Genomic 5-Methyldeoxycytidine Decreases with Age*

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Vincent L. Wilson‡, Ruth A. Smith‡, Shuyi Ma§, and Richard G. Cutler¶

From the ‡Laboratory of Human Carcinogenesis, Division of Cancer Etiology, National Cancer Institute, Bethesda, Maryland 20892 and the §National Institute on Aging, Gerontology Research Center, Baltimore, Maryland 21224

Significant losses of DNA 5-methyldeoxycytidine residues in old age could disrupt cellular gene expression and contribute to the physiological decline of the animal. Thus, the 5-methyldeoxycytidine content of DNAs, isolated from the tissues of two rodent species of various ages, were determined. Mus musculus lost DNA methylation sites at a rate of about 4.7 × 10⁻⁴ (approximately 0.012% of the newborn level)/month. Peromyscus leucopus lost DNA 5-methyldeoxycytidine residues at a rate of only 2.3 × 10⁻⁴ (approximately 0.006% of the newborn level)/month. Since P. leucopus generally live twice as long as M. musculus, the rate of loss of DNA 5-methyldeoxycytidine residues appears to be inversely related to life span. Similar losses in genomic 5-methyldeoxycytidine content were also observed to correlate with donor age in cultured normal human bronchial epithelial cells.

Alterations in DNA 5-methyldeoxycytidine (5mdCyd) patterns have been implicated in many of the basic processes of mammalian cells, including embryogenesis, differentiation, and carcinogenesis (for reviews see Refs. 1–4). Holliday (5) also proposed that changes in DNA 5mdCyd patterns may be involved in the aging process. DNA methylation patterns appear to be the first level of control of gene expression for at least some mammalian genes (6, 7) and maintain the state of differentiation of the cell (3), so that losses of DNA 5mdCyD sites in aging cells, such as that observed in mammalian cell cultures (8, 9), may disrupt gene regulation and perturb the normal state of cellular differentiation in old cells. If the development of old age is the result of such a dysdifferentiation process (10), then longer-lived species must have evolved a superior means of maintaining or stabilizing the proper differentiation state of cells. Species with greater maximum life span potentials, therefore, should have more stable DNA 5mdCyd patterns. The fact that cultured normal human cells have more stable DNA methylation patterns than rodent cells (8) supports this conclusion.

Changes in the genomic content of 5mdCyd of tissues of two rodent species of widely different life span potentials were monitored in the present study. Although some evidence has been reported suggesting an age-related decrease in genomic 5mdCyd levels in mammalian tissues (11, 12), neither the rate of DNA 5mdCyd changes nor species comparisons have been determined. The results presented here demonstrate not only that genomic 5mdCyd levels decline with in vivo age, but that the rate of DNA 5mdCyd loss also appears to be inversely proportional to the maximum life span potential.

EXPERIMENTAL PROCEDURES

Four-week-old C57 BL/6J mice (Mus musculus) from Jackson Laboratory (Bar Harbor, ME) were maintained in the Gerontology Research Center animal colony until use. White-footed mice (Peromyscus leucopus) were housed and bred in the Gerontology Research Center animal facilities. Six mice of the same age were killed by cervical dislocation; the brains, livers, and small intestinal mucosae were pooled; and the DNAs were isolated by standard methods as previously described (13).

Normal human bronchial epithelial cell outgrowths (first passage) were obtained from freshly excised bronchial tissue from immediate autopsies when postmortem delay was less than 1 h. The normal human bronchial epithelial cells were grown on surface-coated (10 pg/ml fibronectin, 10 pg/ml bovine serum albumin, 30 µg/ml Vitrogen® collagen) 100-mm plastic culture dishes with serum-free LHC-9 medium as previously described (14). Second passage cells were seeded at 2–5 × 10⁴ cells/surface-coated T-75 flask. Log phase normal human bronchial epithelial cells were harvested 48 to 72 h later, and the DNAs were isolated by standard methods as previously described (13).

The level of 5mdCyd was quantitated by ³²P postlabeling as previously described (13). Briefly, the DNA was digested to 3'-monophosphate nucleotides by micrococcal nuclease (Sigma) and calf spleen phosphodiesterase (Boehringer Mannheim). These 3'-monophosphate nucleotides were converted to ³²P-labeled 5',3'-bisphospho-nucleotides by the action of T₄ polynucleotide kinase (3'-phosphatase-free, Du Pont-New England Nuclear) in the presence of [γ-³²P]ATP (5000 Ci/mmol, Amersham Corp.). The ³²P-phosphate was removed by nuclease P₁ (Sigma), the labeled 5'-monophosphate nucleotides were separated by two-dimensional thin layer chromatography, and the relative amounts of individual nucleotides were determined by scraping and counting the radioactivity (13). The 5mdCyd content of DNA was calculated from the radioactivity found in 5mdCMP and dCMP by the following equation % 5mdCyd = 5mdCMP/(5mdCMP + dCMP) × 100.

RESULTS AND DISCUSSION

To characterize DNA methylation changes during the normal aging process in vivo, genomic 5mdCyd levels were measured in DNA isolated from the tissues of two rodent species of similar size and basal metabolic rate (16), C57 BL/6J mice (Mus musculus) and white-footed mice (Peromyscus leucopus). DNA 5mdCyd levels were measured utilizing our modifications of the Randerath et al. (17) ³²P postlabeling methods (13). Significant reductions in the genomic 5mdC content of brain, liver, and small intestinal mucosa were observed to correlate with the chronological age of the C57 BL/6J mice (Fig. 1). Similar age-related decreases in liver and small intestinal mucosa DNA 5mdCyd residues were also observed in white-footed mice (Fig. 2). The total number of genomic 5mdCyd residues were reduced by more than 10% or greater than 5.0% 5mdCyd residues/cell (Table I) over the ages monitored in each of the two rodent species. Genomic 5mdCyd levels declined about twice as fast in the C57 BL/6J mice as in the white-footed mice (Table I). The maximum life span potentials of C57 BL/6J and white-footed mice have been reported to be 42 and 96 months, respectively (18). Thus, the rate of DNA 5mdCyd loss appears to be inversely proportional to the maximum life span potential.

Assuming the rate of loss of DNA 5mdCyd residues was...
constant throughout the life span of these rodents, the total maximum reduction in genomic 5mCyd is approximately 20% of the newborn DNA methylation level. Extrapolation to human tissues would predict an estimated rate of loss of DNA methylation of 0.007% of the newborn genomic 5mCyd content/year, based on a maximum life span potential of 95 years (19). This is in agreement with the results of monitoring the genomic 5mCyd content of cultured, second passage normal human bronchial epithelial cells obtained from autopsy donors of various ages (Fig. 3). Linear regression analysis provided a slope of \(-0.004%\)/year, which amounts to a loss of about 1.6 \(\times\) 10^5 5mCyd residues/year/human epithelial cellular genomic complement (Table I).

These 5mCyd losses in aging tissues might be partially accounted for by selective loss of highly repetitive DNA sequences (20, 21), which are known to contain the highest percentage of methylation sites (22). However, the loss of highly repetitive sequences is not sufficient to account for the total reduction in 5mCyd content observed in these studies (8).

Unlike the intimate relationship of decreases in DNA methylation with cell division observed in vitro (8, 9), the present results suggest that 5mCyd reductions were not necessarily related to cell division in vivo. Three separate tissues of widely varying physiological functions and mitotic index had similar rates of 5mCyd reductions (Table I). Adult neural tissue and hepatic tissue have relatively low mitotic indexes, while mucosa epithelial cells constantly replenish themselves, so that the loss of DNA 5mCyd residues may occur in nondividing cells. This conclusion is further supported by the fact that the number of cell divisions epithelial cells undergo in vivo age may be less than expected due to the possibility of clonal succession (23) in tissues such as intestinal mucosa. Alternatively, the rate of DNA 5mCyd residue loss could reflect inefficient maintenance of methylation patterns following DNA replication (8) in the proportion of cells which were dividing in the tissue. Although adult neurons may not turnover, the majority of brain tissue is composed of supporting cells, e.g. astrocytes and other neuroglia cells, which do divide. This explanation seems unlikely since tissues of both low and high mitotic indexes were found to have similar rates of DNA 5mCyd reductions. The possibility that these reductions in DNA methylation represent the continuous selection of a specific cell type or a population of cells containing lower genomic 5mCyd content, however, cannot be ruled out.
The persistent loss of 5mdCyd residues may be random, so that the probability of the loss of this controlling component from specific sites in DNA will increase with time. Disturbance of controlled genetic expression due to the loss of 5mdCyd residues from promoter sequences of previously quiescent genes alters the balanced functions of a cell (6, 7).

Ectopic expression of globin and murine leukemia virus related mRNAs has been observed in brain and liver tissues from old age mice (28). Age-dependent derepression of murine mammary tumor virus has also been reported (29). Thus, increasing numbers of cells will cease to fulfill their developmentally programmed functions in old aged animals due to accumulated losses of 5mdCyd residues from DNA.

Numerous reports have also linked DNA 5mdCyd levels and pattern changes with cancer (1–3, 30). Inhibition of DNA 5mdCyd formation by chemical carcinogens may be directly involved in the initiation of carcinogenesis (15, 31, 32), while alterations in DNA 5mdCyd patterns have been found to be important to the progression of the oncogenic process (33).

The results presented here demonstrate that an average loss of 5mdCyd from a specific sequence or site will increase with time. The probability of perturbation of selective 5mdCyd sites by a single carcinogen treatment, however, will remain the same throughout the life span of the animal, increasing only with chronic exposure to the carcinogen or carcinogens. Thus, the progressive age-dependent loss of genomic 5mdCyd content may also account for why many cancers are diseases of old age (35). In addition, this programmed or environmentally induced process of accumulated genomic 5mdCyd content may be important to the progression of the oncogenic process (33).

The loss of 5mdCyd residues in nontargeting DNA could occur by demethylase activity or by faulty enzymatic maintenance of methylation sites following DNA repair. The former is unlikely since the presence of 5mdCyd demethylase activity in mammalian cells has been suggested (24), but not confirmed (1–3). Kastan et al. (25) demonstrated that human fibroblasts demethylate repaired DNA with less than 90% fidelity. Considering the amount of repair that constantly takes place in the animal genome due to oxidative damage alone (26), it may not be unreasonable that DNA 5mdCyd levels decline at the rates determined here for low mitotic tissues. Decreases in 5mdCyd may, thus, be a possible consequence of environmental exposure to oxidative processes and other DNA damaging events inherent with the metabolic processes of life (26, 27).

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