt1-deficient mice and controls. Survival was compared in cohorts consisting of 45 male Dnmt1 heterozygous deficient mice and 52 male wild-type controls. The y-axis represents survival fraction, and the x-axis represents time in years.

ApoAII deposition was seen under polarizing light, consistent with amyloid deposition. Similar Congo Red staining of the brain, kidney, heart, liver, lung, pancreas, and spleen demonstrated no significant amyloid deposition, although there were scant amounts in the loose connective tissue surrounding the esophagus and other tissues. Thus the deposition was largely confined to the jejunum.

Amyloid Characterization

To identify the amyloid subtype, protein was solubilized from formalin-fixed, paraffin-embedded jejunal tissue sections with guanidine hydrochloride and digested with trypsin, and the resulting peptides were fractionated by reverse-phase high performance liquid chromatography (HPLC). Edman analysis of one eluted peak yielded the sequence: Thr-Ser-Glu-Ile-Gln-Ser-Gln-Val-Lys. Basic Local Alignment Search Tool (BLAST) analysis of the sequence showed that it was identical to mouse ApoAII precursor residues 54–62 or mature plasma ApoAII residues 31–39. The identity of the amyloid was verified by immunohistochemistry using anti-ApoAII, provided by Dr. K. Higuchi. Figure 4A shows heavy ApoAII deposition in the jejunum of a Dnmt1 heterozygous mouse 32 months of age. Figure 4B shows absence of staining in a control mouse 31 months of age, and C and D of Figure 4 show patchy and significantly less intense ApoAII deposition in jejunal tissue from another control 32 months of age. The results thus indicate that the amyloid is derived from ApoAII as occurs in the senescence-accelerated mouse and occasionally in other strains (13), and that its deposition is accelerated in the Dnmt1 heterozygous strain.

We considered the possibility that the amyloid deposition contributed to the demise of the animals through malnutrition. This possibility was tested by comparing weights of mice dying from amyloidosis to mice dying from developing jejunal amyloidosis was significant ($p < .02$ by chi-square analysis, $p < .04$ with Bonferroni correction for the malignancy comparison). The average age at death of mice with amyloidosis was $2.5 \pm 0.1$ years in the Dnmt1$^{+/−}$ and $2.4 \pm 0.3$ years in the controls, whereas the average age of death in mice with lymphoma was $2.3 \pm 0.3$ years in the Dnmt1$^{+/−}$ and $2.5 \pm 0.3$ years in the controls (mean ± standard deviation [SD]). The similarity in age of mice dying with these conditions may contribute to the lack of differential survival between groups.

Figure 2 shows a representative jejunal specimen from a heterozygous Dnmt1-deficient mouse with amyloid stained with hematoxylin and eosin, and demonstrating increased amounts of proteinaceous material distributed diffusely throughout the villi. Figure 3 shows a jejunal section stained with Congo Red and viewed under conventional (A) and polarized (B) light. Bright apple green birefringence is seen under polarizing light, consistent with amyloid deposition. Similar Congo Red staining of the brain, kidney, heart, liver, lung, pancreas, and spleen demonstrated no significant amyloid deposition, although there were scant amounts in the loose connective tissue surrounding the esophagus and other tissues. Thus the deposition was largely confined to the jejunum.

Table 1. Histologic Abnormalities

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<th>Dnmt1 Knockout (N = 29)</th>
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<tr>
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<td>10</td>
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malignancies. In the Dnmt1⁺⁺/⁻ group, the average weight at death of the mice with amyloid was 23.8 ± 2.9 grams (mean ± SD), and the average weight of the mice with malignancies was 29.5 ± 6.1 grams. Similarly, the mean weight of the control mice with amyloid was 20.3 ± 3.7 grams, and 31.7 ± 8.2 grams in the mice with malignancies. Combining all mice with malignancies and mice with amyloid, the decreased weight of the mice with amyloid was significant (p = .001 by t test).

**Effect of Aging on DNA Methyltransferase and MeCP2 Expression in Dnmt1-Deficient and Control Mice**

The other goal of these studies was to compare the magnitude of DNA methyltransferase increases following mitogenic stimulation in adult and old Dnmt1-deficient mice and controls as well as changes in MeCP2 levels with aging in the same groups. As noted above, our earlier work demonstrated that total genomic d⁰C content was approximately the same in T cells from 11-month-old control and Dnmt1-deficient mice, and decreased significantly by 18 months of age in the control mice as reported by others for other murine tissues (14), but not in the Dnmt1 knockout mice. There were no significant shifts in memory and naïve or CD4⁺ and CD8⁺ subsets in either group of mice during this time (7), arguing for an intrinsic effect on the mechanisms maintaining DNA methylation levels rather than an effect due to changes in T-cell subsets. In the present studies we therefore compared DNA methyltransferase and MeCP2 transcripts in unstimulated and stimulated T cells from adult and old Dnmt1-deficient mice and controls.

Splenic T lymphocytes were isolated from 4–5 adult (11.2 ± 1.2 months, mean ± SD) or old (19.4 ± 1.4 months) heterozygous Dnmt1-deficient mice and their wild-type littermates. The purified T cells were stimulated with anti-CD3 + anti-CD28, and cells were harvested at 0, 6, and 24 hours. Dnmt1 transcripts were measured relative to histone H4, as others have reported that levels are linked to the cell cycle (15). In contrast, Dnmt3a and Dnmt3b are not cell cycle linked (9), and were measured relative to β-actin. Figure 5 shows the effect of age on the Dnmt transcripts in unstimulated and stimulated T cells from the wild-type and Dnmt1⁺⁺/⁻ mice. In the control mice (left panels), Dnmt1 levels were higher in unstimulated cells from the adult mice relative to the old, and remained higher at all time points tested (p = .001 overall). The relative decrease in Dnmt1 expression with age was also observed in the unstimulated (T = 0) cells when transcripts were quantitated relative to actin (0.96 ± 0.10 vs 0.27 ± 0.04, adult vs old, mean ± SEM, p = .001 by t test). In contrast, Dnmt3a and Dnmt3b transcripts were essentially identical in unstimulated T cells from the adult and old control mice, but increased significantly more in the old control group following stimulation.
Relative amounts of the methyltransferases between the increases in the de novo methyltransferases. Further, there were no compensatory levels alone as causing progressive DNA demethylation. This sustains DNA methylation argues against decreases in Dnmt1. This sustained DNA methylation argues against decreases in Dnmt1 levels alone as causing progressive DNA demethylation with age in these mice. Further, there were no compensatory increases in the de novo methyltransferases.

We considered the possibility that differences in the relative amounts of the methyltransferases between the control and Dnmt1+/− mice could contribute to the differences in DNA methylation. Figure 6 shows the same data analyzed to compare the transcripts in adult and old control and Dnmt1+/− mice. There is no significant difference in Dnmt1 or Dnmt3a transcripts in the adult control and the Dnmt1+/− mice at any time point (left panels). However, there were significantly greater amounts of Dnmt3b 6 hours after stimulation of the Dnmt1+/− mice relative to controls (p = .04). The increase in Dnmt3b in the adult Dnmt1+/− mice suggests a possible explanation for the increase in T-cell DNA methylation seen between 6 and 11 months of age in these mice previously reported by our group (7). The right panels compare the transcripts in the old cohorts. There were no statistically significant differences between the two groups by 20 months of age for any of the three transcripts at any time point, although there is a trend toward increased expression in stimulated cells from the controls. The observation that none of the methyltransferases were significantly increased in the old Dnmt1+/− mice relative to controls argues against the possibility that DNA methylation levels are maintained in the aging Dnmt1+/− mice by increased levels of one or more of the DNA methyltransferases.

Our earlier study found that MeCP2 levels decrease with age in the brains of the control but not the Dnmt1-deficient mice (7). Others reported that MeCP2 maintains DNA methylation by binding Dnmt1 and hemimethylated DNA, focusing methyltransferase activity on the hemimethylated substrate and restoring fully methylated DNA (16). We therefore compared MeCP2 transcript levels in T cells from adult and old controls and from Dnmt1-deficient mice (Figure 7). MeCP2 transcript levels were not significantly different in the adult controls and Dnmt1+/− mice. There was a small but not statistically significant decrease in MeCP2 levels with age in the control mice (p = .22, adult vs old controls by analysis of variance and post hoc testing with Bonferroni correction). In contrast, the transcript levels increased significantly with age in the Dnmt1+/− mice (p = .004 adult vs old). The difference between the old controls and Dnmt1+/− mice was also significant (p = .03). There was no significant effect of stimulation on MeCP2 transcript levels (not shown). These observations raise the possibility that MeCP2 overexpression might contribute to the stability of DNA methylation patterns with age in the Dnmt1+/− group by tethering Dnmt1 to hemimethylated sites, despite an overall decrease in maintenance Dnmt1 levels (vide infra).

**DISCUSSION**

The major conclusions from this study are that the heterozygous Dnmt1-null mutation does not significantly modify overall longevity in C57BL/6 mice, that the Dnmt1+/− mice develop jejunal ApoAI amyloidosis more frequently than do controls, that aging affects DNA methyltransferase and MeCP2 expression differently in the control and Dnmt1+/− mice, and that de novo methyltransferase transcripts increase with age in dividing T cells from normal mice. Recent reports indicate that C57BL/6 mice die most commonly from malignancies, with lymphoma the most frequent and occurring in as many as 60% (12). Lymphoma
was the most commonly encountered malignancy in our control group, although fewer (~25%) had lymphoma at death than were reported by others (12). As the overall survival in the present study compares favorably to others, the reason for the decreased incidence of malignancy in the controls is uncertain. The Dnmt1-deficient mice developed somewhat fewer malignancies, particularly lymphomas, although the difference was not statistically significant. The lack of statistical significance may be due to the lower-than-expected incidence of lymphoma and therefore lack of statistical power, because DNA hypomethylation has been reported to contribute to genomic instability and lymphoma development in mice (17,18), and DNA demethylates in the controls but not the Dnmt1+/− mice (7). Interestingly, heterozygous Dnmt1 deficiency can decrease the incidence of intestinal tumor formation in Apc(Min/+; mice (19), although only one was observed in our study, and was in a Dnmt1-deficient mouse.

The development of amyloidosis in the Dnmt1+/− mice was unexpected. The jejunal amyloidosis may have contributed to the death of the affected mice, because their weight was significantly lower than that of the mice dying from cancers. This is also supported by reports that ApoAII deposition shortens murine life span by ~20% (20). No overall effect on survival was noted, though, probably because the amyloidosis developed late in life, and the control mice likely succumbed to other causes at the same age.

Characterization of the amyloid included isolation and sequencing of a fragment of the protein, and indicated that the protein was ApoAII. Mutations in the gene encoding this protein cause a form of hereditary amyloidosis in humans (21). A peptide corresponding to ApoAII residues 31–39 showed a valine at position 38. This indicates ApoAIIA, the allele occurring in C57BL/6 mice (13). Others have reported similar ApoAII deposition in old C57BL6 mice (13). However, the deposition is highly dependent on the conditions under which the mice are raised, with ApoAIIA deposition detected in C57BL/6 and BDF1 mice aged in some laboratories and not others (13). Our observation that deposition was increased in the Dnmt1-deficient mice relative to wild-type littermates, raised in adjacent cages in the same facility, suggests that epigenetic changes in DNA methylation patterns may contribute to variability in onset between institutions. These changes could reflect differences

Figure 4. Peroxidase immunostaining of jejunal amyloid. A, Jejunal section from a 32-month-old Dnmt1+/− mouse with amyloid was stained with anti-apolipoprotein AI (ApoAII) and immunoperoxidase. Brown staining indicates immunoreactivity. B, Jejunal tissue from a 31-month-old control mouse similarly stained, showing no immunoreactivity. C and D, Jejunal tissue from a 32-month-old control mouse showing mild ApoAII in one section (C) and absence of deposition in another section (D).
Figure 5. DNA methyltransferase transcript levels in adult versus old control and Dnmt1-deficient mice. T cells from adult (11 month) and old (19 month) wild-type (control, left column) and heterozygous Dnmt1-deficient mice (knockout, right column) were stimulated with anti-CD3 + anti-CD28, and transcripts of Dnmt1 (top), Dnmt3a (middle), and Dnmt3b (bottom) quantitated at the indicated times. Dnmt1 is expressed relative to histone H4 transcripts, and Dnmt3a and 3b are expressed relative to β-actin. The significance of the overall differences between groups was tested using the residual maximum likelihood (REML) method (N.S. = not significant).
Figure 6. DNA methyltransferase transcript levels in control versus Dnmt1-deficient (KO) adult and old mice. The results shown in Figure 5 are plotted to permit comparison of control and Dnmt1$^{+/+}$ (KO) mice. Dnmt1, Dnmt3a, and Dnmt3b results are shown in the top, middle, and bottom rows, respectively, as in Figure 5. Left column: results from the adult mice; right column: results from the old mice. Overall differences between groups were not significant. The significance of the increase in Dnmt3b in the adult Dnmt1$^{+/+}$ mice at 6 hours was determined using Student's $t$ test.
increasing pools of such as folic acid, methionine, B12, choline, and others in nutritional factors affecting transmethylation reactions (23). Others have proposed that DNA methylation changes may predispose to deposition of other types of amyloid with aging, including demethylation of the amyloid A4 precursor gene in Alzheimer’s disease (24); the present findings support this mechanism.

The effects of heterozygous Dnmt1 deficiency on DNA methyltransferase expression in aging is also of interest. We previously demonstrated that total genomic dmC content may increase in the livers of these enzymes (28). How Dnmt1 recognizes and methylates hemimethylated DNA at sites distant from the replication fork, such as might occur during replication when Dnmt1 levels are limiting and CG pairs aberrantly missed, or during DNA repair, is not explained by the replication fork model. MeCP2 binds Dnmt1 and both fully methylated as well as hemimethylated DNA. Further, the Dnmt1–MeCP2 complex is capable of methylating hemimethylated DNA to which the MeCP2 is bound. This fact supports a model in which MeCP2 targets Dnmt1 to hemimethylated DNA occurring elsewhere than the replication fork to maintain methylation patterns (16). This model thus predicts that methylation patterns not faithfully replicated during mitosis, resulting in a methylated parent strand and an unmethylated daughter strand, can be restored postmitotically by the MeCP2–Dnmt1 complex. This model is consistent with earlier work demonstrating biphasic remethylation kinetics in experimentally demethylated cells, with some sequences rapidly remethylated during mitosis, and others remethylating up to several hours later (29). We propose that age-related demethylation requires both a decrease in Dnmt1 as well as a decrease in MeCP2, and that stable or increased MeCP2 levels are able to target Dnmt1 to hemimethylated sequences even if Dnmt1 levels are limiting during mitosis.

Finally, the increases in Dnmt3a and 3b following stimulation in T cells from the old controls are of interest. CpG islands in the promoters of some tumor suppressor genes aberrantly methylate with aging in tissues such as colonic epithelium, contributing to the development of malignancies (4). The mechanism is unknown. The present results suggest that overexpression of de novo methyltransferases during mitosis may contribute to the de novo methylation of some CpG islands with age. This hypothesis is supported by reports of CpG hypermethylation in cells overexpressing these enzymes (30,31). Further, others have reported that total genomic dmC content may increase in the livers of
C57BL/6 mice after 24 months of age (32), possibly due to similar increases in de novo methyltransferases.

Together these results further support the hypothesis that changes in maintenance and de novo DNA methylation contribute to diseases such as amyloidosis, autoimmunity, and possibly cancer with aging, and suggest new and intriguing hypotheses for further investigation into the role that DNA methylation plays in diseases important in aging.

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Address correspondence to Bruce Richardson, MD, PhD, 5310 Cancer Center and Geriatrics Center Bldg., Ann Arbor, MI 48109-0940. E-mail: brichard@umich.edu

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