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Short Communication

High-performance liquid chromatographic analysis of methylation changes of CCGG sequence in brain and liver DNA of mice during pre- and postnatal development

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Abstract

The change of the methylation of CpG in the CCGG sequence of brain and liver DNAs of mice during late fetal and suckling periods was determined by high-performance liquid chromatography using a reversed-phase column and 0.1 *M* phosphate buffer (pH 6.0) as the mobile phase. The tissue DNA was digested with the restriction enzyme, *MspI*, and was labeled at the 5′-end with [γ-^{32}P]ATP. The cpm% of deoxycytidine 5′-monophosphate (5mdCMP) in total CpG dinucleotides was calculated from the equation 5mdCMP/total CCGG (cpm%) = $(5mdCMP)_{MspI,cpm}/\{(5mdCMP)_{MspI,cpm} + (dCMP)_{MspI,cpm}\} \times 100$. The brain DNA exhibited a significant decrease in CpG methylation at prenatal day 18 but little change after birth. This marked decline of 5mdCMP in the CCGG sequence may be associated with the increase of enzymes before birth. The liver DNA showed considerable change during the late prenatal period. The observed changes of CpG methylation in liver DNA are indicative of the corresponding alterations of enzymes, multinucleate cells and hepatocytes. The results obtained indicate that both brain and liver cells have the development-associated changes in the conformation and transition of DNA around the time of birth.

1. Introduction

The switches of particular gene expression during the development processes of mammals are generally referred to as epigenetic interactions of DNA with highly specific proteins, producing either transient changes or permanent patterns in gene activities. In general, it is assumed that one essential feature of these interactions may depend on methylation at the 5-position of deoxycytidine (5-methyldeoxycytidine, 5mdC) as a post-synthetic chemical modification of the DNA base. There have been many studies tracing age-associated and tissue-specific differences in the level of 5mdC in DNA from various mammals [1–6]. Recently, we investigated the total methylation change in brain and liver DNA of mice during pre- and postnatal periods, and found large alterations near birth for both organs [7]. Although quantitative changes of 5mdC in DNA seem to be an integral part of the differentiative processes of the cells, their relationship with gene expression is still unclear. Therefore, it is necessary to know

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whether any changes of 5mdC of CG sequences in DNA are responsible for any different stages during the development of mammals, because DNA methylation of mammalian cells occurs predominantly on C in CpG sites.

High-performance liquid chromatography (HPLC) has been widely applied to the analysis of bases, nucleosides and nucleotides in DNA [8–11]. The high selectivity and sensitivity of this method has made possible the detection and identification of very low amounts of 5mdC, far less than 1% relative to the other deoxynucleosides in DNA [7].

In the present work we applied this method to the quantitation of the sequence-specific methylation degree of brain and liver DNA of mice during the late fetal and suckling periods of CpG dinucleotides, especially in CCGG sequences, by using the methyl-insensitive restriction enzyme (*MspI*) digestion and ^{32}P-5′-end labeling with [γ-^{32}P]ATP and polynucleotide kinase.

2. Experimental

2.1. Chemicals

The deoxynucleotide 5′-monophosphate (dNMP) used as standard was purchased from Sigma (St. Louis, MO, USA). The λ-phage DNA and its 5mdC-free DNA were obtained from Toyobo (Osaka, Japan). All other chemicals were of analytical-reagent grade. Distilled water was filtered through a Milli-Q II water purification system (Nippon Millipore, Tokyo, Japan).

2.2. Animals and preparation of tissue DNA

The C57BL/6NJCL mice at ten weeks age were obtained from CLEA Japan (Tokyo) and mated in our laboratory. The day of mating was taken as day 0 of gestation, which was judged by the presence of a vaginal plug. DNA was isolated from brain and liver tissues of mice at different ages (from 14 day gestation to one month old after birth) by essentially the same method as described previously [2,12]. To remove RNA contamination from DNA, RNase A (bovine pancreas, Sigma) and RNase T_1 (*Aspergillus oryzae*, Sigma) were used. Both single and double strand breaks in the tissue DNA were checked using 0.8% agarose gel electrophoresis by the method of Kohen *et al.* [13] (data not shown).

2.3. Enzymatic digestion and ^{32}P-5′-end labeling of DNA fragments

Aliquots (5 μg) of tissue DNA were digested overnight at 37°C with restriction endonuclease, *MspI* or *HpaII* (Toyobo) at 3 U/μg DNA. To ensure complete digestion, the following experiment was done: first, 5 μg of 5mdC-free λDNA was methylated *in vitro* with 4 U of *HpaII* methylase (Toyobo), and the degree of methylation of the central C of CCGG sites was evaluated. The completeness of digestion was assessed by comparison of digestion patterns with the undigested ones on 0.8% agarose-gel electrophoresis (data not shown). After complete digestion, DNA fragments were labeled with [γ-^{32}P]adenosine triphosphate (ATP) (~222 TBq/mmol, Amersham-Japan, Tokyo, Japan) by a kinase reaction using 5 U of alkaline phosphatase (*E. coli*, Toyobo) and 10 U of T_4 polynucleotide kinase (phage T_4 infected *E. coli*, Toyobo) according to the standard method [12]. To separate the ^{32}P-5′-end-labeled fragments from the unreacted [γ-^{32}P]ATP, the mixture was applied to a Sephadex-G50 (Pharmacia, Uppsala, Sweden) column equilibrated with 10 m*M* Tris–HCl–1 m*M* EDTA (TE) buffer (pH 8.0). The column was then washed six times with 100 μl aliquots of TE, and 500 μl of the eluates containing ^{32}P-5′-end-labeled fragments were collected. The ^{32}P-labeled DNA fragments were degraded to dNMPs by the addition of 50 μl of 30 m*M* sodium acetate (pH 5.3) and 7 μl of nuclease P_1 (*ca.* 0.3 U/μl in 30 m*M* sodium acetate (pH 5.3), *Penicillium citrinum*, Boehringer-Mannheim, Mannheim, Germany). The reaction mixture was incubated at 37°C for 2 h.

2.4. HPLC analysis

The HPLC system consisted of a Model CCPM liquid chromatograph multi-pump (Tosoh, Tokyo, Japan) equipped with both a Model UV-8000 UV detector and a Model RS-8000 radio-LC detector. All data were processed with a Model CP-8080 data processor (Tosoh). The dNMPs were separated on a column (150 × 4.6 mm I.D.) packed with Cosmosil C_{18} (particle size 5 μm) (Nakalai Tesque, Kyoto, Japan). The mobile phase was 0.1 *M* $Na_2P_2O_4$–0.1 *M* phosphoric acid (pH 6.0). The flow-rate was 0.5 ml/min. The UV absorbance of the column effluent was monitored at 273 nm at a sensitivity of 0.005 AUFS (1.0 AUFS/V), and the radioactivity was measured with a Model RS-8000 solid scintillator double type-I Teflon cell (45 × 2 mm I.D.) containing 300–400 mesh CaF_2(Eu) (Tosoh). A 50-μl volume of the hydrolysate sample was injected. All measurements were carried out at room temperature (25°C) under isocratic conditions.

3. Results and discussion

3.1. HPLC separation of deoxynucleoside 5′-monophosphates

By hydrolysis of brain or liver DNAs of mice, the five dNMPs, deoxycytidine 5′-monophosphate (dCMP), deoxyadenosine 5′-monophosphate (dAMP), deoxyguanosine 5′-monophosphate (dGMP), thymidine 5′-monophosphate (TMP) and 5-methyldeoxycytidine 5′-monophosphate (5mdCMP) were separated on the reversed-phase column using 0.1 *M* phosphate buffer (pH 6.0) as the mobile phase (Fig. 1). The peak of each dNMP was identified by the retention time, and by co-chromatography using dNMP standards singly under identical conditions. With RNase treatment, ribonucleoside 5′-monophosphates in contaminating RNA from dNMPs were separated under the present chromatographic conditions. Also, other modified deoxynucleosides or deoxynucleotides did not interfere with these dNMPs under the present conditions [7].

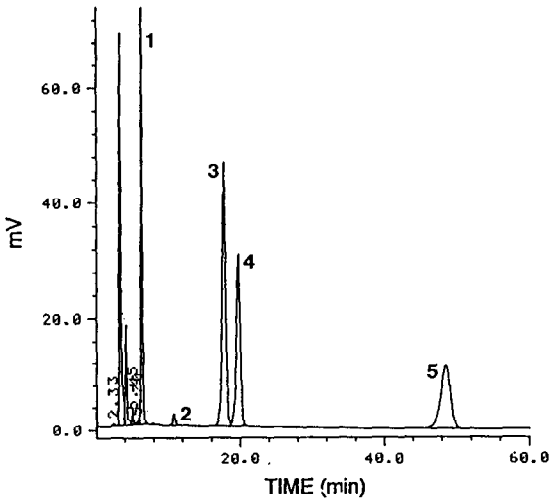

Fig. 1. HPLC separation of deoxynucleoside 5′-monophosphates from hydrolysed DNA restricted fragments digested as described in Experimental. The eluent was 0.1 *M* phosphate buffer (pH 6.0) at a flow-rate of 0.5 ml/min and was monitored at 273 nm. Peaks: 1 = deoxycytidine 5′-monophosphate; 2 = 5-methyldeoxycytidine 5′-monophosphate; 3 = thymidine 5′-monophosphate; 4 = deoxyguanosine 5′-monophosphate; 5 = deoxyadenosine-5′-monophosphate.

Fig. 2 shows the separation profile of dNMPs from an enzymic hydrolysate of ^{32}P-5′-end-labeled DNA fragments.

It is recognized that many 5′-ends that have not arisen from specific cleavage of DNA by *MspI* are contained in digested DNA [14,15], as shown in Fig. 2. In this study, it was considered that 5mdCMP of randomly generated 5′ ends in *MspI* restricted DNA were the same as in *HpaII*

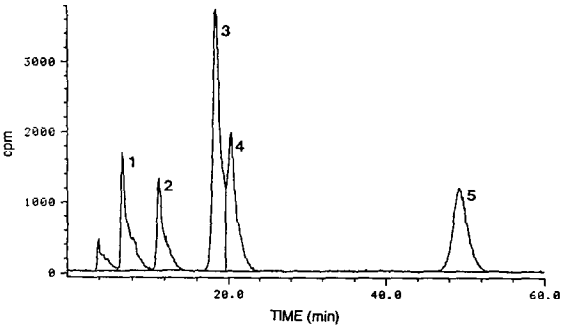

Fig. 2. HPLC separation of ^{32}P-labeled deoxynucleoside 5′-monophosphates from hydrolysed DNA restricted fragments. Approximately $1.5 \cdot 10^5$ cpm were injected into the column. Chromatographic conditions and peak numbers as for Fig. 1.

(an isoschizomer of *MspI*) restricted DNA, and that its dCMP in *MspI* restricted DNA was the same as in non-restricted DNA. The contributions of non-specifically generated 5'-ends of 5mdCMP and dCMP to the cpm in the 5mdCMP and dCMP peaks, respectively, are listed in Table 1. As the cpm% of randomly generated 5mdCMP in total DNA was small, the cpm% of 5mdCMP in CpG dinucleotides of total CCGGs was calculated as follows:

$$\text{5mdCMP/total CCGG (cpm\%)} = (\text{5mdCMP})_{MspI,\text{cpm}} / \{(\text{5mdCMP})_{MspI,\text{cpm}} + (\text{dCMP})_{MspI,\text{cpm}}\} \times 100$$

3.2. CpG methylation change of brain DNA around the birth

The results of HPLC analysis are shown in Fig. 3 and Table 2. The 5mdCMP in total CCGGs of brain DNA for each age group was calculated as the cpm% by the above equation. The brain DNA exhibited a significant decrease in CpG methylation at prenatal day 18 (−1D in Fig. 3), and little change after birth up to the adulthood. The constant level (*ca.* 66%) of overall CpG methylation in DNA before birth

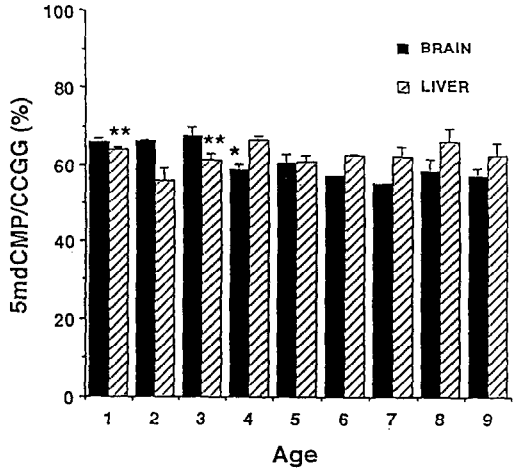

Fig. 3. The changes of 5mdCMP level in the CCGG sequence of mouse brain and liver DNA digested with *MspI*, before and after birth. The 5mdCMP contents were calculated as cpm%, as described in the text. Ages: 1 = −5D; 2 = −3D; 3 = −2D; 4 = −1D; 5 = 0D; 6 = 2D; 7 = 5D; 8 = 9D; 9 = 1M (as in Table 2). Single and double asterisks indicate significantly different $P < 0.01$ from −2D and $P < 0.05$ from −3D, respectively, by analysis of variance with Student's *t*-test.

was almost identical with the value of the 5mdC:dC ratio (1.84:1) at CCGG sites, which was estimated for the naked DNA digested with *MspI* [22]. Though the endogeneous nucleases

Table 1
Percentage cpm of 5mdCMP and dCMP based on ^{32}P-5'-end-labeling of non-specific DNA cleavage

Tissue DNA	Enzyme treatment	5mdCMP (10^3 cpm)	dCMP (10^3 cpm)	Total DNA (10^3 cpm)	5mdCMP/Total DNA (cpm%)	dCMP/Total DNA (cpm%)
Brain	Non-restricted		5.590	64.55		8.660
			2.824	39.66		7.121
						7.89 ± 1.09
	Hpa II	0.630		99.84	0.631	
		1.669		196.58	0.849	
		1.393		191.61	0.727	
					0.736 ± 0.109	
Liver	Non-restricted		12.627	121.53		10.390
			10.947	108.17		10.120
						10.26 ± 0.19
	Hpa II	1.007		409.35	0.246	
		1.820		279.57	0.651	
		2.730		667.48	0.435 ± 0.203	

Table 2
5mdCMP contents in CCGG sequences of mouse brain and liver DNA, calculated by HPLC measurements using ^{32}P-5′-end-labeling

Age[a]	5mdCMP/total CCGG (cpm%)[b]	
	Brain cpm(%)[c]	Liver cpm(%)[c]
−5D	66.0 ± 0.94 (4)[c]	64.0 ± 0.46 (4)[e]
−3D	66.2 ± 0.28 (3)	55.8 ± 3.45 (3)
−2D	67.6 ± 2.17 (3)	61.1 ± 1.88 (3)[e]
−1D	59.0 ± 1.37 (5)[d]	66.4 ± 1.28 (3)
0D	60.6 ± 2.25 (4)	60.8 ± 1.93 (4)
2D	52.9 ± 6.29 (2)	62.6 ± 0.35 (2)
5D	55.4 (1)	62.1 ± 2.83 (2)
9D	58.5 ± 3.18 (2)	66.3 ± 3.18 (2)
1M	57.3 ± 2.12 (4)	62.7 ± 3.18 (2)

[a] −D = prenatal days; D = postnatal days; M = postnatal month.
[b] cpm% is given as 5mdCMP*cpm*/{5mdCMP*cpm* + dCMP*cpm*} × 100.
[c] Values in parentheses represent the number of mouse individuals examined.
[d] Significantly different ($P < 0.01$) from −2D by analysis of variance with Student's *t*-test.
[e] Significantly different ($P < 0.05$) from −3D by analysis of variance with Student's *t*-test.

are less active in the brain, the CpG methylation decreased after prenatal day 18. This observation is interesting, when compared with the previous results for the 5mdC level in brain DNA of mice during the same period [7]. It is known that the decrease in CpG methylation before birth may correlate with the minor and progressive decline of neuronal and ependymal cell turnover from mid-gestation to about postnatal 4 weeks [16]. However, this correlation might be fortuitous, especially in view of the lack of a general relationship between cell turnover rates and DNA methylation levels. Because it is generally assumed that a high content of 5mdC in the tissue DNA is associated with its high functional activity [17], the marked decrease of 5mdC in the CCGG of brain DNA prior to birth, and its low level after birth, may show that a number of enzymes rise either before birth or shortly after [18–20].

3.3. CpG methylation change of liver DNA around birth

The 5mdCMP in the total CCGGs in liver DNA varied from 55.8 to 66.4 cpm% during late fetal and early suckling periods (Table 2). Considerable changes in the level of CpG methylation also occurred, especially during late prenatal development. This was particularly apparent at prenatal day 16 (−3D), as shown in Fig. 3, which is in good agreement with the changes of 5mdC in DNA observed previously [7]. These changes were assumed not to be associated with the changes in DNA synthesis, because it was reported that rat liver exhibited a significant increase in DNA methylation between prenatal day 20 and postnatal 4 weeks, but that the exponential rate of DNA accumulation was constant from prenatal day 15 to postnatal day 44 [16]. Both birth and weaning periods are times of drastic environmental alteration in the liver of many mammals [18]. The late fetal and the suckling periods would be expected to be associated with numerous alterations of enzyme profiles in various tissues. Therefore, the observed changes of CpG methylation before birth may reflect the increase in the various enzyme concentrations in liver. They may be also associated with the failure of many liver cells to divide following DNA replication to multinucleate cells. They may be also associated with the proportion of hepatocytes at the expense of hematopoietic cells that occur around the time of birth and the changes in liver function. For example, it has been found that *ca.* 50% of liver cells are hemopoietic from day 12 to day 17 before birth and *ca.* 12% at two days after birth [16]. This is consistent with the change of CpG methylation shown in Fig. 3.

Methylation changes in mammalian cells usually occur at CpG sites, the so-called CpG island, that are frequently clustered at the 5′-end of genes. These clusters are associated with the gene activation. Therefore, the CpG island may regulate the activation of housekeeping and some tissue-specific genes through its methylation [23]. DNA methylation is also correlated with alteration of the chromatin structure [22].

The present results may reflect gestational changes in the percentage of brain or liver chromatin in the template-active region, which is the transcriptionally active portion of the genome DNA. The template-active change has been found in brain chromatin of rats of varying ages, from prenatal sixteen days to postnatal six days [24,25]. However, the physiological significance of large differences in the 5mdCMP content in the CpG sequence of liver and brain DNA around the birth is unknown.

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